European Journal of Neuroscience, Vol. 14, pp. 1522–1532, 2001 *© Federation of European Neuroscience Societies*

Histamine H_3 receptor-mediated impairment of contextual fear conditioning and *in-vivo* inhibition of cholinergic transmission in the rat basolateral amygdala

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Keywords: ACh, amygdala, conditioned freezing, H_2 receptor, H_3 receptor, memory, microdialysis, scopolamine

Abstract

We investigated the effects of agents acting at histamine receptors on both, spontaneous release of ACh from the basolateral amygdala (BLA) of freely moving rats, and fear conditioning. Extensive evidence suggests that the effects of histamine on cognition might be explained by the modulation of cholinergic systems. Using the microdialysis technique in freely moving rats, we demonstrated that perfusion of the BLA with histaminergic compounds modulates the spontaneous release of ACh. The addition of 100 mM KCl to the perfusion medium strongly stimulated ACh release, whereas, 0.5 μM tetrodotoxin (TTX) inhibited spontaneous ACh release by more than 50%. Histaminergic H_3 antagonists (ciproxifan, clobenpropit and thioperamide), directly administered to the BLA, decreased ACh spontaneous release, an effect fully antagonized by the simultaneous perfusion of the BLA with cimetidine, an H_2 antagonist. Local administration of cimetidine alone increased ACh spontaneous release slightly, but significantly. Conversely, the administration of H_1 antagonists failed to alter ACh spontaneous release. Rats receiving intra-BLA, bilateral injections of the H_3 antagonists at doses similar to those inhibiting ACh spontaneous release, immediately after contextual fear conditioning, showed memory consolidation impairment of contextual fear conditioning. Post-training, bilateral injections of 50 μg scopolamine also had an adverse effect on memory retention. These observations provide the first evidence that histamine receptors are involved in the modulation of cholinergic tone in the amygdala and in the consolidation of fear conditioning.

Introduction

Considerable evidence indicates that the basolateral nuclei of the amygdala (BLA) are implicated in memory of adverse events in a wide variety of learning situations (Pitkånen *et al*., 1997; LeDoux, 2000; McGaugh, 2000; Nader *et al*. 2000). For example, the BLA appears to be involved in the consolidation of both contextual and auditory fear (Sacchetti *et al*., 1999). Moreover, pharmacological manipulation of the BLA, with drugs influencing GABAergic, opioid or cholinergic systems, affects memory consolidation for adverse events (Helmstetter & Bellgowan, 1994; McGaugh *et al*., 1996; Wilensky *et al*. 2000). In particular, muscarinic receptor activation within the amygdala appears to be critical in the memory for adverse stimuli (McGaugh *et al*., 1996). Indeed, intra-amygdala post-training injection of oxotremorine, a muscarinic agonist, enhanced memory in an inhibitory avoidance task (Introini-Collison *et al*., 1996) and in contextual fear conditioning (Vazdarjanova & McGaugh, 1999). Conversely, intra-amygdala post-training injections of atropine, a muscarinic antagonist, prevented memory enhancement produced by systemic administration of muscarinic agonists (Dalmaz *et al*., 1993; Introini-Collison *et al*., 1996). Thus, neurotransmitters that affect BLA cholinergic activity might also influence memory consolidation, as is the case for the cholinergic systems in the cortex and in the hippocampus (Blandina *et al*., 1996; Passani & Blandina, 1998; Pepeu & Blandina, 1998).

The BLA is innervated by cholinergic fibers that originate in the nucleus basalis magnocellularis (Mesulam *et al*., 1983) and receives the major input from cortical and subcortical sensory areas (Davis *et al*., 1994). In addition, it receives histaminergic projections from the hypothalamus (Inagaki *et al*., 1988; Panula *et al*., 1989). Histaminergic modulation of basal forebrain cholinergic neurons has been demonstrated and the mechanisms included both interactions on target cell bodies (Mochizuki *et al*., 1991; Katheb *et al*., 1995; Gorelova & Reiner, 1996; Cecchi *et al*. 2001) and also modulation of ACh release from nerve endings (Arrang *et al*., 1995; Clapham & Kilpatrick, 1992). For example, locally applied histamine decreased the cholinergic tone in the cortex of freely moving rats by activating H_3 receptors (Blandina *et al*., 1996), through a mechanism which involved GABAergic interneurons (Giorgetti *et al*. 2000). This interaction may have functional relevance, as systemic administration of H_3 receptor agonists impaired rat performance in cognitive tasks at the same doses that reduced ACh release from freely moving rat cortex (Blandina *et al*., 1996). This finding is in agreement with the observations that reduced availability of ACh in the synaptic cleft may result in cognitive deficits (Quirion *et al*., 1995). It is also consistent with reports of H_3 receptor antagonists' procognitive

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Received 30 May 2001, revised 18 September 2001, accepted 21 September 2001

effects in normal (Prast *et al*., 1996; Ligneau *et al*., 1998) and scopolamine-impaired rats (Giovannini *et al*., 1999), as well as in senescence-accelerated mice (Meguro *et al*., 1992). Indeed, there is much evidence suggesting that the effects of histamine on cognition might be explained by the modulation of cholinergic systems (Passani & Blandina, 1998; Nakazato *et al*. 2000; Passani *et al*. 2000a). The current study investigates the neuromodulatory effect of brain histamine on ACh release from the BLA of freely moving animals, and on contextual fear conditioning. Some of these results have been reported in abstract form (Passani *et al*. 2000b).

Materials and methods

Animal housing

Male Wistar rats (250–280 g) were housed in groups of three in a temperature-controlled room (20–24 °C), allowed free access to food and water, and kept on a 12-h light : 12-h dark cycle. All the experiments were performed in strict compliance with the EEC recommendations for the care and use of laboratory animals (86/609/CEE), and were approved by the Animal Care Committee of the 'Dipartimento di Farmacologia Preclinica e Clinica' of the 'Universitá di Firenze'.

Surgery and microdialysis

The rats were anaesthetized with chloral hydrate (400 mg/kg i. p.) and placed in a stereotaxic frame (Stellar, Stoelting Co., Wood Dale, IL). Each rat was implanted with a guide cannula (Metalant, Sweden) according to the following coordinates from bregma (Paxinos & Watson, 1982): AP, –2.8 mm, L, –4.9 and H, 3.8. A surgical screw served as an anchor and the guide cannula was fixed to the skull with acrylic dental cement. After surgical manipulations, rats were replaced in their cages (one rat per cage) to recover from surgery. Each rat was used for a single experiment. The microdialysis experiments were performed 24 h after surgery. The stylet was removed from the guide cannula and a microdialysis probe (1.5 mm dialysing membrane molecular weight cut-off at 6000 Da; Metalant, Sweden) was lowered into the BLA. The tip of the probe extended 4.5 mm beyond the tip of the guide cannula. The microdialysis probe was perfused at a rate of 1.35 μL/min using a microperfusion pump (Carnegie Medicine, Sweden; mod. CMA/100) with Ringer solution (in mM: NaCl, 147; $CaCl_2$, 1.2 and KCl, 4.0 at pH 7.0). To recover detectable dialysate concentrations of ACh, a cholinesterase inhibitor (0.1 μM neostigmine bromide) was added to the medium perfusing the BLA. Solutes with molecular weight lower than 6000 Da were allowed to cross the dialysis membrane according to their concentration gradient. Hence, both suitable endogenous molecules and exogenous compounds could feasibly be collected and administered, respectively. Drugs, alone or in combination, were added to the BLA perfusing medium. After an equilibration period of 2 h, fractions were collected at 20-min intervals. The BLA was perfused with control medium in the first four fractions to measure ACh spontaneous release and drugs were then added to the medium.

Histology

Accurate placement of microdialysis membranes was verified histologically. The rats were killed by an overdose of chloral hydrate, the brains were removed and stored in 10% formaline for 10 days. Forty-μM sections were sliced on a cryostat, mounted on gelatin-coated slides and then stained with cresyl violet for light microscopy observation. Data from rats in which the membranes were not correctly positioned were discarded.

Assay of ACh

Acetylcholine was determined by HPLC-electrochemical detection as described previously (Giorgetti *et al*. 2000). Briefly, ACh was separated on the cation exchange column. ACh was hydrolysed by acetylcholinesterase to form acetate and choline in the post-column enzyme reactor, then choline was oxidized by choline oxidase to produce betaine and hydrogen peroxide. Hydrogen peroxide was detected by a platinum electrode with the potential set at 0.5 V. Peaks were identified by comparison of their retention times with those of the standards.

Quantification of ACh

The levels of ACh in the perfusate were calculated by comparison of sample peak heights with external standard peak height and expressed as pmol/20 min. Calibration curves for ACh were constructed by plotting the heights of peaks against the concentrations. Regression lines were then calculated and determination of unknown samples was carried out by the method of inverse prediction. Acetylcholine spontaneous release was calculated for each experiment by averaging the mean of the four 20-min samples of perfusate collected before drug treatment. Acetylcholine release was expressed as a percent of its spontaneous release value. The *in vitro* recovery of ACh from the dialysis membrane was about 60% at room temperature. Values reported here were not corrected for recovery.

Statistical analysis

All values are expressed as means ± SEM and the number of rats used in each experiments is also indicated. The presence of significant treatment effects was first determined by a one-way or a two-way analysis of variance (ANOVA) followed by Bonferroni's or Scheffe's and Neuman–Keuls tests, as appropriate. For all statistical tests, $P < 0.05$ was considered significant. Scheffe's procedure for post-hoc comparisons performed all possible comparisons of the means. For clarity purposes and for its biological relevance we reported in figures and figure legends only the significant differences vs. the last sample before drug treatment. Statistical analysis was performed using StatView (Abacus Concepts, Inc., Berkeley, CA) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

Behavioural experiments

Experiments were performed on 70-day-old male albino Wistar rats (average body-weight 290 g). The animals were individually housed in stainless steel cages in a room with a natural light : dark cycle (windows) and constant temperature of 20 ± 1 °C. The rats had free access to food and water throughout the experiments.

Apparatus

A basic Skinner box module (Modular Operant Cage, Coulbourn Instruments Inc.) was used to induce contextual conditioned freezing as in previous experiments (Sacchetti *et al*., 1999). Box dimensions were 29 × 31 × 26 cm. The top and the two opposite sides were made of aluminium panels. The other two sides were made of transparent plastic. The floor was made of stainless steel rods connected to a shock delivery apparatus (Grid Floor Shocker, model E13-08, Coulbourn Instruments Inc.). The apparatus was connected to a stimulus programming device (Scatola di comando Arco 2340, Ugo Basile, Italy) to predetermine the number and duration of the electric shocks and the duration of the intervals between them. The apparatus was placed in an acoustically insulated room kept at a constant temperature of 20 ± 1 °C. Illumination inside the room was 60 lux.

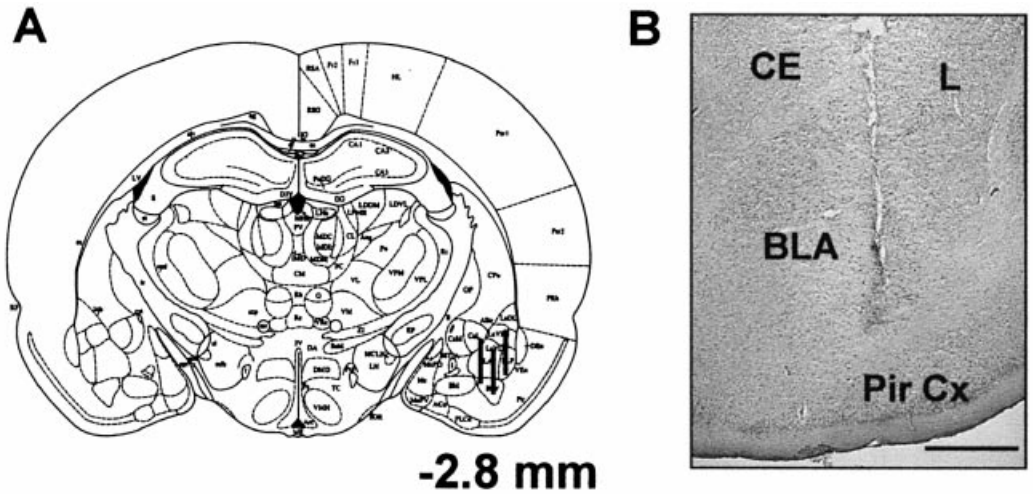

FIG. 1. Placement of dialysing membranes within the BLA. (A) Schematic drawing illustrating the placement of three probes representing the most lateral, medial and ventral positions. The length of the bar corresponds to the extent of the dialysing membrane. The number indicates the relative position of the coronal section posterior to bregma according to Paxinos & Watson (1982). (B) Photomicrograph of the probe tip location. CE, central nucleus; BLA, basolateral complex; L, lateral nucleus; Pir Cx, piriform cortex. Scale bar, 0.5 mm.

Contextual fear conditioning procedure

The rat was gently taken manually from the home cage, placed in a bucket and carried from the housing room to the soundproof room. Once there, it was placed inside the conditioning apparatus. The rat was left undisturbed for 3 min. After this time seven 1- s, 1-mA electric footshocks were administered at 30 s intervals. Two minutes after the end of the stimulation, the rats were removed, thus spending a total time of 8 min inside the conditioning apparatus.

Conditioned freezing measurements

Freezing duration was measured 72 h after conditioning by an experimenter blind to the animal's treatment. To measure freezing, the animals were again placed inside the conditioning apparatus and left for 3 min, during which they did not receive electrical stimulation. After that time, they were brought back to the home cage. Each rat's behaviour was recorded by means of a closed circuit television system. Freezing was defined as complete absence of somatic mobility, with the exception of respiratory movements. Freezing time was measured with a stop-watch. All behavioural testings were performed between 10.00 am and 12.00 pm.

Surgery and drug administration

Injections into the BLA were performed immediately after the training. Briefly, drugs dissolved in physiological saline were injected under general anaesthesia (ketamine, 100 mg/kg i.p.) into the BLA of rats restrained in a stereotaxic apparatus. The same coordinates were used as for the implantation of the microdialysis probes [according to Paxinos & Watson (1982)]. The tip of the needle was placed 8.7 mm ventral to bregma. The injection needle (outside diameter, 0.3 mm) was connected with a short piece of polyethylene tubing to a Hamilton syringe that was fixed to an electrode holder. Solutions (0.5 μL per side) were injected over a 1–2-min period and the needle was left in place for another min before withholding it. Control groups received bilateral injections of saline.

Histology

At the end of the experiments, rats were deeply anaesthetized and perfused intracardially with 5% paraformaldehyde. Brain sections were cut with a freezing microtome and Nissl-stained to verify injection sites. Animals in which histological evidence was not adequate were discarded.

Data analysis

For each session, data were expressed as seconds spent freezing within the 3 min of testing. One-way ANOVA and Neuman–Keuls *post hoc* test were used. For all statistical tests, $P < 0.05$ was considered significant.

Chemicals

The substances used in this study included cimetidine, neostigmine bromide, thioperamide maleate, triprolidine dihydrochloride (RBI, Natick, MA, USA); tetrodotoxin (Sigma), scopolamine hydrobromide (Sigma), clobenpropit dihydrobromide was provided by Drs R. Leurs and H. Timmerman and ciproxifan was provided by Dr W. Schunack. All other reagents and solvents were of HPLC grade or the highest grade available (Sigma, UK).

Results

Three examples of the medial, ventral and lateral-most locations of the dialysing membrane within the BLA are shown in Fig. 1A. A photomicrograph of a representative probe placement in the BLA is illustrated in Fig. 1B.

Spontaneous release of ACh from the BLA of freely moving rats and its modulation by potassium and TTX

Twenty-four hours after implantation of the dialysis probe, the microdialysis membrane was perfused with Ringer solution containing 0.1 μM neostigmine. After 120 min of equilibration, the rat BLA spontaneously released ACh at a stable rate, 0.32 ± 0.07 pmol/20 min ($n = 62$). Spontaneous ACh release was calculated for each experiment by averaging the mean of the four 20-min samples of perfusate collected before drug treatment and did not decrease significantly with time. The introduction of 0.1 μM neostigmine, a cholinesterase inhibitor, into the perfusing medium was necessary to recover detectable dialysate concentrations of ACh. This concentra-

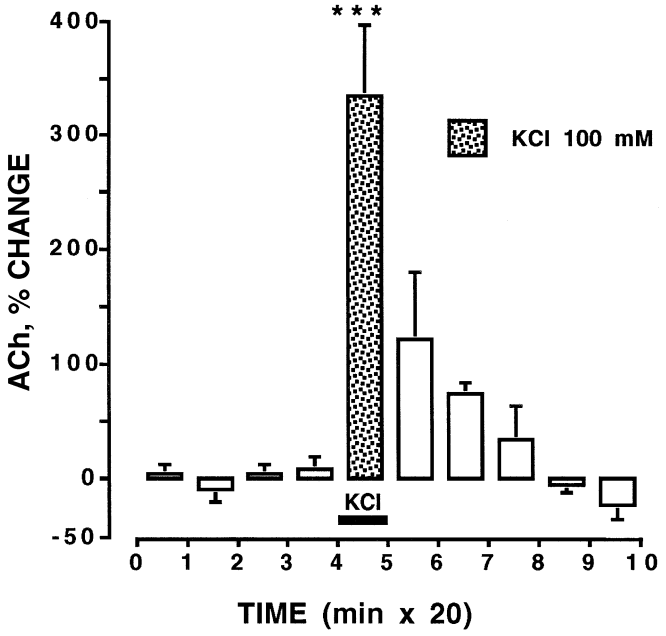

FIG. 2. Spontaneous- and 100 mM KCl-evoked release of ACh from the BLA of freely moving rats. Twenty-four h after implantation of the dialysis fibre, ACh was measured in fractions collected every 20 min, beginning 2 h after the onset of the perfusion. Spontaneous release of ACh was calculated for each experiment by averaging the mean of the four, initially collected, 20-min samples of perfusate. At 80 min the perfusion medium was changed from 4 to 100 mM KCl for 20 min. Isotonicity was maintained by reducing NaCl concentration. Acetylcholine release was expressed as percentage of its spontaneous release value. The mean spontaneous release was 0.36 ± 0.03 pmol/20 min. The black bar shows the period of 100 mM KCl application to the BLA. Shown are means ± SEM of five rats. ***$P < 0.001$ vs. the last sample before drug treatment (ANOVA and Scheffe's test).

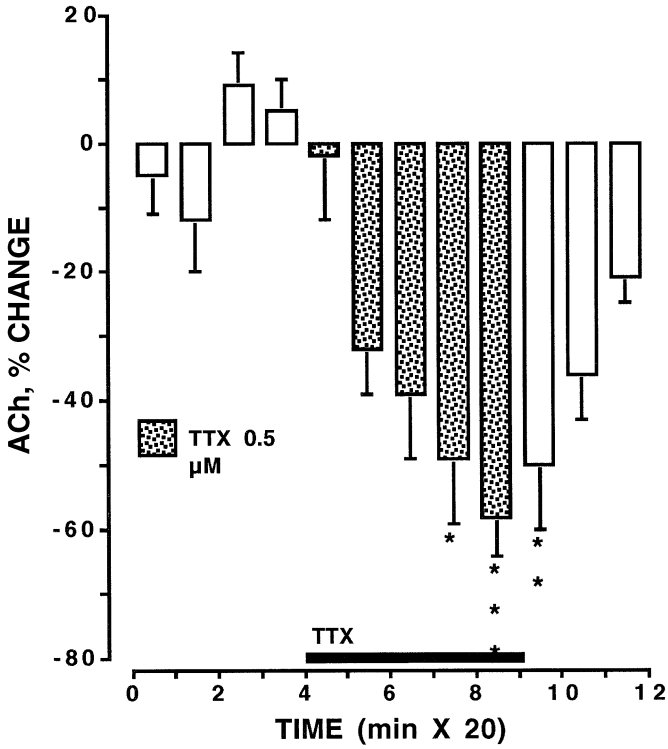

FIG. 3. Influence of TTX on spontaneous ACh release from the BLA of freely moving rats. Twenty-four h after implantation of the dialysis fibre, ACh was measured in fractions collected every 20 min, beginning 2 h after the onset of the perfusion. At 80 min, 0.5 µM TTX was added to the perfusing medium and administered for 100 min. The control value for ACh spontaneous release was calculated as in Fig. 2. and ACh release was expressed as percentage of its spontaneous release value. The mean spontaneous release averaged 0.28 ± 0.02 pmol/20 min. The black bar indicates the period of TTX application. Shown are means ± SEM of seven rats. ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$. Differences were significant vs. the last sample before drug treatment (ANOVA and Scheffe's test).

tion of neostigmine was chosen on the basis of pilot data showing that 0.05 µM neostigmine was insufficient to produce consistently detectable dialysate concentrations of ACh, whereas 0.5 µM neostigmine was less effective than 0.1 µM, probably due to the activation of negative feedback mechanisms (Marshall & Wurtman, 1993).

Depolarization, induced by perfusing the BLA with a medium containing 100 mM potassium for 20 min, strongly stimulated the release of ACh, producing a maximal increase of 335 ± 62% (Fig. 2). The maximal effect occurred in the 20-min fraction collected during perfusion with 100 mM potassium, and the level of the spontaneous release of ACh was restored during the subsequent perfusion with control medium. During the potassium pulse, the rats did not show any abnormal behaviour. The spontaneous release in this set of experiments was 0.36 ± 0.03 pmol/20 min ($n = 5$).

The infusion of 0.5 µM tetrodotoxin (TTX), a voltage-dependent sodium-channel blocker, for 100 min into the amygdala through the dialysis probe reduced spontaneous ACh release by more than 50% (Fig. 3). Spontaneous release averaged 0.28 ± 0.02 pmol/20 min ($n = 7$). ACh release slowly returned to basal values upon washout (Fig. 3). During TTX exposure the rats did not show any abnormal behaviour.

Local administration of H_3 receptor antagonists in the BLA decreased spontaneous release of ACh

Thioperamide, a selective H_3 receptor antagonist (Arrang *et al.*, 1987a), added to the BLA-perfusing medium for 40 min at a concentration of 30 nM, significantly modulated spontaneous ACh release, thus causing a maximal decrease of 37 ± 2% (Fig. 4). The spontaneous release of ACh averaged 0.29 ± 0.07 pmol/20 min ($n = 4$). An identical perfusion with 300 nM thioperamide elicited a 53 ± 7% maximal decrease in ACh release (Fig. 4). The spontaneous release of ACh averaged 0.26 ± 0.05 pmol/20 min ($n = 4$). The maximal effect was always achieved in the 20-min fraction collected after thioperamide was withdrawn from the perfusing medium. Spontaneous ACh release level was restored after withdrawal of thioperamide from BLA perfusion medium. The inhibition of ACh release caused by perfusion with medium containing 30 nM thioperamide was not significantly different from that caused by perfusion with 300 nM thioperamide-containing medium (Two-way ANOVA and Bonferroni's test).

An identical BLA perfusion with 300 nM clobenpropit, another H_3 receptor antagonist (VanderGoot *et al.*, 1992), caused a 62 ± 10% maximal decrease of ACh spontaneous release (Fig. 5). Eventually, ACh release was restored to control levels during subsequent perfusion with control medium. The spontaneous release of ACh averaged 0.28 ± 0.05 pmol/20 min ($n = 4$). As with thioperamide, there was a time delay between the onset of drug perfusion and the achievement of the maximal drug effect, that occurred in the second 20-min fraction collected after clobenpropit was withdrawn from the perfusing medium (Fig. 5).

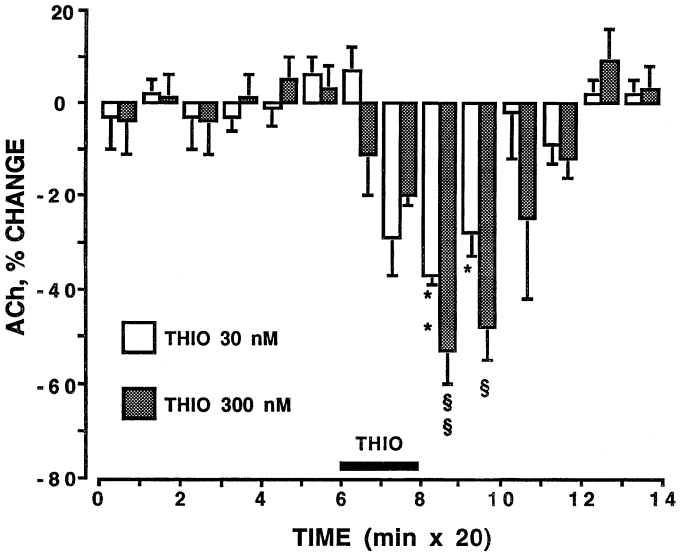

FIG. 4. Time course of thioperamide-induced inhibition of spontaneous ACh release from the BLA of freely moving rats. Acetylcholine was measured in fractions collected every 20 min, beginning 2 h after the onset of the perfusion. The control value for spontaneous ACh release was calculated as in Fig. 2. and ACh release was expressed as percentage of its spontaneous release value. Thioperamide (THIO, 30 and 300 nM) was administered into the BLA through the dialysis probe at 120 min and stopped at 160 min. The mean spontaneous release was 0.29 ± 0.07 pmol/20 min in the experiments with 30 nM thioperamide, and 0.26 ± 0.05 pmol/20 min in those with 300 nM thioperamide. The black bar indicates the period of thioperamide application. Shown are means ± SEM of four rats in each group. §§, $^{**}P < 0.01$ and §, $^{*}P < 0.05$ vs. last sample before drug treatment (ANOVA and Scheffe's test).

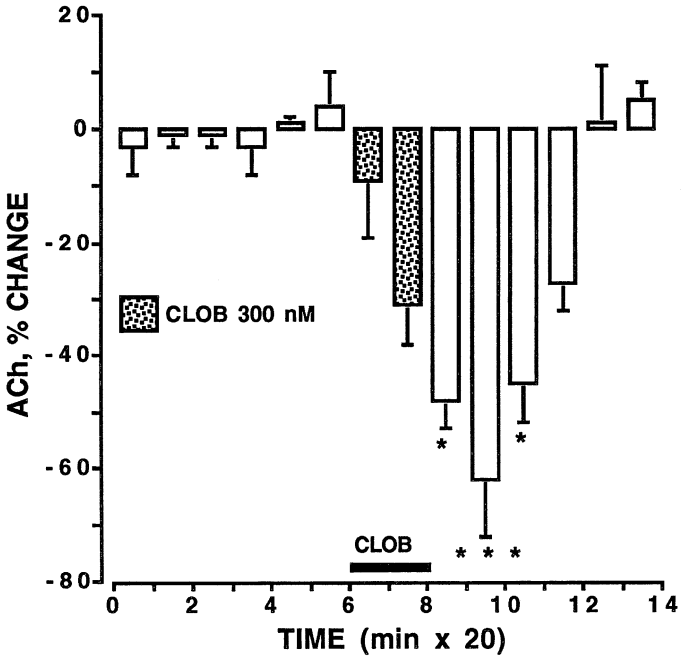

FIG. 5. Clobenpropit-induced inhibition of spontaneous ACh release from the BLA of freely moving rats. Acetylcholine was measured in fractions collected every 20 min, beginning 2 h after the onset of the perfusion. After 120 min, clobenpropit (CLOB, 300 nM) was administered through the dialysing membrane for 40 min. Spontaneous ACh release was calculated for each experiment by averaging the mean of the four 20-min samples of perfusate collected before drug treatment. Acetylcholine release was expressed as percentage of its spontaneous release value. The mean spontaneous release averaged 0.28 ± 0.05 pmol/20 min. The black bar indicates the period of clobenpropit application. Shown are means ± SEM of four rats. $^{***}P < 0.001$ and $^{*}P < 0.05$ vs. the last sample before drug treatment (ANOVA and Scheffe's test).

A significant decrease in spontaneous ACh release from the BLA was also observed following the 40 min perfusion with medium containing 50 nM ciproxifan, another potent and selective H_3 receptor antagonist (Ligneau *et al.*, 1998). Ciproxifan elicited a 60 ± 6% maximal decrease of spontaneous ACh release with a time course similar to that obtained with both thioperamide and clobenpropit (Fig. 6). As for thioperamide and clobenpropit, the ciproxifan effect was completely reversible (Fig. 6). The spontaneous release of ACh was 0.6 ± 0.2 pmol/20 min ($n = 3$).

H_2 receptor antagonism increased the spontaneous release of ACh and antagonized the inhibition produced by H_3 receptor antagonists

The effect of blocking H_2 receptors on ACh spontaneous release was tested by perfusing the BLA with 100 μM cimetidine, a selective H_2 antagonist (Durant *et al.*, 1977). After collecting four 20-min fractions, cimetidine was perfused for 40 min (Fig. 7). Cimetidine produced a small but significant increase in spontaneous ACh release in the second 20-min fraction after the onset of BLA perfusion with this H_2 antagonist (Fig. 7). ACh release was restored to control levels during subsequent perfusion with control medium (Fig. 7). The spontaneous ACh release averaged 0.3 ± 0.03 pmol/20 min ($n = 12$).

Figures 8 and 9 show the time course of ACh release from the BLA evoked by the local administration of thioperamide or clobenpropit, respectively, in the presence of cimetidine. After collection of four 20-min baseline samples, 100 μM cimetidine was added to the perfusing medium for 40 min, and was then administered in combination with either 300 nM thioperamide (Fig. 8) or 300 nM clobenpropit (Fig. 9) for an additional 40 min. Medium containing 100 μM cimetidine was then restored for the following 40 min. Washout with control medium was carried out for the final four 20-min fractions. In the presence of cimetidine, the inhibitory effect of thioperamide on ACh release was completely abolished (Fig. 8). The data shown in Fig. 8 A were compared with those presented in Fig. 4. There was a significant effect of both concentration–time interaction ($F_{1,98} = 3.789$, $P < 0.0001$) and drug treatment ($F_{1,13} = 2.723$, $P < 0.0001$) (Two-way ANOVA). Both ninth and tenth 20-min fractions shown in Fig. 8 were significantly different when compared to the corresponding 20-min fractions of Fig. 4 ($P < 0.001$, Bonferroni's test).

A similar effect was observed when clobenpropit was infused into the BLA in the presence of cimetidine. Figure 9 shows that the inhibitory effect of clobenpropit (300 nM) on ACh release was fully counteracted by cimetidine (100 μM). The data shown in Fig. 9 were compared with those presented in Fig. 5. There was a significant effect of both concentration–time interaction ($F_{1,112} = 7.105$, $P < 0.0001$) and drug treatment ($F_{1,13} = 2.582$, $P < 0.0001$) (Two-way ANOVA). Both ninth ($P < 0.01$, Bonferroni's test) and tenth ($P < 0.001$, Bonferroni's test) 20-min fractions shown in Fig. 9 were significantly different when compared to the corresponding 20-min fractions of Fig. 5. Spontaneous ACh release averaged 0.42 ± 0.05 pmol/20min ($n = 5$) in the experiments with thioperamide and cimetidine, and 0.45 ± 0.05 pmol/20 min ($n = 6$) in those with clobenpropit and cimetidine.

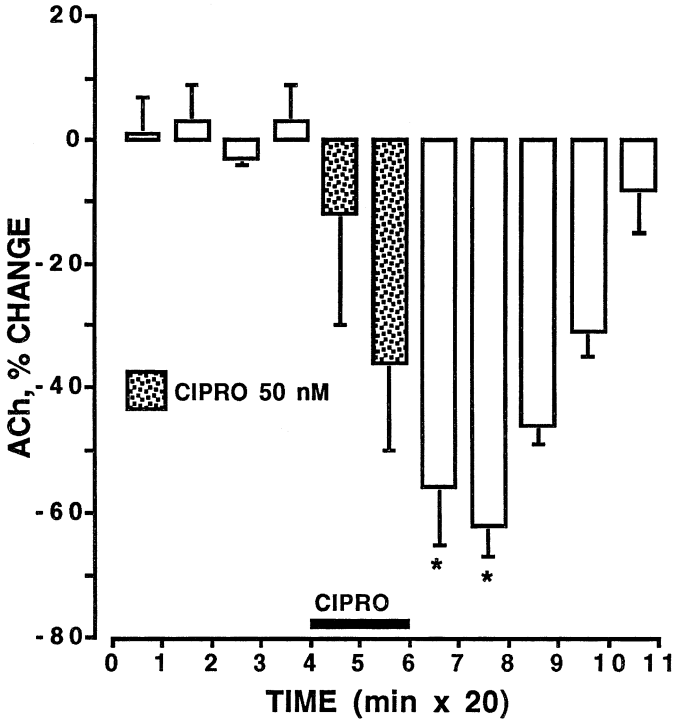

FIG. 6. Ciproxifan-induced inhibition of spontaneous ACh release from the BLA of freely moving rats. Acetylcholine was measured in fractions collected every 20 min, beginning 2 h after the onset of the perfusion. After 80 min, ciproxifan (CIPRO 50 nM) was added to the perfusing medium and administered for 40 min. The control value for spontaneous ACh release was calculated as in Fig. 2. and ACh release was expressed as percentage of its spontaneous release value. The mean spontaneous release averaged 0.6 ± 0.2 pmol/20 min. The black bar indicates the period of ciproxifan application. Shown are means ± SEM of three rats. $*P < 0.05$ vs. last sample before drug treatment (ANOVA and Scheffe's test).

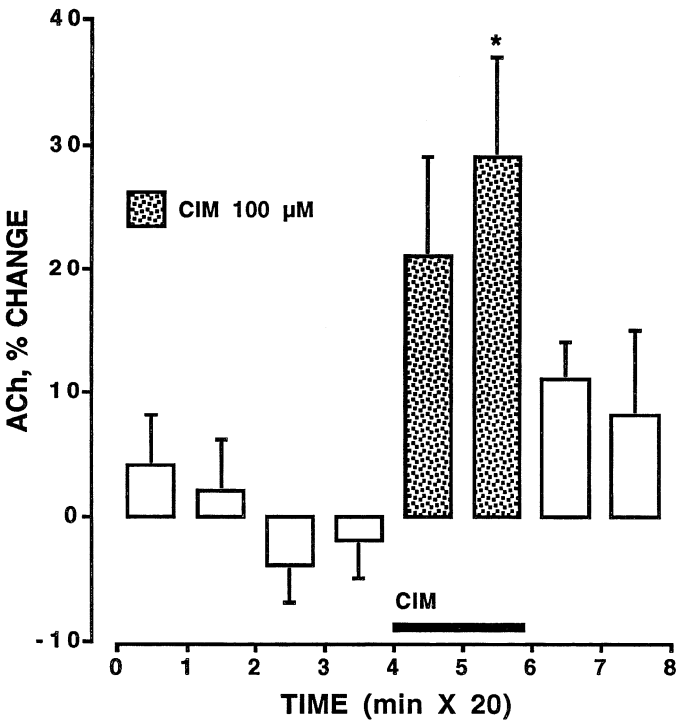

FIG. 7. Effect of cimetidine on spontaneous ACh release from the BLA of freely moving rats. Acetylcholine was measured in fractions collected every 20 min, beginning 2 h after the onset of the perfusion. Cimetidine (CIM, 100 µM) was added to the perfusing medium after collecting four 20-min fractions, and administered for the following 40 min as indicated by the bar. The control value for spontaneous ACh release was calculated as in Fig. 2, and ACh release was expressed as percentage of its spontaneous release value. The mean spontaneous release averaged 0.3 ± 0.03 pmol/20 min ($n = 12$). The black bar indicates the period of cimetidine application. Shown are means ± SEM of twelve rats. $*P < 0.05$ vs. last sample before drug treatment (ANOVA and Scheffe's test).

Effects of H_1 receptor antagonism on spontaneous release of ACh

Introduction of 300 nM triprolidine, an H_1 receptor antagonist (Ison & Casy, 1971), into the BLA perfusing medium had no significant effect on spontaneous ACh release. After collection of four 20-min baseline samples (spontaneous release was 0.17 ± 0.02 pmol/20 min; $n = 3$), triprolidine was administered through the dialysis membrane for 40 min. Following the drug perfusion period, three 20-min control samples were collected during washout. The changes in spontaneous ACh release were always within the range of variability (less than ± 20%) observed between individual 20-min collection periods during BLA perfusion with control medium (data not shown).

Memory for contextual fear conditioning was impaired by post-training infusion of H_3 receptor antagonists and scopolamine in the BLA

In these experiments, we examined the effects of post-training bilateral injections into the BLA of scopolamine, a muscarinic antagonist, thioperamide or clobenpropit on contextual fear conditioning. Figure 10 shows a representative location of the needle tips of an operated animal. When either one or both cannulae were outside the ranges outlined in Fig. 1, the animals were discarded. Each drug was diluted in saline to permit the bilateral intra-BLA injection of a constant volume (0.5 µL) to each rat. As 50 µg of scopolamine injected into the rat amygdala impaired working memory in the double-Y maze (Ingles *et al*., 1993), the same dose was used in this study. On the other hand, the doses of thioperamide and clobenpropit injected were based upon the results of the microdialysis experiments. For example, 0.5 µL of a 300-nM solution of thioperamide corresponded to a total amount of 44 pg, and a 0.5-µL of a 3-µM solution of clobenpropit corresponded to 464 pg. During training, there were no differences in the level of freezing between the control rats (to be injected with saline) and those to be injected with drugs after training (data not shown). Rats that received bilateral intra-BLA infusions of the histaminergic compounds failed to show any modification in their behaviour (locomotor and exploratory activities, grooming, yawning and rearing) during the three days between training and testing. Analysis of variance on the freezing behaviour revealed a significant treatment effect ($F_{7,67} = 5.419$, $P < 0.0001$; Fig. 11). Neuman–Keuls *post hoc* analysis showed that rats receiving 44 pg thioperamide ($n = 9$), 4.4 pg thioperamide ($n = 9$), 464 pg clobenpropit ($n = 9$) and 50 µg scopolamine ($n = 8$) spent significantly less time freezing than saline-injected controls ($n = 11$; $P < 0.01$ in all cases). There were no significant differences between animals treated with 0.44 pg thioperamide ($n = 9$), 46.4 pg clobenpropit ($n = 8$) and 10 µg scopolamine ($n = 6$) and controls.

Discussion

The results of the present study provide the first evidence that intra-BLA administration of histaminergic compounds alters the rate of

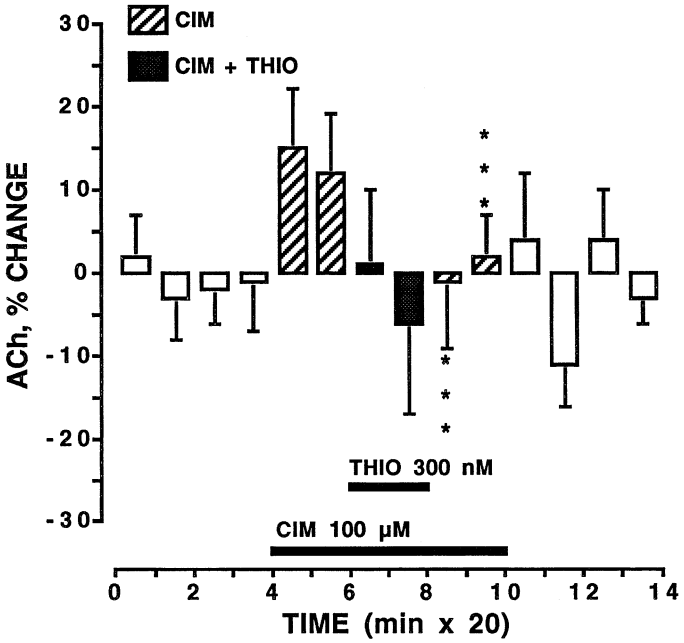

FIG. 8. Reversal by cimetidine of thioperamide induced inhibition of ACh release from the BLA of freely moving rats. Acetylcholine was measured in fractions collected every 20 min, beginning 2 h after the onset of the perfusion. Spontaneous ACh release was calculated for each experiment by averaging the mean of the four 20-min samples of perfusate collected before drug treatment. Thioperamide (300 nM) was administered at 120 min into the BLA through the dialysis probe for 40 min in the presence of cimetidine (100 µM), that was added to amygdala-perfusing medium 40 min before administration of thioperamide, and kept in the medium for 40 additional min after thioperamide was withdrawn. Acetylcholine release was expressed as percentage of its spontaneous release value. Spontaneous ACh release was 0.42 ± 0.05. The black bars indicate the period of drug applications. Each point represents the mean value ± SEM of five rats. Statistical comparison was performed with the experiments shown in Fig. 4. There was a significant effect of concentration–time interaction ($F_{1,98}$ = 3.789, $P < 0.0001$) and of drug treatment ($F_{1,13}$ = 2.723, $P < 0.0001$) (Two-way ANOVA). ***$P < 0.001$ vs. corresponding 20-min fractions shown in Fig. 4. (Bonferroni's test).

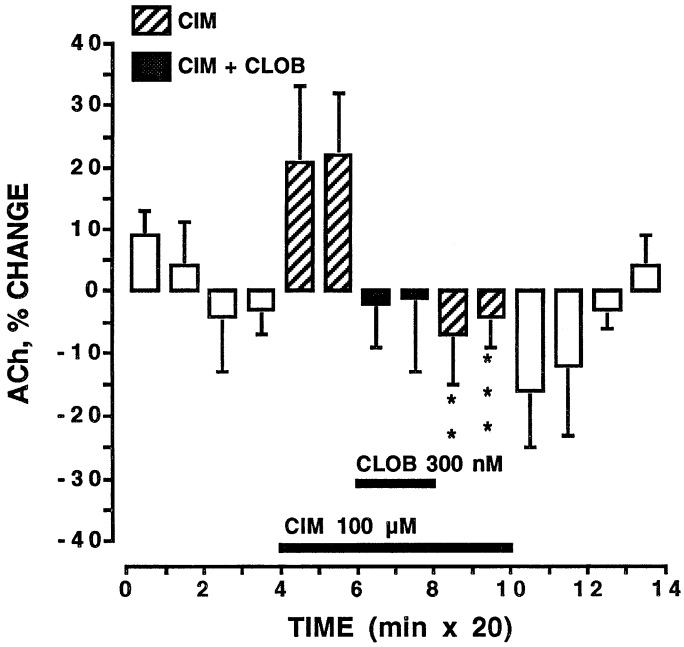

FIG. 9. Reversal by cimetidine of clobenpropit induced inhibition of ACh release from the BLA of freely moving rats. Acetylcholine was measured in fractions collected every 20 min, beginning 2 h after the onset of the perfusion. Spontaneous ACh release was calculated for each experiment by averaging the mean of the four 20-min samples of perfusate collected before drug treatment. Clobenpropit (300 nM) was administered at 120 min into the BLA through the dialysis probe for 40 min in the presence of cimetidine (100 µM), that was added to amygdala-perfusing medium 40 min before administration of clobenpropit, and kept in the medium for 40 additional min after clobenpropit was withdrawn. Acetylcholine release was expressed as percentage of its spontaneous release value. Spontaneous ACh release was 0.45 ± 0.05. The black bars indicate the period of drug applications. Each bar represents the mean value ± SEM of six rats. Statistical comparison was performed with the experiments shown in Fig. 5. There was a significant effect of concentration–time interaction ($F_{1,112}$ = 7.105, $P < 0.0001$) and of drug treatment ($F_{1,13}$ = 2.582, $P < 0.0001$) (Two-way ANOVA). **$P < 0.01$ and ***$P < 0.001$ vs. corresponding 20-min fractions shown in Fig. 5 (Bonferroni's test).

spontaneous ACh release and impairs memory for contextual fear conditioning. The BLA receives cholinergic innervation from the nucleus basalis magnocellularis (NBM) (Mesulam *et al.*, 1983; Carlsen *et al.*, 1985). Only sparse cholinergic cell bodies are found in the anterior amygdala (Nitecka & Frotscher, 1989). The use of the microdialysis technique was validated by perfusing the BLA with 100 mM KCl to increase BLA ACh extracellular levels. This concentration of KCl may appear high, however, low potassium recovery through the microdialysis membrane, and its rapid dilution into the extracellular space after crossing the membrane necessitate high concentrations in the perfusion medium (Westerink & DeVries, 1996). Moreover, administration of TTX decreased extracellular ACh levels by about 50%, thus indicating the presence of spontaneous impulse activity in NBM–BLA cholinergic neurons at rest.

The use of cholinesterase inhibitors may be criticized because they may alter modulation of ACh release, as in the case of dopamine on striatal ACh release (DeBoer & Abercrombie, 1996). However, the amygdala, as well as the cortex (Herrera-Marschitz *et al.*, 1990), release much lower levels of ACh compared to the striatum, thus requiring the presence of cholinesterase inhibitors in the perfusion fluid. It is noteworthy that concentrations of neostigmine up to 0.5 µM failed to alter cortical ACh release patterns qualitatively in response to behavioural activation (Himmelheber *et al.*, 1998).

Inhibition of ACh release caused by thioperamide, clobenpropit or ciproxifan appears to be mediated by H_3 receptors localized in the BLA. This release was inhibited by perfusion with medium containing 30 and 300 nM thioperamide or 300 nM clobenpropit, both competitive antagonists at H_3 receptors with pA_2 values in cortical slices of 8.96 (Arrang *et al.*, 1987a) and 9.5 (Kathmann *et al.*, 1993). Another highly selective H_3-antagonist, ciproxifan (Ligneau *et al.*, 1998) – about 10 × more potent than thioperamide – at 50 nM, caused a decrease in amplitude and time course of ACh release similar to that produced by thioperamide or clobenpropit. The involvement of H_3 receptors is further demonstrated by the presence of H_3 receptor binding (Pollard *et al.*, 1993) and with the distribution of mRNA encoding for H_3 receptors (Lovenberg *et al.*, 1999) in the BLA. H_3 receptors, initially detected as presynaptic autoreceptors inhibiting histamine release (Arrang *et al.*, 1983; Arrang *et al.*, 1987b), were shown subsequently to act also as presynaptic heteroreceptors in non-histamine-containing neurons (for a review, see Hill *et al.*, 1997). H_3 receptors are G-protein-coupled receptors that inhibit cAMP formation (Lovenberg *et al.*, 1999). However, the

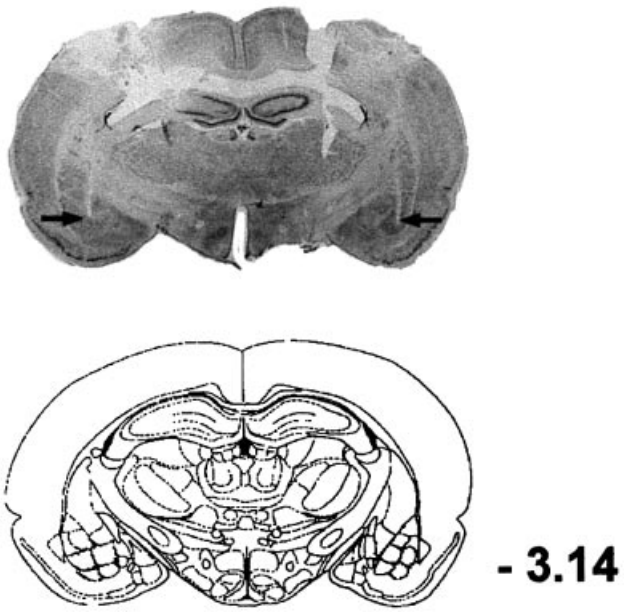

FIG. 10. Bilateral placement of injecting needles in the BLA. The photomicrograph is representative of an operated brain. Arrows indicate the end of the bilateral needle tracks. The number indicates the relative position of the coronal section posterior to bregma according to Paxinos & Watson (1982).

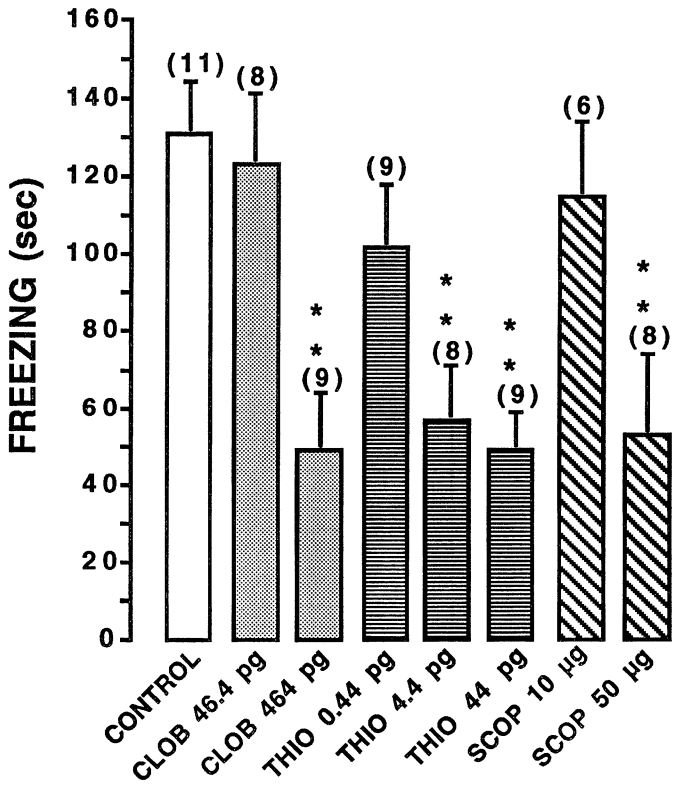

FIG. 11. Effects of post-training bilateral injections of H_3 receptor antagonists or scopolamine, into the BLA, on contextual fear conditioning. Rats were injected immediately after training under general anaesthesia (ketamine, 100 mg/kg i.p.). Each drug was freshly prepared and diluted in sterile saline to permit the injection of a constant volume of 0.5 µL per side to each rat. A 300-nM solution of thioperamide corresponded to 44 pg/0.5 µL, and a 3-µM solution of clobenpropit to 464 pg/0.5 µL. Solutions were injected over a 1–2-min period and the needle was left in place for 1 min before withdrawing it. Controls were injected with saline. Seventy-two h after training, freezing was measured during the first 3-min period of the re-testing. Each bar represents the mean value + SEM of (*n*) rats. $^{**}P < 0.01$ vs. control (ANOVA and Neuman-Keuls's test).

identification of isoforms with distinct CNS expression profiles and coupling to adenylylcyclase or MAP-kinase signalling pathways (Drutel *et al*. 2001), adds more complexity to the understanding of their functions. Pretreatment with cimetidine, at a concentration sufficient to fully block H_2 receptors (Durant *et al*., 1977), completely abolished the effect of thioperamide and clobenpropit. These observations, together with the demonstration that, in the BLA H_3-receptor binding is strictly associated with the presence of histaminergic fibers (Anichtchik *et al*. 2000), suggest a role for endogenous histamine in the control of BLA ACh release. The inhibition elicited by H_3-antagonists could be most simply explained by a blockade of H_3 autoreceptors, which is accompanied by an increase in endogenous histamine release (Arrang *et al*., 1983). Histamine, activating postsynaptic H_2 receptors, might inhibit ACh release. The BLA receives the most abundant histaminergic innervation in the brain (Ben-Ari *et al*., 1977), and displays both high H_2-receptor binding and its gene transcripts (Vizuete *et al*., 1997). The anatomical arrangement of H_2 receptors in the amygdala is unknown. Several hypotheses based on the intracellular cascade of events brought about when activating H_2 receptors can be proposed to explain inhibition of ACh release. H_2 receptors are positively coupled with adenylylcyclase (Johnson, 1982) and reduce a calcium-activated potassium current in the hippocampus, thus increasing the amplitude of population spikes (Haas, 1984; Haas & Greene, 1986). However, they also negatively modulate outward currents through phosphorylation of Kv3.2-containing potassium channels, hence lowering the neuronal maximal firing frequency (Atzori *et al*. 2000). They also elicite inhibitory postsynaptic potentials in supraoptic oxytocin-neurons, presumably by opening chloride channels (Yang & Hatton, 1994). Therefore, H_2 receptors might inhibit ACh release directly, acting at BLA cholinergic terminals, or indirectly through activation of inhibitory interneurons, largely present in the amygdala (Kemppainen & Pitkånen, 2000). Interestingly, histamine inhibition of amygdaloid kindled seizures (Ishizawa *et al*. 2000) and histaminergic inhibition of cortical ACh release both involve GABAergic transmission (Giorgetti *et al*., 1997).

Conversely, modulation of ACh release from the amygdala did not involve H_1 receptors, as perfusion with the H_1-antagonist, triprolidine at a concentration sufficient to fully occupy H_1 receptors (Ison & Casy, 1971), failed to modify ACh release.

In addition to preventing H_3-antagonist-elicited inhibition, administration of cimetidine alone increased ACh spontaneous release, thus indicating the occurrence of a histaminergic tonic inhibition. However, this effect was rather weak, suggesting a minor participation of endogenous histamine in the tonic regulation of ACh release. This finding seems to be in contrast with the hypothesis that the robust decrease in ACh release elicited by H_3 antagonists was due to antagonism of endogenous histamine. Recent evidence demonstrates that native brain H_3 receptors, analogously with other metabotropic receptors (Lefkowitz *et al*., 1993), exhibit spontaneous receptor activity, undergoing an allosteric conformation change capable of binding G-proteins in the absence of agonists (Morisset *et al*. 2000). This activity is accompanied by the occurrence of inverse agonism exerted by thioperamide and ciproxifan (Morisset *et al*. 2000). Clobenpropit also behaves as an inverse agonist (Professor R. Leurs, Leiden University/Vrije Universiteit, Amsterdam, NL, personal communication). Therefore, in our experiments, antagonism of H_3-

autoreceptor stimulation by endogenous histamine might have only partially contributed to the potential histamine releasing effect of these drugs.

This is the first report showing that intra-BLA post-training injection of scopolamine produced an amnesic effect on fear conditioning. This indicates a crucial role for the amygdaloid cholinergic system in the retention of fear memories, and is consistant with earlier observations of the effects of intra-amygdala infusion of oxotremorine, alone or associated with atropine, on memory storage (Dalmaz *et al*., 1993; Introini-Collison *et al*., 1996; Vazdarjanova *et al*., 1999). Moreover, if cognitive deficits are related to reduced availability of ACh in the synaptic cleft (Quirion *et al*., 1995), and H_3-antagonists reduce ACh release, the same compounds would be expected to impair cognition. This study substantiates this prediction; intra-BLA injections of thioperamide and clobenpropit impair retention for a contextual fear-conditioned response. Post-training administration of these compounds excludes any influence of the treatment on acquisition and on other processes that indirectly affect learning (McGaugh & Izquierdo, 2000), thus suggesting that H_3-antagonists directly modulate memory consolidation processes. Amnesic animals displayed residual freezing, which is in agreement with previous observations indicating that Pavlovian fear conditioning may be still acquired when either major intra-BLA circuits (Selden *et al*., 1991; Vazdarjanova & McGaugh, 1999), or the whole amygdala (Cahill *et al*. 2000) have been damaged.

We have reported previously that unoperated rats and ketamine-anaesthetized rats that received intra-amygdala saline injections exhibited similar freezing responses to context and acoustic conditioned stimuli (Sacchetti *et al*., 1999), thus suggesting that post-trial ketamine anaesthesia and surgical procedures do not influence negatively avoidance responses by interfering with some late stages of memory consolidation. In this regard, it is noteworthy that frequency-specific receptive field plasticity induced by pavlovian fear conditioning was expressed in the brain of ketamine-anaesthetized animals (Lennartz & Weinberger, 1992). Therefore, it is conceivable that under our experimental conditions, the amnesic effects caused by H_3 antagonists and scopolamine are independent of ketamine-induced NMDA blocking action. As H_3-antagonists cause amnesia and reduction of ACh release in the same dose range, it is conceivable that memory impairment occurs as a consequence of cholinergic tone inhibition. This finding seems difficult to reconcile with reported pro-cognitive effects of thioperamide and clobenpropit in a passive avoidance response and in object recognition (Meguro *et al*., 1995; Giovannini *et al*., 1999). However, these tests require an intact cholinergic system (Goldman-Rakic, 1987; Petrides, 1994) in the cortex. Regional differences in cytoarchitectonic and H_3-receptor location may explain the discrepancy. Functional evidence suggests that H_3 receptors are not localized on cortical cholinergic terminals in the cortex (Arrang *et al*., 1995; Blandina *et al*., 1996; Bacciottini *et al*., 1999). Consistently, very low expression of H_3 receptor-encoding mRNA has been detected in the basal forebrain nuclei from which cholinergic neurons project to the amygdala, the cortex and the hippocampus (Drutel *et al*. 2001). If so, the effects of pharmacological manipulation of H_3 receptors on ACh release might be indirect, and vary according to the regional difference of receptor arrangements. Indeed, perfusion of the rat cortex with clobenpropit (Blandina *et al*., 1996) or thioperamide (Giorgetti *et al*., 1996) failed to alter directly spontaneous ACh release.

Consistent with previous findings (Vazdarjanova *et al*., 1999), this study stresses the importance of BLA muscarinic receptors for consolidation of fear memories, and strongly suggests that the histaminergic system influences this process by regulating cholinergic activity within the BLA. The same mechanism has been proposed for other neurotransmitters (Introini-Collison *et al*., 1996). The histaminergic perikarya are found exclusively in the posterior hypothalamus (Panula *et al*., 1984; Watanabe *et al*., 1984), from where they project to all parts of the CNS, including the BLA (Inagaki *et al*., 1988; Panula *et al*., 1989). The hypothalamus may integrate and coordinate the behavioural expression of affective states. Thus, it is conceivable that histaminergic neurons might represent at least one of the neural bases for hypothalamic influences in the storage of emotionally based memories, providing the fine-tuning to ACh release necessary to produce the adequate behavioural response.

Acknowledgements

This work was supported by grants 40% and 60% from M.U.R.S.T- Universitá di Firenze (Italy). The authors thank Prof. Rob Leurs and Prof. Henk Timmerman for the gift of clobenpropit as well as Prof. Walter Schunack for providing ciproxifan. We are grateful to Prof. Joseph Goldfarb and Prof. Giancarlo Pepeu for reading the manuscript.

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