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ARTICLE

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Histaminergic ligands injected into the nucleus basalis magnocellularis differentially affect fear conditioning consolidation

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Abstract

The role of the nucleus basalis magnocellularis (NBM) in fear conditioning encoding is well established. In the present report, we investigate the involvement of the NBM histaminergic system in consolidating fear memories. The NBM was injected bilaterally with ligands of histaminergic receptors immediately after contextual fear conditioning. Histaminergic compounds, either alone or in combination, were stereotaxically administered to different groups of adult male Wistar rats and memory was assessed as conditioned freezing duration 72 h after administration. This protocol prevents interference with NBM function during either acquisition or retrieval phases, hence restricting the effect of pharmacological manipulations to fear memory consolidation. The results presented here demonstrate that post-training H_3 receptors (H_3R) blockade with the antagonist/inverse agonist thioperamide or activation with immepip in the NBM potentiates or decreases, respectively, freezing response at retrieval. Thioperamide induced memory enhancement seems to depend on H_2R, but not H_1R activation, as the H_2R antagonist zolantidine blocked the effect of thioperamide, whereas the H_1R antagonist pyrilamine was ineffective. Furthermore, the H_2R agonist ampthamine improved fear memory expression independently of the H_3R agonist effect. Our results indicate that activation of post-synaptic H_2R within the NBM by endogenous histamine is responsible for the potentiated expression of fear responses. The results are discussed in terms of activation of H_3 auto- and heteroreceptors within the NBM and the differential effect of H_3R ligands on fear memory consolidation in distinct brain regions.

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Introduction

We recently provided evidence that the functional integrity of the nucleus basalis magnocellularis (NBM) during memory processing is necessary to consolidate fear memories (Baldi *et al.* 2008). In addition to a population of γ-aminobutyric acid (GABA)ergic neurones, the NBM contains a large number of cholinergic neurons that provide the main cholinergic afferents to the cortex and amygdala (Mesulam *et al.* 1983), organized in distinct pathways (Heckers *et al.* 1994). The integrity of the NBM is necessary for general learning and memory mechanisms, as shown by permanent electrolytic or excitotoxic lesions (Vale-Martínez *et al.* 2002). Irreversible lesions of NBM cholinergic neurones indicated that these neurones may be important in mediating selective aspects of aversive states with a strong component of anxiety (Knox & Berntson, 2006; Power & McGaugh, 2002).

The use of reversible inactivation or infusions of ligands selective for neurotransmitter systems of interest makes it possible to block brain areas at any stage of memory formation and to understand some of the regulatory mechanisms underlying cognitive processes. For example, reversible inactivation with tetrodotoxin showed that NBM integrity is necessary at different post-acquisition time-points for the

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consolidation of either contextual or acoustic fear memories (Baldi *et al.* 2007).

The NBM receives histaminergic afferents from the tuberomamillary nucleus (TMN) in the posterior hypothalamus, the only source of histaminergic projections (Panula *et al.* 1984). The histaminergic system constitutes a major, wake-promoting system (Lin *et al.* 2011) and is mainly responsible for cortical activation and cognitive activities during wakefulness (Anaclet *et al.* 2009). The specificity of action of histamine on cognitive processes depends on the localization of histaminergic receptor subtypes, the brain region and the nature of the cognitive task involved and the activation of specific intracellular pathways (Passani *et al.* 2007; Passani & Blandina, 2011). Local administration of H_3R antagonists, such as thioperamide (Giannoni *et al.* 2009) or GSK-189254 (Giannoni *et al.* 2010), augments histamine release within the NBM presumably by blocking H_3 autoreceptors. Activation of histaminergic H_1 receptor (H_1R), but not H_2R, in the NBM depolarizes cholinergic neurons (Khateb *et al.* 1995) and increases acetylcholine (Ach) output in the cortex of freely moving rats (Cecchi *et al.* 2001). It seems, therefore, that H_2R in the NBM do not contribute to the neuromodulation of cholinergic corticopetal projections. These findings are apparently in agreement with reports that blockade of H_1R with chlorepheniramine within the NBM impairs behavioural tasks that presumably engage the cortical cholinergic system (Privou *et al.* 1999). Chlorpheniramine, however, lacks specificity at the high doses used by Privou *et al.* as it shows anti-muscarinic properties (Kubo *et al.* 1987) and is a potent serotonin and noradrenaline uptake inhibitor (Carlsson & Lindqvist, 1969). These results, therefore, are not conclusive.

In the present study, we evaluated the effects of histaminergic compounds injected in the NBM on the formation of emotional memories. Our aim was to dissect out the components that may affect fear memories consolidation. Using selective agonists and antagonists of the H_1, H_2 and H_3 receptors we established the relevance of the histaminergic system in controlling selective pathways originating in the NBM that are involved in fear memory consolidation.

Method

Animals

Adult male Wistar rats were individually housed in a room with a natural light/dark cycle and constant temperature (20 ± 1 °C) and had free access to food and water throughout the experiments. Animals used in this study were cared for in accordance with the guidelines of the European Community recommendations (86/606/CEE) and were approved by the Animal Care Committee of the Università di Firenze.

Behavioural experiments

Contextual fear conditioning was induced in a Skinner box module ($29 \times 31 \times 26$ cm, Modular Operant Cage; Coulbourn Instruments Inc., USA), equipped with a grid floor connected to a shock-delivery apparatus (Modular Operant Cage/Grid Floor Shocker E13-08; Coulbourn Instruments) and placed in an acoustically insulated room at 20 ± 1 °C, as in previous experiments (Baldi *et al.* 2007). The number of the electric shocks and the inter-shock interval duration was predetermined by a stimulus programming device (Scatola di comando Arco 2340, Italy). Illumination inside the room was 60 lux. The rat was left undisturbed for 3 min and subsequently seven 1-s, 1-mA electric footshocks were administered at 30-s intervals. The footshock intensity was chosen according to previous published data from our laboratory (Cangioli *et al.* 2002; Giovannini *et al.* 2003). This is a strong enough footshock to guarantee retention at 72 h post-acquisition without inducing generalization (Baldi *et al.* 2004). The rat was removed 2 min after the end of the stimulation, therefore spending a total time of 8 min inside the conditioning apparatus.

Surgery and intra-NBM drug administration

Rats were anaesthetized immediately after the training session with nembutal (50 mg/kg i.p.) and bilateral injections took place within the following 20 min. Animals were restrained in the stereotaxic apparatus and insertion of the injection needle was performed according to the coordinates: anterior–posterior -1.5 mm, lateral ± 2.8 mm, dorsoventral 7.2 mm from bregma (Paxinos & Watson, 1998). Drugs were dissolved in physiological saline and injected bilaterally into the NBM in nine different groups of rats and they received the following solutions: physiological solution; H_3R antagonists/inverse agonist thioperamide (0.3 μM); H_3R agonist immepip (1 μM); H_1R antagonist pyrilamine (0.9 μM); H_2R antagonist zolantidine (0.1 μM); thioperamide + pyrilamine; thioperamide + zolantidine; H_2R agonist ampthamine (10 μM); ampthamine + immepip. The concentrations chosen were within the range of selectivity for the various compounds and/or according to their efficacy in microdialysis or behavioural experiments (Calcutt *et al.* 1988; Cangioli *et al.* 2002; Cecchi *et al.* 2001;

Passani *et al.* 2001). The injection needle had an outside diameter of 0.3 mm and was connected with a short piece of polyethylene tubing to a Hamilton syringe fixed to an electrode holder. Solutions (0.4 μl per side) were injected over a 1–2 min period and the needle was left in place for another min before withdrawal. After surgery, rats were returned to their home cages. During the following 3 d before testing, the animals displayed a normal behaviour that did not differ from that of controls. A group of rats was fear conditioned without receiving Nembutal anaesthesia. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Intra-NBM injected animals recovered from surgery and general anaesthesia within 24 h and in the following 48 h did not show any abnormal behaviour.

Freezing measurement

Seventy-two hours after conditioning, rats were again placed inside the conditioning apparatus in the soundproof room and left undisturbed for 6 min. The rats' behaviour was recorded by means of a closed circuit television system by an experimenter unaware of the animal's treatment. Freezing (immobility) was defined as the complete absence of somatic motility, with the exception of respiratory movements (Sacchetti *et al.* 1999). Measurements were performed with a stopwatch by personnel who did not know to which experimental group each animal belonged. Total cumulated freezing time (i.e. total seconds spent freezing during each 3 min period) was calculated and results expressed as percentage of freezing time. All behavioural tests were performed between 10:00 and 12:00 hours to avoid interference with the circadian rhythm (Kamin, 1957).

Rat locomotor and exploratory activity

After measuring conditioned freezing, rats were gently placed on a round table with a diameter of 1.5 m and their behaviour observed for 3 min according to Giovannini *et al.* (2003). Data from rats showing abnormal locomotor and exploratory activities, grooming, yawning and rearing, were not included (four rats out of 94). Non-quantitative criteria were used to exclude rats. An experienced observer estimated whether rats' behaviour in the open field was within the norm for animals of that age.

Histology

At the end of the experiments, rats were deeply anaesthetised with Nembutal, the brains were removed and stored in 10% formalin for 10 d. Brain sections were cut with a freezing microtome and Nissl-stained to verify injection sites. Only animals with correct placement of the injection needles within the boundaries of the NBM were used for data analysis (Fig. 1).

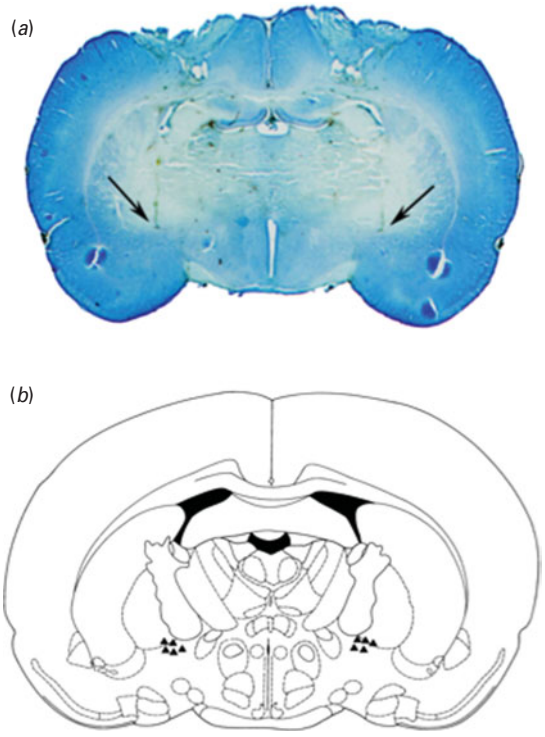

Fig. 1. (*a*) The photomicrograph shows an example of the bilateral needle tracks in the nucleus basalis magnocellularis (NBM) of the rat brain. Arrows point to the regions of perfusion. (*b*) Schematic drawing of a rat brain coronal section showing sites of injection within the NBM.

Data analysis

For each session, data were expressed as seconds spent freezing within the 6 min of testing. One-way analysis of variance (ANOVA) and the Neuman–Keuls *post-hoc* test were used based on the only source of variation, i.e. pharmacological treatment. Results of each set of experiments were analysed independently. For all statistical tests, $p < 0.05$ was considered significant.

Results

Post-acquisition injections of H_3R ligands in the NBM modulate contextual fear memory consolidation. Rats of all experimental groups did not show differences in spontaneous locomotor and explorative behaviour in the initial 3 min of exploration during acquisition training. Control rats that did not receive Nembutal

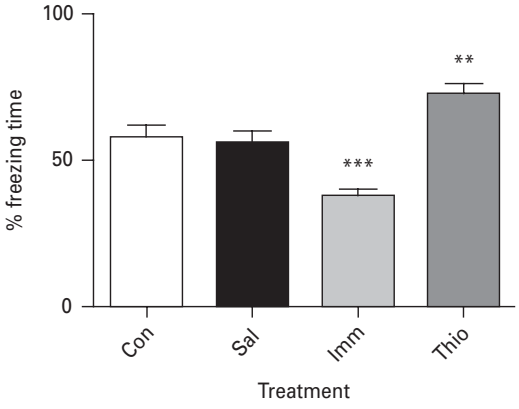

Fig. 2. Post-acquisition injections of H_3R ligands in the nucleus basalis magnocellularis (NBM) affect contextual fear memory consolidation. Thioperamide (Thio; 0.3 μM), immepip (Imm; 1 μM) or saline (Sal) were injected bilaterally in the NBM immediately after contextual fear conditioning. Control (Con) rats were not anaesthetized and did not receive intra-NBM injections. All animals were tested for fear retention 72 h after conditioning. Bars represent mean values $\pm$S.E.M. *** $p<0.001$ *vs.* Con; one-way analysis of variance and Newman–Keuls *post-hoc* analysis.

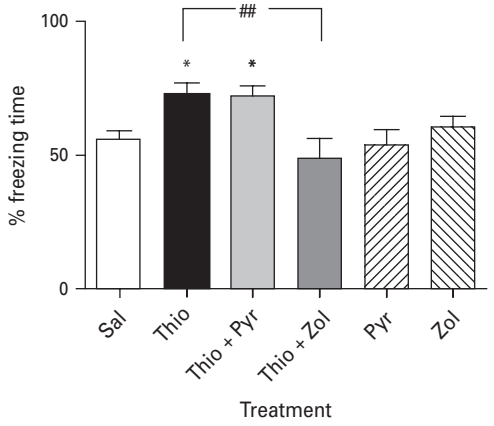

Fig. 3. H_2R but not H_1R activation in the nucleus basalis magnocellularis (NBM) improves memory consolidation of fear memory. Compounds were injected bilaterally in the NBM immediately after contextual fear conditioning. Rats were tested for fear retention 72 h after conditioning. The H_2R antagonist zolantidine blocks the H_3R antagonist-mediated memory impairment. Thioperamide (Thio; 0.3 μM); pyrilamine (Pyr; 0.9 μM); zolantidine (Zol; 0.1 μM). * $p<0.05$ *vs.* saline (Sal); ## $p<0.05$ Thio *vs.* Thio + Zol; one-way analysis of variance and Newman–Keuls *post-hoc* analysis.

after contextual conditioning showed levels of freezing comparable to rats anaesthetized and infused with saline in the NBM ($58\pm4\%$ *vs.* $56\pm4\%$; $n=10$ and $n=12$, respectively; Fig. 2). The effects of post-training bilateral injections of H_3R ligands in the NBM are shown in Fig. 2. One-way ANOVA for freezing time revealed an overall significant difference between groups ($F_{3,45}=17.90$; $p<0.001$). The H_3R antagonist/inverse agonist thioperamide increased freezing time compared to non-anaesthetized and saline-injected rats ($73\pm3\%$; $n=13$; $p<0.001$), whereas the H_3R agonist immepip significantly decreased freezing time ($38\pm2\%$; $n=11$; $p<0.001$). As non-anaesthetized (controls) and saline-injected rats displayed the same freezing levels, further comparisons were carried out against the saline-treated group only.

Blockade of H_2R in the NBM prevents thioperamide-induced modulation of fear memory consolidation. The effect of thioperamide post-acquisition injections in the NBM on freezing time was prevented by co-administration of the H_2R antagonist zolantidine ($48\pm8\%$; $n=12$), but not of the H_1R antagonist pyrilamine ($72\pm4\%$; $n=10$; Fig. 3). One-way ANOVA for freezing time revealed an overall difference between groups ($F_{5,65}=5.558$; $p<0.001$) with significant differences (Newman–Keuls multiple comparison tests) between controls and thioperamide ($p<0.05$) and pyrilamine + thioperamide ($p<0.05$). Also, rats treated with thioperamide differed significantly from rats treated with thioperamide + zolantidine ($p<0.01$). Post-acquisition perfusion of the NBM with either zolantidine ($n=10$) or pyrilamine ($n=10$) alone did not significantly change the freezing time (Fig. 3).

H_2R activation in the NBM improves memory consolidation of fear memory. We then tested the effect of post-acquisition H_2R activation in the NBM on freezing time at retention. As shown in Fig. 4, the H_2R agonist ampthamine increased the freezing time at testing to $75\pm5\%$ ($n=15$; $p<0.01$). This effect was not blocked by co-administration of ampthamine with the H_3R agonist immepip ($74\pm4\%$ s; $n=9$), which significantly inhibits consolidation when given alone (Fig. 4). One-way ANOVA for freezing time showed an overall difference between groups ($F_{2,36}=7.568$; $p<0.01$) with significant differences (Newman–Keuls multiple comparison tests) between controls and both amthamine ($p<0.01$) and amthamine + immepip ($p<0.01$).

Discussion

Post-training injections of H_3R ligands in the NBM modulate the expression of fear memory in opposite fashions, as low concentrations of the H_3R antagonist thioperamide increased, whereas the H_3R agonist immepip impaired freezing time at testing. It is interesting to note that, pre-acquisition, intra-NBM injections of thioperamide also has pro-cognitive effects in

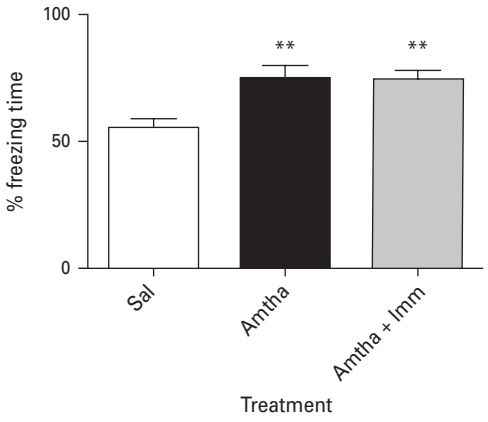

Fig. 4. The H_2R agonist ampthamine improves fear memory independently of H_3R agonist effects. Ampthamine (Amtha; 10 μM); immepip (Imm; 1 μM). Bars represent mean values $\pm$S.E.M. ** $p < 0.01$ *vs.* saline (Sal); one-way analysis of variance and Newman–Keuls *post-hoc* analysis.

a two-trial, delayed, place-recognition task (Orsetti *et al.* 2002). The potentiation of freezing response observed with intra-NBM injections of thioperamide seems to depend on H_2R activation, as zolantidine blocked the effect of thioperamide, whereas the H_1R antagonist pyrilamine was ineffective. Furthermore, intra-NBM injections of the H_2R agonist ampthamine improved freezing response. This effect was independent of H_3R activation with immepip. Consolidation of fear memory *per se* was not affected by blockade of either H_1R or H_2R within the NMB, as rats that received intra-NBM injections of either pyrilamine (H_1R antagonist) or zolantidine (H_2R antagonist) showed freezing behaviour at testing similar to controls that received saline.

The H_3R acts both as an autoreceptor that limits histamine synthesis and its release from histaminergic endings and as a heteroceptor that moderates the release of other neurotransmitters, including ACh, dopamine, GABA, 5-hydroxytryptamine and peptides (Haas *et al.* 2008). The most conservative explanation of the results presented here predicts that blockade of H_3 autoreceptors augments histamine release within the NBM as previously observed (Giannoni *et al.* 2009, 2010) and that histamine in turn activates postsynaptic H_2R responsible for the potentiated expression of fear response. Indeed, activation of H_3R within the NBM that presumably decreased histamine output did not block the pro-cognitive effect of the H_2R agonist ampthamine (Fig. 4). The contribution of H_3 heteroreceptors on axonal endings or neuronal somata, however, cannot be excluded. For example, blockade or activation of H_3 heteroreceptors may directly modulate neurotransmission within the NBM independently of histamine release. This possibility may contribute to the amnesic effect of the H_3R agonist immepip. The NBM also contains GABAergic interneurones that are involved in the consolidation process of inhibitory avoidance learning (Morón *et al.* 2002). However, the presence of histaminergic receptors on GABAergic neurones and their potential contribution to modulating contextual fear memory is not known at present.

Our observations suggest that activation of H_2R within the NBM is not essential for the consolidation or expression of fear memories under our experimental conditions. But when histamine output within the NBM is perturbed, H_2R activation is necessary for increased fear response. The histaminergic modulation of NBM neurones, hence, may become critical under specific circumstances, such as during emotionally relevant experiences. Indeed, augmented histamine release in the central nervous system is classically associated with increased alertness (Lin, 2000). Stress determines a transient increase of histamine release in the rat frontal cortex (Westerink *et al.* 2002), whereas motivated behaviours such as food searching augments histamine output from the TMN of the rat (Valdés *et al.* 2010). The present results demonstrate that post-training blockade of H_3R or activation of H_2R in the NBM provides a mechanism for augmenting conditioned fear response expression.

The present data, together with previous observations from our laboratory, strongly suggest a physiological role for the differential regulation, in a region-specific manner, of histamine and of neurotransmitters that are recognized as major modulators of cognitive processing and motivated behaviours. Histaminergic ligands modulate fear conditioning consolidation in opposite ways when locally administered in different brain regions that control fear memory. For example, post-training injections of H_3R antagonists/inverse agonists in the basolateral amygdala (BLA) decreases the freezing time of trained rats compared to saline-injected controls (Bucherelli *et al.* 2006; Passani *et al.* 2001), whereas intra-BLA administration of H_3R agonists augments the freezing time (Cangioli *et al.* 2002). In our opinion, the controversial role of histamine can be partially reconciled with the observation that H_3R antagonists directly administered to the BLA at the same doses that inhibit fear memory modulate the cholinergic tone, required for fear memory consolidation (McGaugh & Izquierdo, 2000), in the same bimodal fashion as they modify expression of fear memories. In the hippocampus, on the other hand, H_2R and H_3R activation improves the expression of

fear memory by phosphorylating ERK (Giovannini *et al.* 2003), which is required for consolidation of associative memories (Schafe *et al.* 2000). Hence, the specificity of action of histaminergic ligands depends on architectural constraints that separate groups of transmitters in particular brain structures modulating the expression of specific behaviours and on the activation of specific intracellular pathways.

The NBM contains mainly cholinergic neurones that project to the cortex and amygdala (Heckers *et al.* 1994; Mesulam *et al.* 1983). NBM corticopetal neurones affect the psychological process by modulating cortical activity (Baxter & Chiba, 1999) and immunotoxin lesions of these neurones suggest that cholinergic input to the neocortex may be involved in attentional processes (Sarter & Bruno, 1997). The same lesion protocol, however, does not produce a deficit in inhibitory avoidance retention (Power *et al.* 2002; Wenk *et al.* 1994), neither induces general deficit in anxiety-like states in the elevated plus-maze nor open field (Knox & Berntson, 2006; Pizzo *et al.* 2002). The activity of NBM cholinergic neurones projecting to the cortex are modulated by endogenous histamine through H_1R (Cecchi *et al.* 2001) and the results reported here suggest that thioperamide-induced fear memory improvement does not depend on the activation of the NBM-cortical cholinergic pathway under histaminergic H_1R control. Indeed, the H_1R antagonist pyrilamine does not affect thioperamide-induced increase of fear conditioning retention.

Lesions of the cholinergic projections from the NBM to the BLA produce a pronounced consolidation deficit of the aversive inhibitory avoidance task, but spare spatial memory (Power & McGaugh, 2002). The same protocol impairs working memory performance in the double Y-maze, but does not affect reference memory (Mallet *et al.* 1995). In conclusion, our results strongly suggest that activation of post-synaptic H_2R within the NBM by endogenous histamine is responsible for the potentiated expression of fear responses. We therefore speculate that the amygdalopetal cholinergic pathway that originates in the NBM is controlled, at least in part, by H_2R activation. To investigate this suggestion, future research may test the hypothesis of a segregation of H_1R and H_2R expression on different NBM cell populations.

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Statement of Interest

None.

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