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Endogenous histamine in the medial septum–diagonal band complex increases the release of acetylcholine from the hippocampus: a dual-probe microdialysis study in the freely moving rat

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Abstract

The effects of histaminergic ligands on both ACh spontaneous release from the hippocampus and the expression of *c-fos* in the medial septum–diagonal band (MSA-DB) of freely moving rats were investigated. Because the majority of cholinergic innervation to the hippocampus is provided by MSA-DB neurons, we used the dual-probe microdialysis technique to apply drugs to the MSA-DB and record the induced effects in the projection area. Perfusion of MSA-DB with high-KCl medium strongly stimulated hippocampal ACh release which, conversely, was significantly reduced by intra-MSA-DB administration of tetrodotoxin. Histamine or the H_2 receptor agonist dimaprit, applied directly to the hippocampus, failed to alter ACh release. Conversely, perfusion of MSA-DB with these two compounds increased ACh release from the hippocampus. Also, thioperamide and ciproxifan, two H_3 receptor antagonists, administered into MSA-DB, increased the release of hippocampal ACh, whereas R-α-methylhistamine, an H_3 receptor agonist, produced the opposite effect. The blockade of MSA-DB H_2 receptors, caused by local perfusion with the H_2 receptor antagonist cimetidine, moderated the spontaneous release of hippocampal ACh and antagonized the facilitation produced by H_3 receptor antagonists. Triprolidine, an H_1 receptor antagonist, was without effect. Moreover, cells expressing c-fos immunoreactivity were significantly more numerous in ciproxifan- or thioperamide-treated rats than in controls, although no colocalization of anti-c-fos and anti-ChAT immunoreactivity was observed. These results indicate a role for endogenous histamine in modulating the cholinergic tone in the hippocampus.

Introduction

Hippocampal ACh release increases during various behaviours, such as sensory stimulation (Nilsson *et al*., 1990; Inglis & Fibiger, 1995), feeding and drinking (Mark *et al*., 1992), wakefulness (Mizuno *et al*., 1994), exploratory activity (Aloisi *et al*., 1997; Ceccarelli *et al*., 1999) and contextual fear conditioning (Nail-Boucherie *et al*., 2000). The functional significance of these increases is not clear yet; nevertheless, basic and clinical studies have long recognized the importance of cholinergic mechanisms operating within the medial septum–banda diagonalis (MSA-DB) complex in cognitive processes (Everitt & Robbins, 1997; Pepeu & Blandina, 1998). As a general rule, increases of hippocampal ACh release improved performances in hippocampal-related memory tasks (Givens & Olton, 1994; Givens & Sarter, 1997; Dickinson-Anson *et al*., 1998). Conversely, lesions of the fimbria–fornix, which conveys MSA-DB cholinergic fibres to the hippocampus (Lewis & Shute, 1967), impaired performances in the same tasks (Brito & Brito, 1990), possibly by preventing behaviour-induced increases of hippocampal ACh (Nilsson *et al*., 1990). Indeed, hippocampal ACh release depends upon impulse flow in neurons localized in the MSA-DB, which provide the cholinergic innervation to the hippocampus (Mesulam *et al*., 1983; Nicoll, 1985; Gaykema *et al*., 1990). Several neurotransmitters modulate the activity of these neurons, thus influencing learning and memory processes (Decker & McGaugh, 1991; Moor *et al*., 1998; Passani & Blandina, 1998). The current study focuses on the neuromodulatory action of histamine, which is released from axon varicosities of neurons located in the tuberomammillary nucleus of the hypothalamus (Panula *et al*., 1984; Watanabe *et al*., 1984). These cells send efferents to brain regions (Takeda *et al*., 1984; Wada *et al*., 1991) and, in the MSA-DB, histaminergic projections create dense networks of fibres (Inagaki *et al*., 1988; Panula *et al*., 1989). Histamine exerts its effects in the CNS by binding to its own specific receptors H_1, H_2 and H_3, and to the polyamine site on the NMDA receptor complex (Brown *et al*., 2001). An early study indicates that ACh release from the CA1–CA3 region of hippocampus of urethane-anaesthetized rats could be modulated by endogenous histamine (Mochizuki *et al*., 1994). Indeed, an electrical stimulation applied to the tuberomammilary nucleus greatly increased histamine and ACh release from both MSA-DB and hippocampus. ACh release was inhibited by i.p. administration of zolantidine, an H_2 receptor antagonist (Calcutt *et al*., 1988), but not of mepyramine, an H_1 receptor antagonist (Ison & Casy, 1971), thus suggesting an H_2 receptor involvement. However, systemic drug injections do not reveal sites of action, whereas

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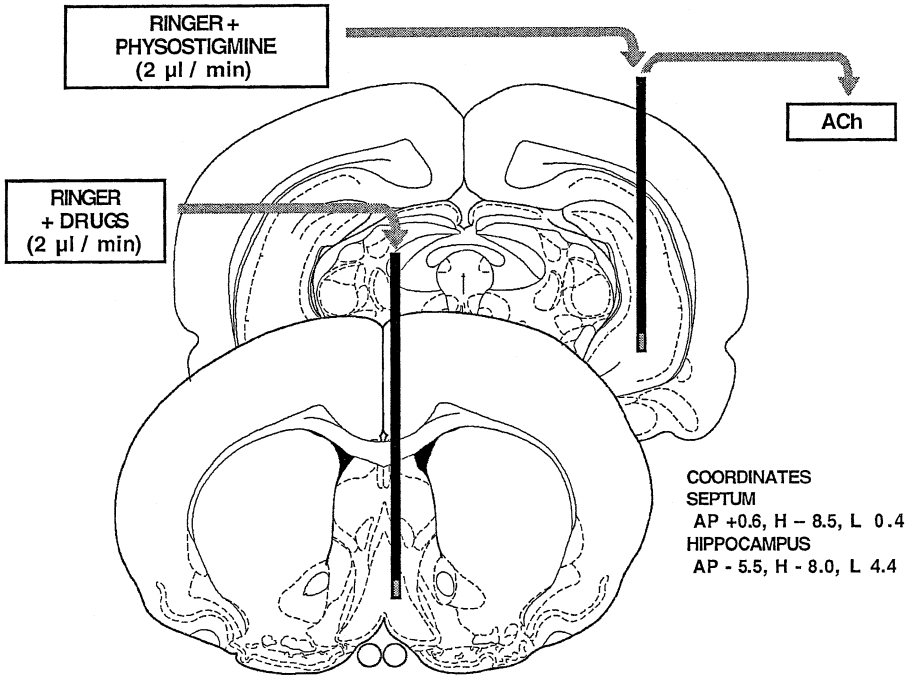

FIG. 1. Schematic diagram showing the position of the dialysis membrane in the MSA-DB and the hippocampus.

localized infusions of drugs to specific brain regions provide evidence of their site of action. In this study we used the dual-probe microdialysis technique to apply histaminergic drugs to the MSA-DB complex and to record the induced effects on ACh release from the projection area, the hippocampus, of freely moving rats. Moreover, it was investigated whether local administration of histaminergic drugs influenced c-fos protooncogene expression. c-Fos-like immunoreactivity may identify areas of neuronal activity and map functionally related neuronal pathways. Some of these results have been reported in abstract form (Bacciottini *et al*., 1999).

Materials and methods

Animal housing

Male Wistar rats (225–275 g body weight) 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, USA). Each rat was implanted with vertical microdialysis probes in both the MSA-DB to deliver drugs locally, and the ipsilateral hippocampus to measure the output of ACh (Fig. 1). In a subset of experiments rats were implanted with a single probe in the hippocampus to investigate the effects of local drug administration on hippocampal ACh release. The microdialysis tubes were made of AN 69 membrane (Dasco, Italy), 220 µm internal diameter and 310 µm external diameter, molecular weight cut-off 15 000 Da. The coordinates used for the implantation of the microdyalisis tubes were as follows: for the hippocampus AP –5.5 mm, L 4.4 and H –8 mm from bregma; for the MSA-DB AP –0.6 mm, L 0.4 and H –8.5 mm. All coordinates (Paxinos & Watson, 1982) were measured on the bone surface and referred to bregma, with bregma and lambda on a horizontal plane. Both cannulae were fixed to the skull with acrylic dental cement. After surgical manipulations rats were replaced in cages (one rat per cage) to recover from surgery.

The microdyalisis experiments were performed 24 h after surgery. The microdialysis probes were perfused at a rate of 2 µL/min using a microperfusion pump (Carnegie Medicine, mod. CMA/100, Sweden) with Ringer's solution (in mM: NaCl, 147; $CaCl_2$, 2.5; KCl, 3.0; pH 7.0). To recover detectable dialysate concentrations of ACh, a cholinesterase inhibitor (physostigmine sulphate, 7 µM) was added to the medium perfusing the hippocampus, but not to that perfusing the MSA-DB. Solutes < 15.000 Da molecular weight 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 dissolved into the MSA-DB perfusing medium. Drug addition did not modify the pH of the medium. After an equilibration period of 60 min, 40-µL fractions (resulting from 20-min intervals of perfusion) were collected. Rat MSA-DB was perfused with control medium in the first four fractions to measure hippocampal ACh spontaneous release; drugs were then added to the medium. Accurate placement of microdialysis membranes was verified post-mortem by observation under light microscopy visualization (Leitz, Dialva EB) of coronal sections cut through the probe paths. China ink (5 µL) was injected through the probes at the end of each experiment to rats anaesthetized with urethane (1.2 g/kg i.p.), before decapitation. Data from rats in which the membranes were not correctly positioned (fewer than 10% of the animals) were discarded.

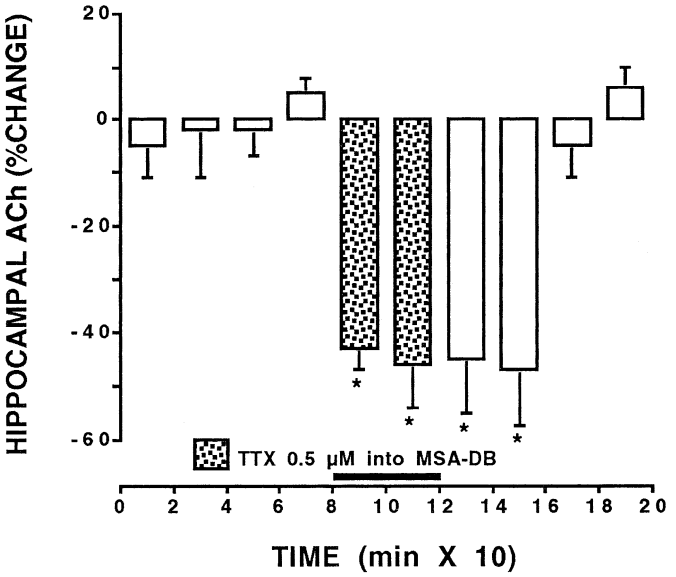

FIG. 2. Influence of administration into MSA-DB of TTX on spontaneous release of ACh from the hippocampus of freely moving rats. Twenty-four hours after implantation of the dialysis fibres, ACh was measured in hippocampal fractions collected every 20 min beginning 60 min after the onset of the perfusion. Spontaneous release of ACh was calculated for each experiment by averaging the mean of the four initially collected 20-min samples of perfusate, and ACh release was expressed as percentage of its spontaneous release value. At 40 min, 0.5 µM TTX was introduced into the MSA-DB perfusing medium for 40 min. The mean spontaneous release was 0.51 ± 0.21 pmol/20 min ($n = 4$). The bar shows the period of TTX application. Shown are means ± SEM of four experiments. *$P < 0.05$ vs. last sample before TTX treatment (ANOVA and Scheffé's test).

Assay and quantification of ACh

ACh was determined by HPLC-electrochemical detection as described previously (Giorgetti *et al*., 2000). The HPLC apparatus consisted of a pump (Mod. 1350, Bio-Rad, Richmond, CA, USA), a presaturation column (Chromspher 5 C18, 100 × 3 mm, Chrompack, Middleburg, The Netherlands), an injector (Mod. 7125, Rhehodyne, Cotati, CA, USA), a guard column (reverse phase), an analytical column (Chromspher 5 C18, 100 × 3 mm, Chrompack), an enzyme reactor (10 × 2.1 mm, Chrompack), an electrochemical detector (Mod. LC4C, BioAnalytical System, West Lafayette, IN, USA) and a Perkin Elmer chart recorder. Briefly, ACh was separated on the cation exchange column. ACh was hydrolysed by acetylcholinesterase to form acetate and choline in the postcolumn enzyme reactor, then choline was oxidized by choline oxidase to produce betaine and hydrogen peroxide. Hydrogen peroxide was detected with 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. The levels of ACh in the perfusates 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. The sensitivity limit was 500 fmol. ACh spontaneous release was calculated for each experiment by averaging the mean of the four initially-collected 20-min samples of perfusate. ACh release was expressed as a percentage of its spontaneous release value. All values are expressed as means ± SEM, and the number of experiments (n) is also indicated. The *in vitro* recovery of ACh from the dialysis membrane was ≈60% at room temperature. Values reported here are not corrected for recovery.

C-Fos procedure

In immunohistochemical experiments, drugs dissolved in physiological saline were injected under general anaesthesia (chloral hydrate, 400 mg/kg i.p.) in the MSA-DB 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 7.5 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-min period and the needle was left in place for 2 min before withdrawing it. Control groups received injections of saline. Two hours after drug injections, rats were perfused transcardially with ice-cold 4% paraformaldehyde. The brains were removed and postfixed in the 4% paraformaldehyde for 2 h and then immersed in 18% sucrose for 24 h. Coronal sections were cut at 40 µm on a freezing cryostat, and were collected in phosphate-buffered saline (PBS) until processed for immunohistochemistry. Free-floating sections were incubated in anti c-fos polyclonal antibodies 1 : 1500 in PBS containing 0.3% Triton X-100 (Sigma) and 5 mg/mL bovine serum albumin (BSA), overnight. The immunoreactive product was detected with the avidin–biotin peroxidase system (Vectastain kit; Vector Laboratories, Burlingame, CA, USA), as described previously (Giovannelli *et al*., 1990). After washing with PBS, sections were mounted on gelatin-coated slides, dehydrated in increasing concentrations of ethanol and coverslipped. In double labelling experiments, after c-fos immunostaining, sections were incubated in polyclonal anticholine acetyltransferase (ChAT) antibodies (Chemicon; Temecula, CA, USA) dissolved 1 : 1000 in PBS plus Triton-X 100 and 5 mg/mL BSA, overnight. Sections were washed in PBS, incubated in Cy3-conjugated antirabbit secondary antibodies (Chemicon), mounted on gelatin-coated slides and coverslipped with 70% glycerol in PBS. The fluorescent immunoreaction product was observed with a Zeiss microscope equipped for epifluorescence.

Chemicals

The substances used in this study included histamine hydrochloride, tetrodotoxin (Sigma Chemical Company Ltd, UK); dimaprit dihydrochloride, cimetidine (R)-α-methylhistamine dihydrochloride, physostigmine sulphate, thioperamide maleate, triprolidine dihydrochloride (R.B.I., Natick, MA, USA); ciproxifan was provided by Dr W. Schunack. All other reagents and solvents were of HPLC grade or the highest grade available (Sigma).

Statistical analysis

All values are expressed as means ± SEM and the number of experiments (n) is also indicated. Comparisons between two means were performed by Student's t-test. Differences among groups were first determined by a one-way or a two-way analysis of variance (ANOVA) followed by Bonferroni's or Scheffé's test, as appropriate. Scheffé's procedure for *post hoc* comparisons performed all possible comparisons of the means. For the purposes of clarity and biological relevance, we report in figures and figure legends only the significant differences vs. the last sample before drug treatment. For all statistical tests, $P < 0.05$ was considered significant. Statistical analysis was performed using StatView (Abacus Concepts, Inc., Berkeley, CA, USA) and GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

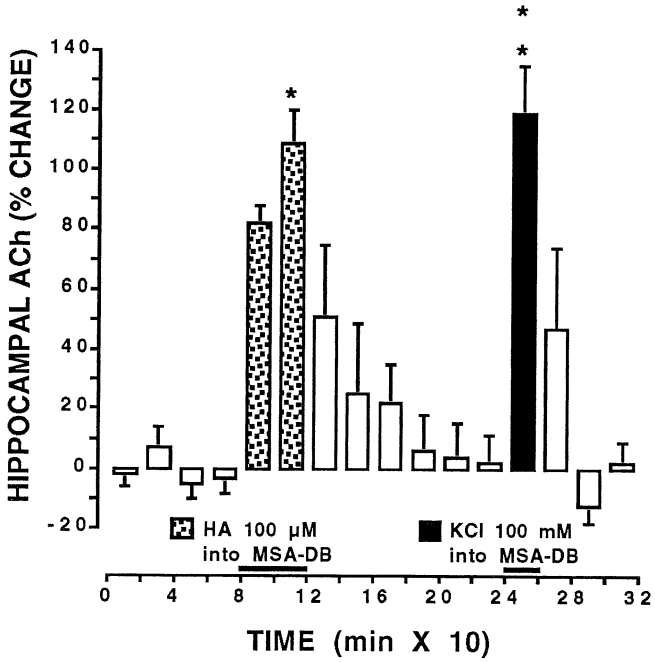

FIG. 3. Influence of administration into MSA-DB of histamine (HA) and 100 mM KCl on spontaneous release of ACh from the hippocampus of freely moving rats. Methods are identical to those described for Fig. 2. At 80 min, histamine (100 µM) was administered for 40 min through the dialysis fibre into the MSA-DB. At 240 min the NBM perfusing medium was changed from 4 to 100 mM KCl for 20 min. Isotonicity was mantained by reducing NaCl concentration. The mean spontaneous release was 0.64 ± 0.1 pmol/20 min (n = 4). The bars show the period of histamine and high-KCl medium applications. Shown are means ± SEM of four experiments. $*P < 0.05$ and $**P < 0.01$ vs. last sample before histamine application (ANOVA and Scheffé's test).

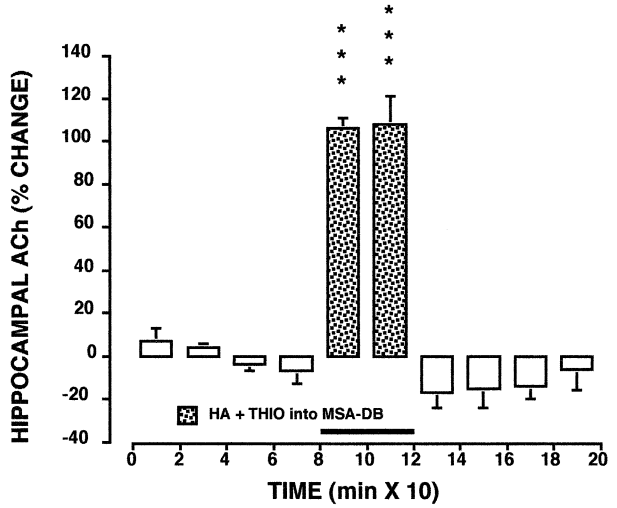

FIG. 4. Influence of simultaneous administration into MSA-DB of 100 µM histamine (HA) and 300 nM thioperamide on spontaneous release of ACh from the hippocampus of freely moving rats. Methods are identical to those described for Fig. 2. At 80 min, histamine (100 µM) and thioperamide (300 nM) were administered for 40 min through the dialysis fibre into the MSA-DB. The mean spontaneous release was 0.31 ± 0.03 pmol/20 min (n = 3). The bars show the period of histamine and thioperamide applications. Shown are means ± SEM of three experiments. $***P < 0.001$ vs. last sample before drug application (ANOVA and Scheffé's test).

Computerized counts of the c-fos-positive nuclei were performed with Image-Pro Plus 3.0 by one of the experimenters unaware of the animals' treatments. Numbers of c-fos-positive nuclei are averages of at least two consecutive sections.

Results

Effects of MSA-DB perfusion with medium containing 1 µM tetrodotoxin on ACh release from rat hippocampus

Twenty-four hours after surgery microdialysis probes were inserted in the implanted guide tubes both in the hippocampus and the MSA-DB. The one implanted in the hippocampus was perfused with Ringer's solution containing 7 µM physostigmine, whereas that implanted in the MSA-DB was perfused with Ringer's solution without physostigmine (Fig. 1). After 60 min of equilibration, the rat hippocampus spontaneously released ACh at stable rates, 0.79 ± 0.05 pmol/20 min (n = 62). Spontaneous release of ACh was calculated for each experiment by averaging the mean of the four initially-collected 20-min samples of hippocampal perfusate, and did not decrease significantly with time.

The infusion of 0.5 µM tetrodotoxin (TTX), a voltage-dependent Na^+-channel blocker, for 80 min into the MSA-DB through the dialysis probe reduced hippocampal ACh spontaneous release by ≈50% (Fig. 2). Spontaneous release averaged 0.51 ± 0.21 pmol/20 min (n = 4). After TTX was withdrawn from septum-perfusing medium, ACh release returned slowly toward basal values (Fig. 2). During the exposure to TTX the rats appeared to be more active but did not show any abnormal behaviour.

MSA-DB perfusion with medium containing 100 µM histamine or 100 mM KCl increased the spontaneous release of ACh from the hippocampus.

Introduction of 100 µM histamine into the medium perfusing the MSA-DB for 40 min increased significantly the release of ACh from the hippocampus (Fig. 3). The maximal effect (109 ± 11%) was achieved in the second 20-min fraction collected after onset of MSA-DB perfusion with histamine. The level of the spontaneous release of hippocampal ACh was restored after withdrawal of histamine from MSA-DB perfusion medium (Fig. 3). A depolarization, induced by perfusing the MSA-DB for 20 min with a medium containing 100 mM KCl, was conducted 120 min after the end of the perfusion with histamine, when hippocampal ACh release returned to basal levels (Fig. 3). It strongly stimulated the release of ACh from the hippocampus, producing a maximal increase of 119 ± 16%, which occurred in the 20-min fraction collected during the perfusion with 100 mM KCl. The level of the spontaneous release of hippocampal ACh was restored during the subsequent MSA-DB perfusion with control medium (Fig. 3). During the perfusion with high-KCl medium, rats appeared to be more active but did not show any abnormal behaviour. The two consecutive exposures to histamine and 100 mM KCl released similar maximal amounts of ACh, the mean ratio of histamine-evoked ACh/KCl-evoked ACh release being 0.9 ± 0.1 (n = 4). The spontaneous release of hippocampal ACh in these experiments averaged 0.64 ± 0.1 pmol/20 min (n = 4).

The pattern of ACh release in response to MSA-DB perfusion with 100 µM exogenous histamine did not change when thioperamide (300 nM) was infused into MSA-DB simultaneously with histamine (Fig. 4). It is noteworthy that exogenous histamine elicited maximal

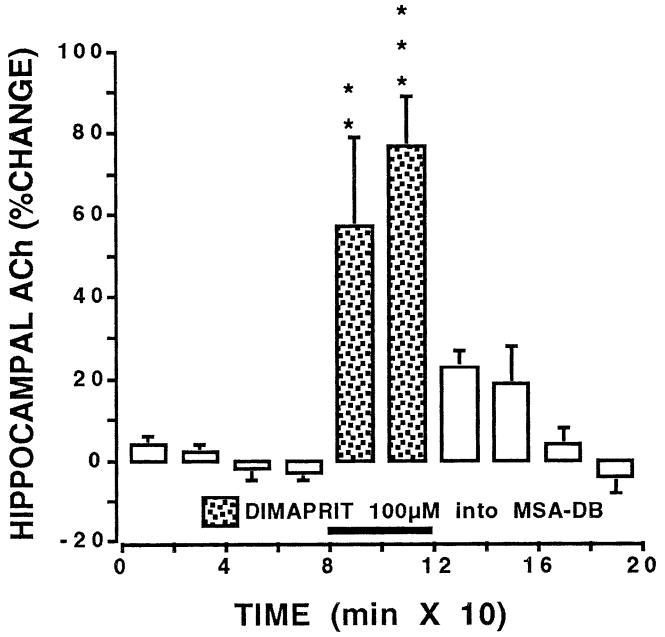

FIG. 5. Time course of release ACh from the hippocampus of freely moving rats evoked by administration into MSA-DB of dimaprit. Methods are identical to those described for Fig. 2. Dimaprit (100 μM) was administered at 80 min into the MSA-DB through the dialysis probe for 40 min. The mean spontaneous release was 0.63 ± 0.2 pmol/20 min (n = 6). The bar shows the period of dimaprit application. Shown are means ± SEM of six experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. last sample before drug treatment (ANOVA and Scheffé's test)

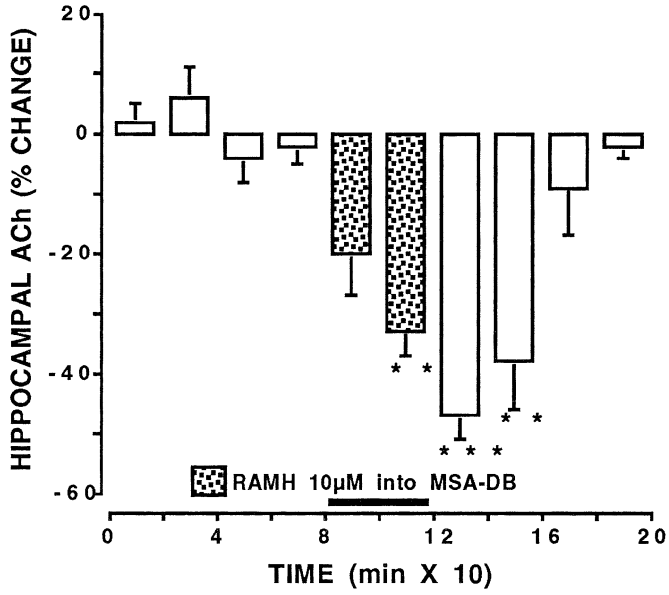

FIG. 6. Time course of release ACh from the hippocampus of freely moving rats evoked by administration of R-α-methylhistamine into MSA-DB. Methods are identical to those described for Fig. 2. R-α-methylhistamine (10 μM) was administered at 80 min into the NBM through the dialysis probe and stopped at 120 min. The bar shows the period of R-α-methylhistamine application. The mean spontaneous release was 0.89 ± 0.11 pmol/20 min (n = 8). Shown are means ± SEM of eight experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. last sample before drug treatment (ANOVA and Scheffé's test).

increases of ACh release of similar amplitude either in the absence (109 ± 11%) or the presence (109 ± 13%) of thioperamide. The spontaneous release of hippocampal ACh in these experiments averaged 0.31 ± 0.03 pmol/20 min (n = 3).

Opposite effects of local administration of histamine H_2 and H_3 receptor agonists into MSA-DB on the spontaneous release of hippocampal ACh

Dimaprit added to the MSA-DB perfusing medium for 40 min at a concentration of 100 μM significantly stimulated hippocampal ACh release, causing a maximal increase of 77 ± 12% (Fig. 5). The spontaneous release of hippocampal ACh averaged 0.63 ± 0.2 pmol/20 min (n = 6). The maximal effect was always achieved in the second 20-min fraction collected after onset of the septum perfusion with dimaprit, and hippocampal ACh release was restored to control levels during subsequent MSA-DB perfusion with control medium (Fig. 5). Conversely, addition of 10 μM R-α-methylhistamine, an H_3 receptor-selective agonist (Arrang *et al.*, 1987) to the MSA-DB perfusing medium for 40 min resulted in a significant decrease in ACh spontaneous release from the hippocampus (Fig. 6). The response to R-α-methylhistamine was of longer duration than the actual presence of the drug in the MSA-DB perfusing medium, and the maximal inhibition (–47 ± 4%) was achieved in the 20-min fraction collected after R-α-methylhistamine was withdrawn from the perfusing medium. Eventually hippocampal ACh release returned to baseline (Fig. 6). In these experiments the spontaneous release of hippocampal ACh averaged 0.89 ± 0.11 pmol/20 min (n = 8).

Local administration of H_3 receptor antagonists into MSA-DB increased the spontaneous release of ACh from the hippocampus

A significant increase in spontaneous release of ACh from the hippocampus was observed following the perfusion of the MSA-DB for 40 min with medium containing 0.3 μM thioperamide, a selective H_3 receptor antagonist (Arrang *et al.*, 1987) (Fig. 7A). The maximal effect, 106 ± 25% increase, was achieved very rapidly, and hippocampal ACh release was restored to control levels during subsequent MSA-DB perfusion with control medium (Fig. 7A). The spontaneous release of hippocampal ACh averaged 0.67 ± 0.15 pmol/20 min (n = 5). An identical MSA-DB perfusion with 0.05 μM ciproxifan, another H_3 receptor antagonist (Ligneau *et al.*, 1998), elicited a 78 ± 5% maximal increase of hippocampal ACh release with a time course comparable to that obtained with thioperamide (Fig. 8A). As for thioperamide, the ciproxifan effect was completely reversible. The spontaneous release of hippocampal ACh averaged 1.6 ± 0.4 pmol/20 min (n = 7).

H_2 receptor antagonism in the MSA-DB moderated the spontaneous release of ACh from the hippocampus and antagonized the facilitation produced by H_3 receptor antagonists.

The effect of blocking H_2 receptors on ACh spontaneous release from the hippocampus was tested by perfusing the MSA-DB with 100 μM cimetidine, a selective H_2 antagonist (Durant *et al.*, 1977). Perfusion through the dialysis probe in the MSA-DB with 100 μM cimetidine for 80 min decreased significantly, although only by ≈30%, the spontaneous release of ACh from the hippocampus (Fig. 9). ACh release was restored to control levels during subsequent perfusion with control medium (Fig. 9). The spontaneous ACh release averaged 0.25 ± 0.03 pmol/20 min (n = 5).

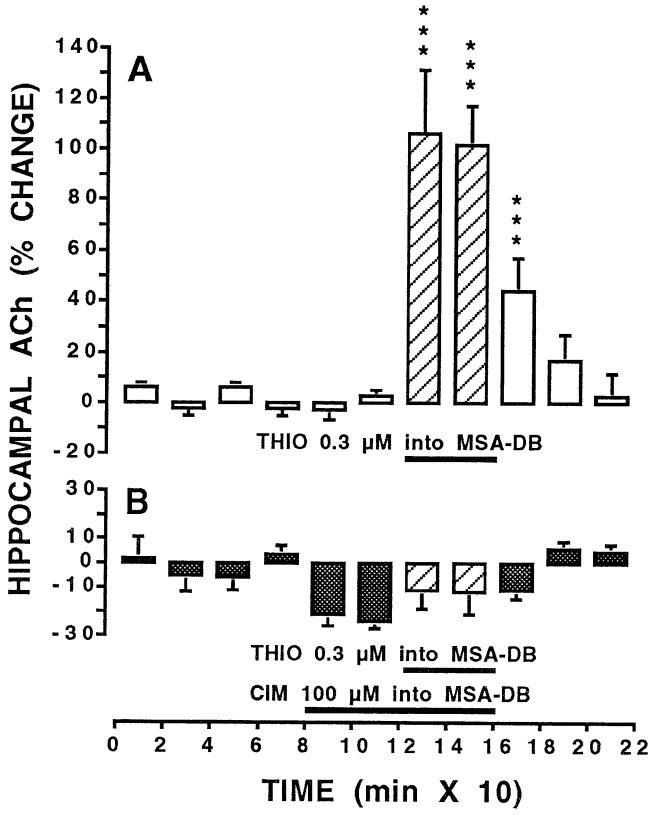

FIG. 7. Time course of release ACh from the hippocampus of freely moving rats evoked by administration into MSA-DB of thioperamide (THIO) in the absence (A)and in the presence (B)of cimetidine (CIM). Methods are identical to those described for Fig. 2. THIO (0.3 μM) was administered at 120 min into the MSA-DB through the dialysis probe for 40 min in (A) the absence or (B) the presence of CIM (100 μM) that was added to MSA-DB perfusing medium 40 min before administration of THIO. Hippocampal ACh spontaneous release averaged 0.67 ± 0.15 pmol/20 min ($n = 5$) in the experiments shown in panel A, and 1.12 ± 0.29 pmol/20min ($n = 5$) in those shown in panel B. The bars show the period of drug applications. There was a significant effect of Concentration × Time interaction ($F_{1,88} = 11.15$, $P < 0.0001$) and of drug treatment ($F_{1,10} = 13.66$, $P < 0.0001$) (two-way ANOVA). Each point represents the mean value ± SEM of (A) five and (B) five experiments. ***$P < 0.001$ vs. THIO + CIM (Bonferroni's test).

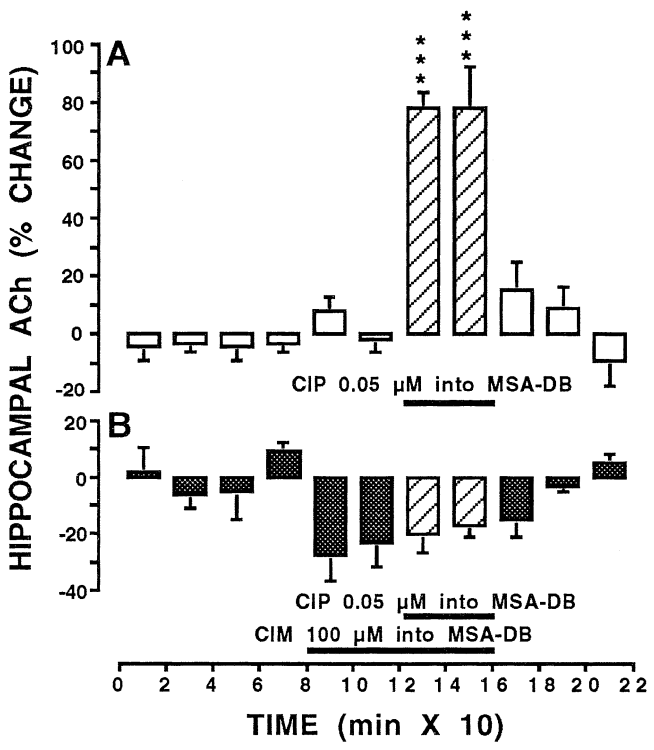

FIG. 8. Time course of release ACh from the hippocampus of freely moving rats evoked by administration into MSA-DB of ciproxifan (CIP) in (A)the absence and (B)the presence of cimetidine (CIM). Methods are identical to those described for Fig. 2. CIP (0.05 μM) was administered at 120 min into the MSA-DB through the dialysis probe for 40 min in (A) the absence or (B) the presence of CIM (100 μM) that was added to MSA-DB perfusing medium 40 min before administration of CIP. Hippocampal ACh spontaneous release averaged 1.6 ± 0.4 pmol/20 min ($n = 7$) in the experiments shown in panel A, and 0.96 ± 0.22 pmol/20 ($n = 3$) in those shown in panel B. The bars show the period of drug applications. There was a significant effect of Concentration × Time interaction ($F_{1,90} = 7.904$, $P < 0.0001$) and of drug treatment ($F_{1,9} = 14.39$, $P < 0.0001$) (two-way ANOVA). Each point represents the mean value ± SEM of (A) seven and (B) three experiments. ***$P < 0.001$ vs. CIP + CIM (Bonferroni's test).

Figure 7B shows the time course of ACh release from the hippocampus evoked by local administration into MSA-DB of thioperamide in the presence of cimetidine. After collection of four 20-min baseline samples from the hippocampus, 100 μM cimetidine was added to the MSA-DB perfusing medium alone for 40 min, and then in combination with 0.3 μM thioperamide for a further 40 min. Washout with control medium was carried out for the final three 20-min fractions. In the presence of cimetidine, the facilitatory effect of thioperamide on ACh release from hippocampus was completely abolished. There was a significant effect of both Concentration × Time interaction ($F_{1,88} = 11.15$, $P < 0.0001$) and drug treatment ($F_{1,10} = 13.66$, $P < 0.0001$; two-way ANOVA). The seventh, eighth and ninth 20-min fractions shown in Fig. 7A were significantly different when compared to the corresponding 20-min fractions of Fig. 7B ($P < 0.001$, Bonferroni's test). A similar effect was observed when ciproxifan was infused into the MSA-DB in the presence of cimetidine. Figure 8B shows that the facilitatory effect of ciproxifan (0.05 μM) on ACh release was fully counteracted by cimetidine (100 μM). There was a significant effect of both Concentration × Time interaction ($F_{1,90} = 7.904$, $P < 0.0001$) and drug treatment ($F_{1,9} = 14.39$, $P < 0.0001$) (two-way ANOVA). Both seventh and eighth 20-min fractions shown in Fig. 8A were significantly different when compared to the corresponding 20-min fractions of Fig. 8B ($P < 0.001$, Bonferroni's test). ACh spontaneous release averaged 1.12 ± 0.29 pmol/20 min ($n = 5$) in the experiments with thioperamide and cimetidine, and 0.96 ± 0.22 pmol/20 min ($n = 3$) in those with ciproxifan and cimetidine.

Local administration of triprolidine into MSA-DB failed to antagonize the release of hippocampal ACh elicited by thioperamide

Perfusion of MSA-DB with 0.5 μM triprolidine, an H_1 receptor antagonist (Ison & Casy, 1971), failed to alter ACh spontaneous release from the hippocampus during a 40-min perfusion. The changes in hippocampal ACh release were always within the range of variability (± 15%) seen between individual 20-min collection periods during MSA-DB perfusion with control medium (data not shown). In these experiments the spontaneous release of hippocampal ACh averaged 0.60 ± 0.04 pmol/20 min ($n = 3$).

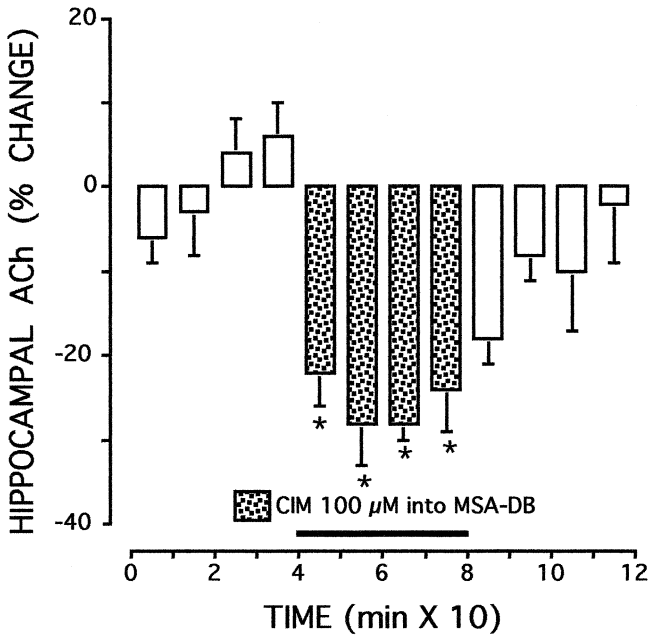

FIG. 9. Time course of release ACh from the hippocampus of freely moving rats evoked by administration into MSA-DB of cimetidine (CIM). Methods are identical to those described for Fig. 2. CIM (100 μM) was administered at 80 min into the NBM through the dialysis probe for 40 min. The mean spontaneous release was 0.77 ± 0.08 pmol/20 min (n = 5). The bar shows the period of cimetidine application. Shown are means ± SEM of five experiments. *P < 0.05 vs. last sample before drug treatment (ANOVA and Scheffé's test).

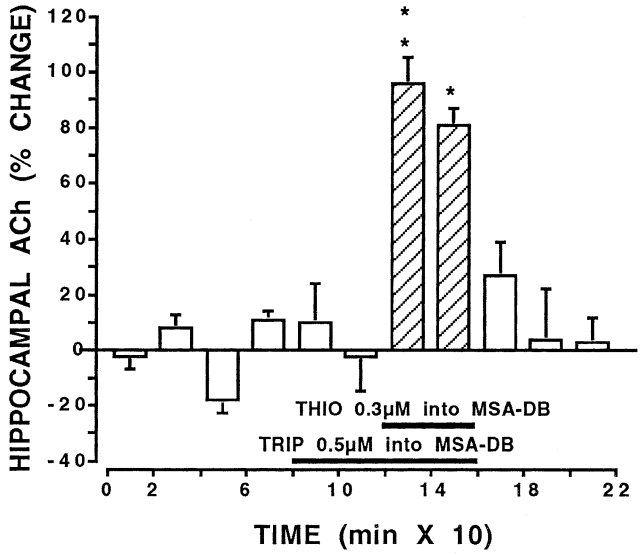

FIG. 10. Time course of release ACh from the hippocampus of freely moving rats evoked by administration of thioperamide (THIO) into MSA-DB in the presence of triprolidine (TRIP). Methods are identical to those described for Fig. 2. THIO (0.3 μM) was administered at 120 min into the MSA-DB through the dialysis probe for 40 min in the presence of TRIP (0.5 μM) that was administered into MSA-DB for 40 min before administration of THIO. Hippocampal ACh spontaneous release averaged 0.44 ± 0.02 pmol/20 min (n = 3). The bars show the period of drug applications. Shown are means ± SEM of three experiments. *P < 0.05 and **P < 0.01 vs. last sample before drug treatment (ANOVA and Scheffé's test).

The effect of 0.5 μM triprolidine on 0.3 μM thioperamide-evoked release of hippocampal ACh was also examined. After collection of four 20-min baseline samples from the hippocampus, triprolidine was added to the MSA-DB perfusing medium alone for 40 min, and then in combination with thioperamide for a further 40 min (Fig. 10). The presence of triprolidine failed to modify thioperamide-evoked release of hippocampal ACh (Fig. 10). In these experiments hippocampal ACh spontaneous release averaged 0.44 ± 0.02 pmol/20 min (n = 3).

Effects of hippocampal perfusion with medium containing 100 μM histamine or dimaprit on ACh release from rat hippocampus

Twenty-four hours after rats were implanted with a microdialysis probe in the hippocampus, the probe was perfused with Ringer's solution containing 7 μM physostigmine. The introduction of 100 μM histamine into hippocampal perfusing medium failed to alter ACh release from the hippocampus during a 40-min perfusion. The changes in hippocampal ACh release were always within the range of variability (± 15%) seen between individual 20-min collection periods during hippocampal perfusion with control medium (data not shown). In these experiments hippocampal ACh spontaneous release averaged 0.71 ± 0.09 pmol/20 min (n = 5). Also, dimaprit, a highly selective H_2 receptor agonist (Parson *et al.*, 1977), added to the hippocampal-perfusing medium for 40 min at a concentration of 100 μM, failed to modify significantly hippocampal ACh release. The spontaneous release of hippocampal ACh averaged 0.76 ± 0.19 pmol/20 min (n = 4).

Thioperamide and ciproxifan modulate c-fos expression in MSA-DB neurons

The counts were taken in sections close to the needle tip and involved the corresponding portions of MSA-DB. As shown in Fig. 11, cells expressing c-fos immunoreactivity were significantly more numerous in thioperamide- and ciproxifan-treated rats than in controls. The photomicrograph shows the cloud of c-fos-positive cell nuclei around the track of the needle tip in thioperamide-treated rats. Double labelling experiments with anti ChAT antibodies were carried out in a subset of experiments in an attempt to characterize the c-fos-positive cell. No colocalization of anti-c-fos and anti-ChAT immunoreactivity, though, was observed (n = 3; data not shown).

Discussion

This study demonstrates that histamine facilitates ACh release from the hippocampus through interactions with H_2 receptors in the MSA-DB. Indeed, intra-MSA-DB, but not intrahippocampal, administration of exogenous histamine or of the H_2 receptor-selective agonist dimaprit increased the spontaneous release of ACh from the hippocampus. Moreover, the blockade of H_3 receptors in MSA-DB by thioperamide and ciproxifan indicates a role for endogenous histamine in modulating the cholinergic tone in the hippocampus. The effects of perfusion of MSA-DB with histaminergic drugs on the rate of hippocampal ACh spontaneous release were investigated by means of a dual-probe microdialysis method. Because both in rats and in humans projections from the MSA-DB provide the majority of cholinergic innervation to the hippocampus (Lewis *et al.*, 1967;

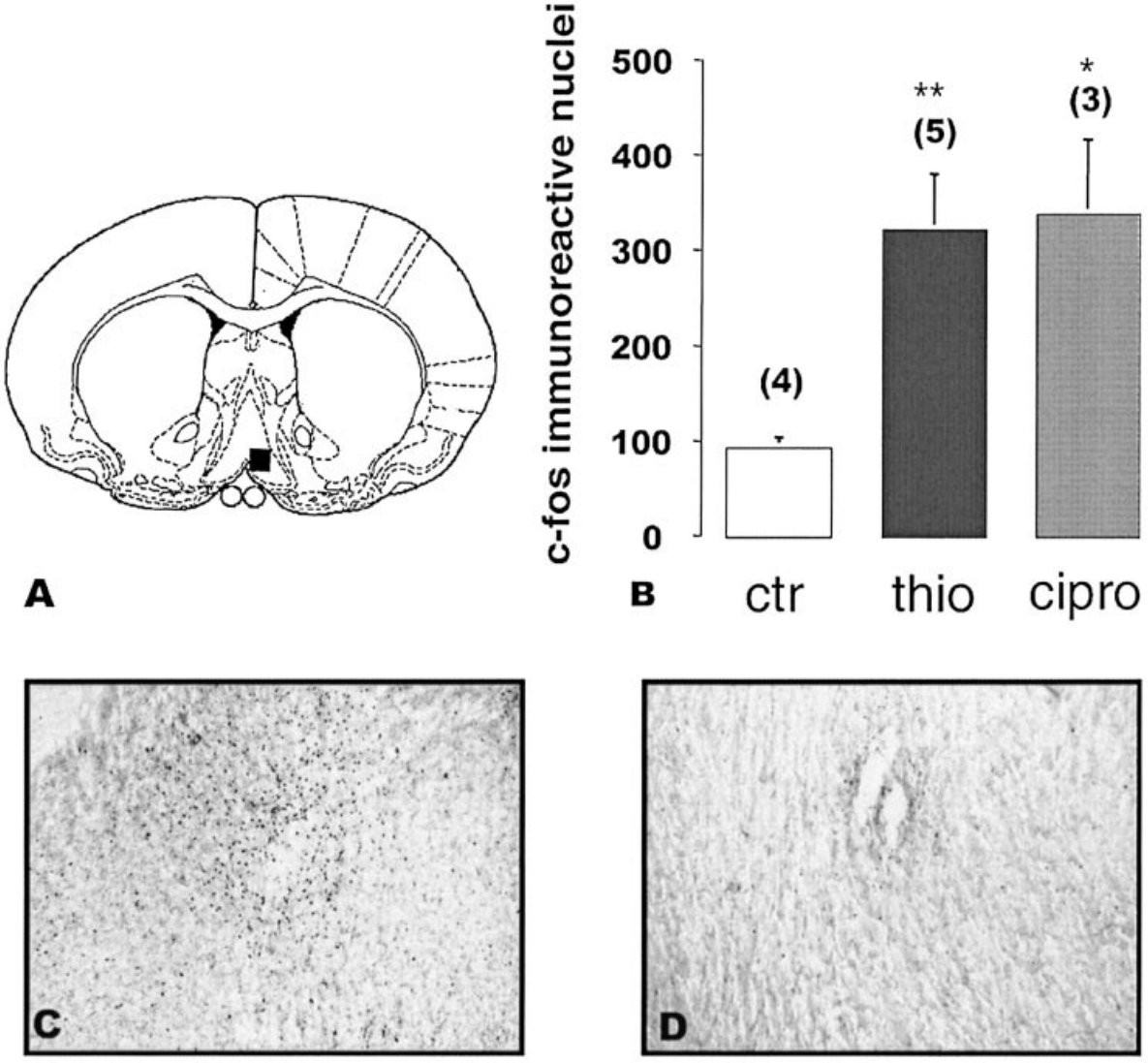

FIG. 11. Injections of H_3 receptor antagonists in the septum increased the expression of c-fos. (A) Schematic drawing of a brain section through the MSA-DB showing the area sampled for c-fos-immunopositive nuclei (black square). (B) The number of cell nuclei expressing c-fos-like immunoreactivity was significantly higher in the septum of animals that received intraseptal injections of either thioperamide (3 μM) or ciproxifan (500 nM). The number of c-fos-positive cells is the average of at least two adjacent sections per animal. (C and D) Photomicrographs of c-fos-like immunoreactivity in the medial septum after (C) thioperamide and (D) saline injection. The track of the needle is visible at the centre of each photomicrograph. Shown are means ± SEM of 3–5 experiments. $*P < 0.05$ and $**P < 0.01$ vs. control (ANOVA and Scheffé's test).

Nyakas *et al.*, 1987; De Lacalle *et al.*, 1994), a first vertical microdialysis probe was implanted in the MSA-DB to deliver locally different drugs, and a second one was inserted in the hippocampus to measure ACh extracellular levels. The feasibility of such an approach was validated by the demonstration that hippocampal ACh extracellular levels depended on the activity of MSA-DB neurons. Indeed, when the KCl concentration in the MSA-DB perfusing medium was changed from 4 to 100 mM KCl for 20 min, it elicited a pronounced and rapid increase in hippocampal ACh release. This concentration of KCl is only apparently very high, considering its rapid dilution into the extracellular fluid and the low recovery of the microdialysis membrane (Westerink & de Vries, 1996). Conversely, administration of the nerve impulse blocker TTX into MSA-DB decreased ACh spontaneous release from the hippocampus by >50%, thus indicating the presence of spontaneous impulse activity in MSA-DB cholinergic neurons at rest. The increase of ACh release produced by 100 mM KCl suggests that *in vivo* the resting MSA-DB cholinergic neurons may increase their firing frequency according to the intensity of the stimulus. This evidence is consistent with the intrinsic membrane properties of these neurons described previously in electrophysiological studies (Segal, 1986; Gorelova & Reiner, 1996; Wu *et al.*, 2000). Therefore, the dual probe is a useful approach for investigating the neurochemical activity of the MSA-DB–hippocampal pathway, and infusion of TTX and high KCl is a reliable method for evaluating the implantation of the microdialysis probes, consistent with earlier observations in the nucleus basalis magnocellularis–cortical pathway (Cecchi *et al.*, 2001).

The presence of physostigmine in the perfusion medium may be criticized because the cholinesterase inhibition might alter the modulation of ACh spontaneous release, as in the case of dopamine on striatal ACh release (DeBoer & Abercrombie, 1996). However, the hippocampus, as well as the amygdala (Passani *et al.*, 2001) and

the cortex (Herrera-Marschitz *et al*., 1990; Consolo *et al*., 1994; Giovannini *et al*., 1998), release much lower levels of ACh than does the striatum, thus requiring the presence of cholinesterase inhibitors in the perfusion fluid. It is noteworthy that concentrations up to 0.5 μM neostigmine, which is ≈10 times more potent than physostigmine, failed to alter qualitatively cortical ACh release patterns in response to behavioural activation (Himmelheber *et al*., 1998).

The increase of ACh release caused by thioperamide or ciproxifan is attributable to blockade of H_3 receptors localized in the MSA-DB. Indeed, comparable increases of hippocampal ACh release were elicited by MSA-DB perfusion with medium containing either 300 nM thioperamide, a competitive antagonists at H_3 receptors with pA_2 values in cortical slices of 8.96 (Arrang *et al*., 1987), or 50 nM ciproxifan, another highly selective H_3-antagonist ≈10 times more potent than thioperamide (Ligneau *et al*., 1998). Conversely, the histaminergic agonist R-α-methylhistamine, added to the MSA-DB perfusing medium at a concentration selective for the H_3 receptor (Arrang *et al*., 1987; Blandina *et al*., 1996), decreased significantly hippocampal ACh release. The H_3 receptor was initially discovered on histaminergic neurons as a presynaptic autoreceptor whose activation inhibited, whilst its blockade increased, the release of histamine (Arrang *et al*., 1983). A physiological function of endogenous histamine in the control of hippocampal ACh release is indicated by the facilitation produced by thioperamide and ciproxifan. Indeed, the increase in hippocampal ACh release could be most simply explained by an interaction with H_3 autoreceptors whose blockade may have produced an increase in endogenous histamine release. Therefore, the present study indicates that activation of histaminergic neurons projecting to the MSA-DB facilitates the cholinergic tone in the hippocampus. Postsynaptic H_2 receptors appear to mediate this effect because pretreatment with cimetidine, at a concentration sufficient to fully block H_2 receptors (Durant *et al*., 1977), completely abolished the effect of both thioperamide and ciproxifan. A different localization of H_2 and H_3 receptors is also suggested by the observation that exogenous histamine (100 μM) produced increases in ACh release of similar amplitude either in the presence or in the absence of a concentration of thioperamide that fully blocks H_3 receptors. Indeed, histamine showed higher affinity for the H_3 (pD2 value of 7.4) than for the H_2 receptor (pD2 value of 6.0) (Leurs *et al*., 1995); thus the increase in ACh release elicited by the infusion with 100 μM exogenous histamine is the result of stimulation of both receptor types. It is conceivable that H_3 receptors are localized on histaminergic terminals. However, a 100-μM concentration of exogenous histamine fully activated postsynaptic H_2 receptors, and any variation of endogenous histamine by H_3 autoreceptor activation had no influence on ACh release. H_2 receptor involvement is also supported by the observation that infusion of MSA-DB with a highly selective H_2 receptor agonist, dimaprit (Parson *et al*., 1977), increased hippocampal ACh release with a pattern similar to that of exogenous histamine. MSA-DB displayed both H_2 receptor binding and its gene transcripts (Vizuete *et al*., 1997; Karlstedt *et al*., 2001). These receptors are positively coupled with adenylyl cyclase (Johnson, 1982), and their activation in neurons led to mainly excitatory effects through blockade of calcium-activated potassium current and modulation of the hyperpolarization-activated cation channel (Brown *et al*., 2001). Stimulation of transmitter release by H_2 receptor activation occurs in other brain regions, because H_2 receptor activation released endogenous noradrenaline from rat hypothalamic slices (Blandina *et al*., 1989) and endogenous enkephalin from mouse striatum (Garbarg *et al*., 1991). Conversely, modulation of ACh release from the hippocampus did not involve H_1 receptors because MSA-DB perfusion with the H_1 antagonist triprolidine, at a concentration >500 times its K_d for the H_1 receptor (Ison & Casy, 1971), failed to modify both spontaneous and thioperamide-evoked release of ACh.

In addition to preventing the stimulation elicited by H_3 antagonists, intra-MSA-DB administration of cimetidine alone decreased hippocampal ACh spontaneous release, thus indicating that MSA-DB histaminergic input tonically facilitated hippocampal cholinergic activity. This hypothesis is consonant with the observation that R-α-methylhistamine moderated hippocampal ACh release. This inhibition may depend on a decrease of endogenous histamine release due to activation of H_3 autoreceptors. It is noteworthy that H_3 receptors are present in the MSA-DB complex (Pollard *et al*., 1993), which exhibits moderate to dense levels of histaminergic fibres (Wouterlood *et al*., 1988; Panula *et al*., 1989). The tonic activation of H_2 receptors was not detected in anaesthetized rats (Mochizuki *et al*., 1994), but differences in administration route of H_2 receptor antagonists (systemically in Mochizuki's study and locally into the MSA-DB in the current study) could account for the discrepancy. Moreover, intrinsic properties of cholinergic septal neurons of anaesthetized rats differ from those of unanaesthetized rats. In anaesthetized rats, about half of these neurons fired rhytmically in single spikes or short bursts (Lamour *et al*., 1984; Dutar *et al*., 1986), whereas in awake rats the proportion of rhytmically bursting neurons is smaller and the mean spontaneous activity is higher (Lee *et al*., 1991; Sweeney *et al*., 1992).

Whether H_2 receptors are located on cholinergic perikarya or on hypothetical interneurons is not clear yet. It has been reported that histamine depolarized MSA-DB cholinergic neurons in a slice preparation of rat brain, producing an increase in sodium conductance which led these neurons to threshold for firing spontaneous action potentials (Gorelova & Reiner, 1996); this observation favours the hypothesis of a direct mechanism. However, this depolarization was attributed to H_1 receptor activation, being significantly reduced by mepyramine and promethazine (Gorelova & Reiner, 1996), whereas Mochizuki *et al*. (1994) and the current study failed to show any effect in modulating hippocampal ACh release by H_1 receptor antagonists. One may argue that H_1 receptor antagonists were employed in the study by Gorelova & Reiner (1996) at concentrations to high for excluding H_1-receptor-unrelated effects (Hill, 1990; Hill *et al*., 1997), but also another explanation could be offered for the lack of effect of H_1 receptor antagonists in microdialysis studies. Depolarization induced by histamine was transient, desensitization within seconds being its prominent feature, and the excitation diminished despite continued application of histamine (Gorelova & Reiner, 1996). If the effect on ACh release was transient as well and very short, it is possible that it became obscured during the attainment of the 20-min perfusion sample, thus explaining the lack of effects of H_1 receptor blockade on hippocampal cholinergic activity.

Systemic administration of thioperamide increased whilst that of R-α-methylhistamine decreased ACh spontaneous release from the hippocampus of anaesthetized rats (Mochizuki *et al*., 1994). The current study extends these findings in freely moving rats, and demonstrates that histaminergic drugs acted in the MSA-DB complex. Indeed, the interaction between histaminergic and cholinergic systems occurs only at this level, because histamine and dimaprit locally applied to the hippocampus failed to alter extracellular levels of local ACh. The report that H_3 receptor-elicited modulation of [^{3}H]-ACh release from rat hippocampal slices was not detectable (Alves-Rodrigues *et al*., 1998) agrees with our findings. Although the hippocampus receives only a weak histaminergic innervation (Wouterlood *et al*., 1988), *in vitro* and *in vivo* studies demonstrated

that histamine exerts in this area many prominent effects which are likely to be important in the induction of synaptic plasticity and consolidation of memory (see review by Brown *et al*., 2001). In this regard, given that MAP kinase activation is necessary for synaptic plasticity (English & Sweatt, 1996), the reports that histaminergic receptors are coupled to MAP-kinase signalling pathways in COS-7 cells (Drutel *et al*., 2001) and CA3 hippocampal neurons (Blandina *et al*., 2001) are intriguing.

The specific behavioural significance of the interactions between histaminergic and MSA-DB cholinergic systems remains to be explored, but a role in cognitive processes is suggested by the observation that post-training intraseptal injection of both H_2 receptor agonists and H_3 receptor antagonists improved retention in a T-maze avoidance task, whilst that of H_3 receptor agonists and H_2 receptor antagonists had the opposite effect (Flood *et al*., 1998). 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). According to our findings, the improvement elicited by H_2 receptor agonists and H_3 receptor antagonists may reflect an enhanced release of hippocampal ACh, whilst cognitive deficits caused by H_3 receptor agonists and H_2 receptor antagonists may occur as a consequence of hippocampal cholinergic tone inhibition. The role of histamine as an important neurotransmitter in the mammalian brain and as a modulator of cognitive processes has recently gained attention (Leurs *et al*., 1998; Passani *et al*., 2000; Bacciottini *et al*., 2001), and the significance of interactions between histaminergic and cholinergic systems in learning and memory processes have been clearly demonstrated in the cortex (Blandina *et al*., 1996; Giovannini *et al*., 1999) and the amygdala (Passani *et al*., 2001).

Intraseptal administration of thioperamide or ciproxifan also increased the number of c-fos-immunopositive cells in MSA-DB. Expression of the c-fos gene is an indirect correlate of increased neuronal activity (Dragunow & Faull, 1989; Herrera & Robertson, 1996); it can therefore be used to detect specific activation in restricted brain sites. The increased c-fos expression does not seem to occur in cholinergic cells within the MSA, as colocalization of anti-ChAT and anti-c-fos antibodies was never observed. As previously mentioned, the cytoarchitectonic relationship between histaminergic and cholinergic cells is not known. In the MSA-DB there are subpopulations of parvalbumin-positive GABAergic neurons, interspersed with the cholinergic cells, that project to the hippocampus (Kiss *et al*., 1990; Van der Zee & Luiten, 1994). In the medial septum there are also peptide-containing neurons (Gall & Moore, 1984), in particular galanin-positive cells, giving rise to septo-hippocampal efferents (Senut *et al*., 1989). Given the complexity of the local circuitry within the medial septum and also in relation to the efferent projections (Onteniente *et al*., 1986; Onteniente *et al*., 1987; Senut *et al*., 1989), several plausible synaptic arrangements can be envisaged to explain the effect of histaminergic compounds on both ACh release in the hipocampus and c-fos expression in the septum.

Increased hippocampal ACh release by histaminergic agents may have implications for the treatment of disorders associated with impaired septo-hippocampal cholinergic functions. Treatment strategies that take advantage of noncholinergic drugs potentiating cholinergic functions may produce beneficial effects on disorders associated with impaired cholinergic functions, such as Alzheimer's disease (Buccafusco & Terry, 2000). This indirect approach appears preferable to cholinomimetic strategies, because cholinergic drugs have resulted in greater stimulation of inhibitory autoreceptors either by increasing the half-life of acetylcholine in the synaptic cleft (Davis *et al*., 1992), or by directly activating these receptors due to the poor selectivity of the agonists available (Gauthier *et al*., 1991). The histaminergic system might well represent a target for these new therapies. The implications of the present study and the report that H_3 receptor antagonists improve learning and memory in senescence-accelerated mice (Meguro *et al*., 1995) and scopolamine-impaired (Giovannini *et al*., 1999) rats support this contention.

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Abbreviations

ACh, acetylcholine; BSA, bovine serum albumin; ChAT, choline acetyltransferase; MSA-DB, medial septum–diagonal band; PBS, phosphate-buffered saline; TTX, tetrodotoxin.

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