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Heterogeneity of histaminergic neurons in the tuberomammillary nucleus of the rat

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Abstract

Histaminergic neurons of the hypothalamic tuberomammillary nuclei (TMN) send projections to the whole brain. Early anatomical studies described histaminergic neurons as a homogeneous cell group, but recent evidence indicates that histaminergic neurons are heterogeneous and organized into distinct circuits. We addressed this issue using the double-probe microdialysis in freely moving rats to investigate if two compounds acting directly onto histaminergic neurons to augment cell firing [thioperamide and bicuculline, histamine H₃- and γ -aminobutyric acid (GABA)_A-receptor (R) antagonists, respectively] may discriminate groups of histaminergic neurons impinging on different brain regions. Intra-hypothalamic perfusion of either drug increased histamine release from the TMN and cortex, but not from the striatum. Thioperamide, but not bicuculline, increased histamine release from the nucleus basalis magnocellularis (NBM), bicuculline but not thioperamide increased histamine release from the nucleus accumbens (NAcc). Intra-hypothalamic perfusion with thioperamide increased the time spent in wakefulness. To explore the local effects of H₃-R blockade in the histaminergic projection areas, each rat was implanted with a single probe to simultaneously administer thioperamide and monitor local changes in histamine release. Thioperamide increased histamine release from the NBM and cortex significantly, but not from the NAcc or striatum. The presence of H₃-Rs on histaminergic neurons was assessed using double-immunofluorescence with anti-histidine decarboxylase antibodies to identify histaminergic cells and anti-H₃-R antibodies. Confocal analysis revealed that all histaminergic somata were immunopositive for the H₃-R. This is the first evidence that histaminergic neurons are organized into functionally distinct circuits that influence different brain regions, and display selective control mechanisms.

Introduction

Somata of aminergic neurons are restricted to discrete cell clusters in distinct brain regions, although their axons innervate nearly the entire CNS. Locus coeruleus, substantia nigra and dorsal raphe are sources of noradrenergic, dopaminergic and serotonergic axons, respectively. These nuclei are comprised of distinct compartments with respect to projection fields, as separate subgroups of noradrenergic (A1–A7), dopaminergic (A8–A17) and serotonergic (B1–B9) neurons give rise to distinct sets of axons innervating separate brain regions (Mason & Fibiger, 1979; Jacobs & Azmitia, 1992). This pattern implies independent functions of sets of neurons according to their origin and terminal projections.

In a comparable architecture, all fibres containing histamine, another aminergic neurotransmitter (Schwartz, 1975), originate within the hypothalamic tuberomammillary nuclei (TMN; Panula *et al.*, 1984; Watanabe *et al.*, 1984), and innervate almost the entire CNS (Inagaki *et al.*, 1988; Panula *et al.*, 1989a; Brown *et al.*, 2001). Histaminergic neurons are grouped in five clusters, E1–E5 (Inagaki *et al.*, 1988), each of which sends overlapping projections throughout the neuroaxis (Ericson *et al.*, 1987). Retrograde tracers injected into different CNS regions labelled histaminergic cell bodies scattered throughout the TMN without a strict topographical pattern (Kohler *et al.*, 1985; Inagaki *et al.*, 1990). Thus, histaminergic neurons appear as a rather homogeneous cell group acting as a regulatory network for whole-brain activity through diffuse projections, and modulating general states of metabolism and consciousness, rather than processing specific functions (Wada *et al.*, 1991). Evidence is beginning to accumulate showing the heterogeneity of histaminergic neurons. Different types of stress (Miklos & Kovacs, 2003) and hypercapnic loading (Haxhiu *et al.*, 2001) activated subgroups of histaminergic neurons. Histamine neurons differ in size, glycine-sensitivity (Serge-

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eva *et al.*, 2001) and expression of γ -subunits of the γ -aminobutyric acid (GABA)_A receptor (GABA_A-R; Sergeeva *et al.*, 2002, 2005). These observations suggest that the histaminergic system is organized into distinct circuits, each triggering specific physiological responses, including arousal, appetite and cognitive processes (Haas & Panula, 2003; Passani *et al.*, 2004; Haas *et al.*, 2008).

The H₃-receptor (H₃-R) is an auto- and a hetero-receptor, controlling release of histamine itself as well as a range of other neurotransmitters (Arrang *et al.*, 1983; Blandina *et al.*, 1998). It shows functional constitutive activity, polymorphisms in humans and rodents with a differential distribution of splice variants, and coupling to different intracellular signal transduction mechanisms (Passani *et al.*, 2004). H₃-R heterogeneity might underlie the diversity of histaminergic neuronal functions. To understand if histaminergic neurons are organized into distinct functional circuits, we used the dual-probe microdialysis technique to block pharmacologically the H₃-R or the GABA_A-R in the TMN and monitor modification of histamine release in histaminergic projection areas. Single-probe microdialysis experiments were also performed to investigate whether blockade of local H₃-Rs in the histaminergic projection areas influenced histamine release within these brain regions. In this study, we demonstrate that histaminergic neurons are characterized by independent control mechanisms and differential output to specific brain regions. This arrangement may influence individually defined behaviours resulting from differential histamine release in various brain regions.

Materials and methods

Eight-week old, male Sprague-Dawley rats (250–280 g body weight; Harlan, Italy) were housed in groups of three in a temperature-controlled room (20–24°C), on a 12 h light : dark cycle (07.00–19.00 h), and were allowed free access to food and water. 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 - Università di Firenze'.

Surgical procedures

Rats, anaesthetized with chloral hydrate (400 mg/kg i.p.) and positioned in a stereotaxic frame (Stellar, Stoelting, Wood Dale, IL, USA), were implanted with guide cannulae (Metalant, Sweden) according to the following coordinates from bregma (Paxinos & Watson, 1998): TMN, AP = -4.3, L = -1.1, DV = -7.2; nucleus basalis magnocellularis (NBM), AP = -0.8; L = -2.8; DV = -6.5; dorsal striatum, AP = 0; L = -4; DV = -4; nucleus accumbens (NAcc), AP = +2.2; L = -1.4; DV = -6.3; prefrontal cortex, AP = +3.2; L = -1.0; DV = -2.8 (Fig. 1). A surgical screw served as an anchor and the cannulae were fixed to the skull with acrylic dental cement. In the dual-probe experiments, one probe was placed in the TMN of every rat, with a second probe in one of four locations (NBM, dorsal striatum, NAcc or prefrontal cortex). In the single-probe experiments each rat was implanted with a single probe in the NBM, dorsal striatum, NAcc or prefrontal cortex.

In vivo microdialysis measurements of histamine

The microdialysis experiments were performed 48 h after surgery, during which rats, housed one per cage, recovered from surgery. Rats

were handled for at least 4 days before experiments began, thus becoming acclimatized to human contact. The stylet was removed from the guide cannulae, and the microdialysis probes (molecular weight cut-off = 6000 Da, Metalant Sweden) were inserted; the dialysing membrane protruded 2 mm from the tip of the cannula. Probes were perfused with Ringer's solution (in mM: NaCl, 147; CaCl₂, 1.2; KCl, 4.0 at pH 7.0) at a flow rate of 2 μ L/min using a microperfusion pump (Carnegie Medicine, Sweden; Mod CMA/100). Histamine release stabilized 2 h after insertion of the microdialysis probes. After this 120-min equilibration period, fractions were collected at 15-min intervals. In the dual-probe experiments, both probes were perfused with control medium in the first four/five 15-min fractions to measure histamine spontaneous release; thioperamide or bicuculline were then added only to the TMN-perfusing medium. Thioperamide or bicuculline were dissolved into the Ringer's solution to reach final concentrations of 300 nM and 10 μ M, respectively. Drugs addition did not modify the pH of the medium. In the single-probe experiments the brain region of interest was perfused with control medium in the first five fractions, and thioperamide (300 nM) was then added to the perfusing medium. Histamine spontaneous release was defined as the average value of histamine levels in the four/five 15-min fractions collected during perfusion with Ringer's solution prior to drug treatment. All fractions were expressed as a percentage of this value. To prevent degradation of histamine, 1.5 μ L of 5 mM HCl was added to each sample. The dialysates were kept at -80°C until analysis. Drugs were applied in Ringer's solution.

Microdialysis and behavioural coupled experiments

To determine whether the sleep/wake behaviour varied with the increase in TMN histamine release induced by local perfusion with thioperamide, we measured the wake time in a set of eight rats implanted with a single microdialysis probe in the TMN and perfused with a medium containing thioperamide (300 nM). Surgical procedures were the same as described above. The collection of perfusate from the TMN started after a 120-min equilibration period. The probe was perfused with control medium for a 60-min fraction to measure histamine spontaneous release; thioperamide (300 nM) was then added to the perfusing medium for the following 60 min. Simultaneously, the behaviour of each animal was visually monitored by experienced individuals, blind to the experimental protocol, and categorized as 'sleep' or 'wake', according to Ko *et al.* (2003). Animals displaying a curled posture with a tucked head and tail and closed eyes were categorized as 'sleep'. Conversely, an animal was categorized as 'wake' if it displayed upright posture or curled posture with open eyes, body movements such as grooming, scratching, eating, standing, sniffing, burrowing, adjusting tail or head position, stretching. The wake time was measured with a stopwatch and reported on special forms. Experiments began at 10.00 h, and ended by 12.00 h, to limit the influence of circadian factors. One rat was excluded from analysis because its time spent in wakefulness during the 60 min under baseline conditions was greater than 2 standard deviations from the group mean.

Determination of histamine concentration by high-performance liquid chromatography (HPLC)-fluorimetry

Histamine contents in the dialysates were determined by HPLC-fluorimetry using a modified version (Cenni *et al.*, 2006) of the protocol by Yamatodani *et al.* (1985). Briefly, the column (Hypersil

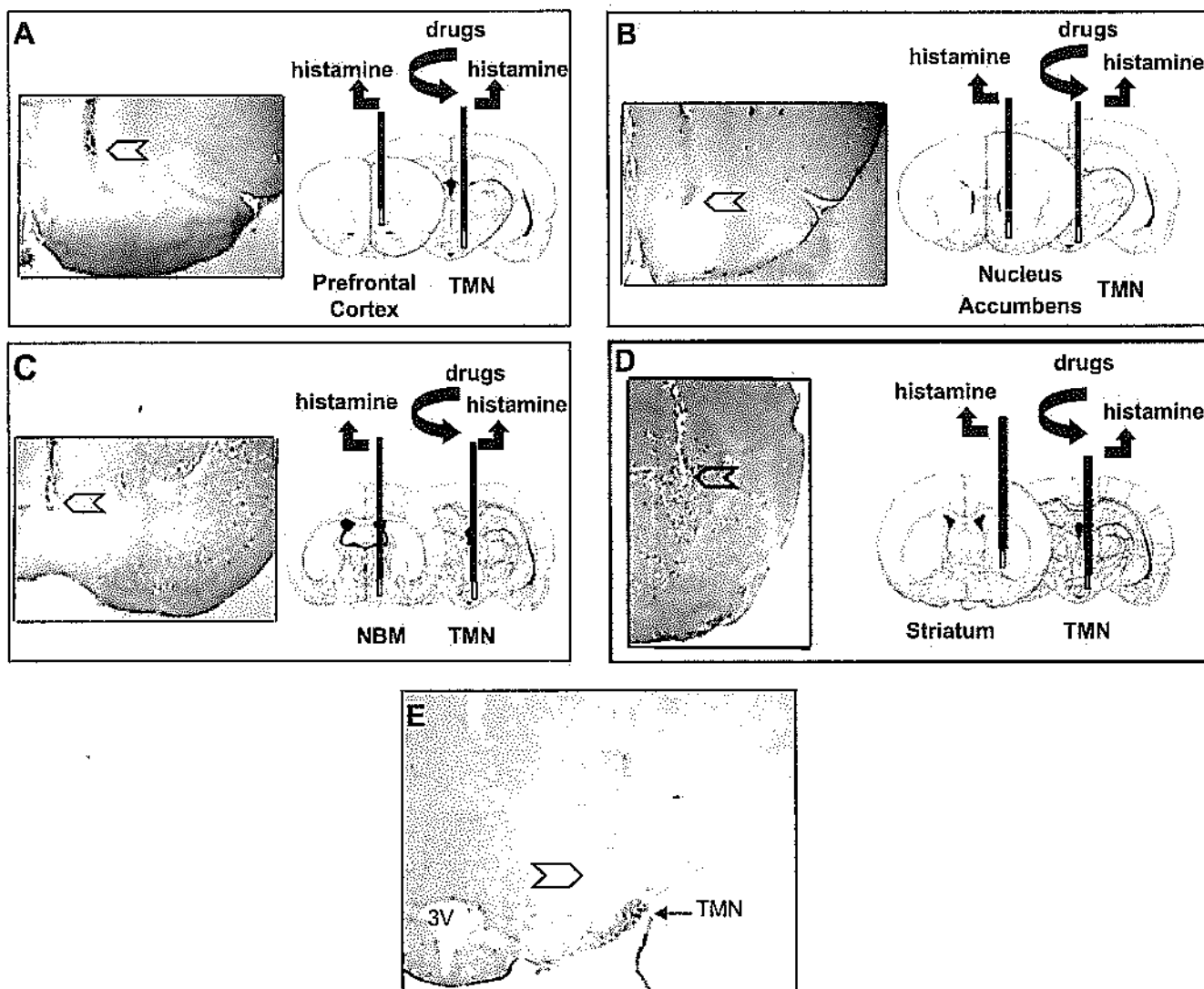


FIG. 1. Schematic diagram and photomicrographs showing the position of the microdialysis probes. In the dual-probe experiments, rats were implanted with one probe in the tuberomammillary nuclei (TMN) to deliver drugs locally and measure neurotransmitter release, and another probe in the prefrontal cortex, the nucleus basalis magnocellularis (NBM), the nucleus accumbens (NAcc) or the dorsal striatum to measure histamine release. In the single-probe experiments, rats were implanted with one probe in the prefrontal cortex, the NBM, the NAcc or the dorsal striatum to deliver thioperamide locally and measure histaminic release. A single probe was implanted in the TMN when assaying the effects of thioperamide on TMN histamine release and the time spent in wakefulness. Representative histological structures showing the actual site of probe placement. Coronal sections of: (A) prefrontal cortex; (B) NAcc; (C) NBM; (D) dorsal striatum; (E) TMN. Hypothalamic sections were immunostained with anti-HDC antibodies to identify the histaminergic neurons (arrow). Arrowheads indicate the microdialysis probe traces.

ODS, 3 μ m, 2.1 \times 100 mm; Thermo Electron, Bellefonte, PA, USA) was eluted with 0.25 M potassium dihydrogen phosphate containing 5% octanesulphonic acid (Sigma) at a flow rate of 0.4 mL/min. The eluate from the column was mixed first with 0.1% *o*-phthalaldehyde solution at a flow rate of 0.1 mL/min and then with a solution containing 4 M sodium hydroxide and 0.2 M boric acid (flow rate 0.137 mL/min) to adjust the reaction mixture to pH 12.5. The reaction took place at 45°C. Then 17% orthophosphoric acid was added to the solution (flow rate 0.137 mL/min) to reach a final reaction mixture at pH 3. The fluorescent intensity was measured with a spectrofluorometer (Agilent series 1100; Waldbronn, Germany) at 450 nm with excitation at 360 nm. The sensitivity limit was 0.010 pmol and the signal/noise ratio was higher than 3.

Histology

The placement of microdialysis membranes was verified *post mortem*. Rats were overdosed with chloral hydrate, the brains removed and stored in 10% formalin for 10 days. Forty-micrometre-thick sections were then cut on a cryostat, mounted on gelatin-coated slides and then stained with Cresyl violet for light microscopic observation. Data from rats in which the membranes were not correctly positioned were discarded (< 10%).

Immunohistochemistry

A specific set of three male Sprague-Dawley rats (250–280 g body weight; Harlan, Italy) was anesthetized with chloral hydrate and

perfused transcardially with 50 mL ice-cold physiological saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Brains were then postfixed in 4% paraformaldehyde in PB for 2 h at 4°C. Brains were cryoprotected in 30% sucrose in PB, and 40- μ m-thick sections were cut on a cryostat microtome and collected in PB. The location of the histaminergic cells with respect to the microdialysis probe was assessed by immunostaining hypothalamic sections with antibodies against histidine decarboxylase (HDC), the histamine-synthesizing enzyme. The sections were incubated with rabbit anti-HDC (1 : 1000; Aeris, Bad Nauheim, Germany) overnight at 4°C, then incubated with biotinylated anti-rabbit IgG antibodies (1 : 400; Vector) followed by horseradish peroxidase-conjugated avidin according to the Vectastain kit (Vector). Immunoreactive products were visualized by incubation with 3,3'-diaminobenzidine.

For double-labelling experiments, all immunostaining procedures were performed on free-floating sections as previously described (Shenton *et al.*, 2005), with modifications. Briefly, H₃-R antigen-retrieval was performed in 50 mM sodium citrate (pH 8.5) at room temperature for 30 min, followed by 30 min at 80°C. Sections were washed in Tris-buffered saline (TBS) containing 0.2% Triton X-100 before blocking in TBS containing 2% foetal calf serum (FCS) and 0.2% Tween 20 for 60 min at room temperature. Sections were then incubated in a cocktail of rabbit anti-H₃-R antibodies (0.5 μ g/mL) and guinea pig anti-HDC (1 : 1000) primary antibodies in TBS containing 1% FCS overnight at 4°C. Rabbit polyclonal antibodies for H₃-R are directed against residues 349–358 of the human and rat H₃-R (Chazot *et al.*, 2001; Cannon *et al.*, 2007; Grandi *et al.*, 2008).

Preabsorption experiments were carried out previously, and described in Chazot *et al.* (2001). After thorough rinsing in TBS, sections were incubated in Cy3-conjugated donkey anti-rabbit IgG (1 : 400; Jackson ImmunoResearch, West Grove, PA, USA) in TBS containing 1% FCS for 90 min at room temperature. After extensive rinses in TBS, sections were incubated in Alexa Fluor 488-labelled, goat anti-guinea pig IgG (Molecular Probes, Eugene, OR, USA) diluted 1 : 500 in TBS containing 1% FCS for 90 min at room temperature. After rinsing, sections were then mounted on glass slides and coverslipped. Elimination of anti-H₃-R primary antibodies resulted in no immunostaining (not shown). Confocal analysis was performed with a Leica TCS SP5 confocal scanning microscope (Leica, Mannheim, Germany), equipped with a HeNe/Ar laser and a Leica Plan Apo \times 63 1.4 oil immersion objective. To avoid bleed-through, green (Alexa 488) and red (Cy3) fluorescent signals were acquired sequentially, using the 488-nm and 543-nm excitation laser line, respectively. Series of optical sections (1024 \times 1024 pixels, pixel size 204 nm) were collected through the cells at intervals of 400 nm. Confocal stacks were then filtered with an FFT-based bandpass filter to cut background noise (structures down to 20 μ m and up to 204 nm) and then z-projected for visualization.

Statistical analysis

All values are expressed as means \pm SEM, and the number of rats used in each experiment is also indicated. The presence of significant treatment effects was first determined by a one-way ANOVA followed

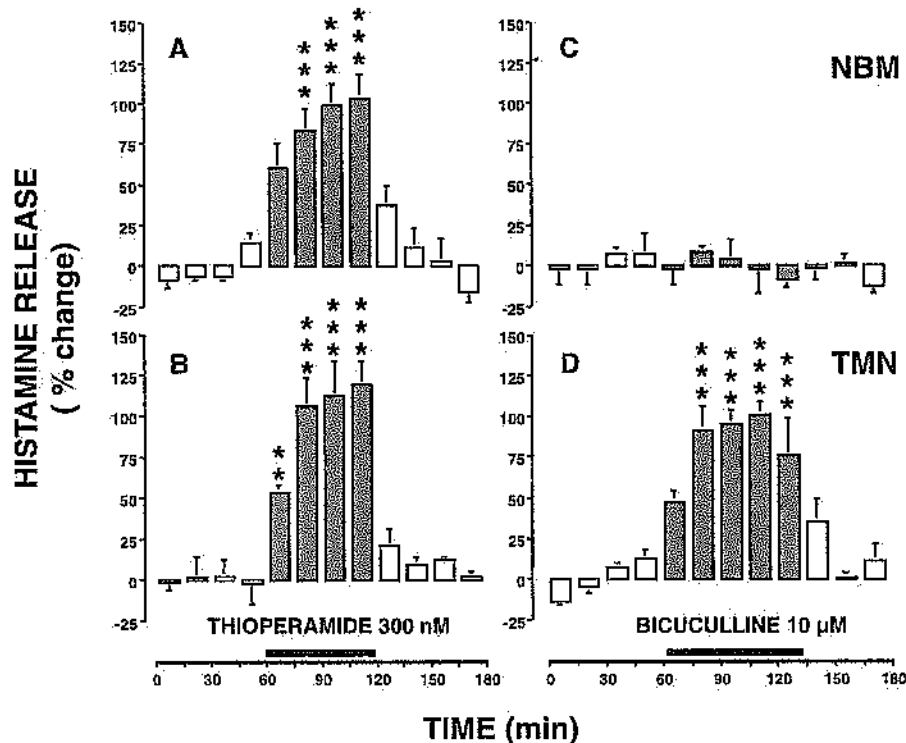


Fig. 2. Influence of thioperamide or bicuculline administration into the tuberomammillary nucleus (TMN) on histamine release from the TMN and the nucleus basalis magnocellularis (NBM) of freely moving rats. Histamine was measured in 15-min fractions and expressed as a percentage of spontaneous release. Control values of spontaneous release were calculated for each experiment by averaging the mean of four initially collected 15-min samples. (A and B) Thioperamide, at a concentration of 300 nM, was infused into the TMN and histamine release was measured from the NBM (A) and the TMN (B). (C and D) Bicuculline, at a concentration of 10 μ M, was infused into the TMN, and histamine release was measured in the NBM (C) and TMN (D). Bars indicate the period of drug application. Shown are means \pm SEM of four (A and B) and five (C and D) experiments. *** P < 0.001; ** P < 0.01 vs. last sample before drug treatment (ANOVA and Bonferroni's test).

by Bonferroni's test. Comparisons using unpaired *t*-tests were used to assess differences within 'sleep' or 'wake' groups. For all statistical tests, $P < 0.05$ was considered significant. For clarity, we reported in figures and figure legends only the significant differences from the last sample before drug treatment. However, differences were significant from all baseline samples. Statistical analysis was performed using STATVIEW (Abacus Concepts; Berkeley, CA, USA).

Chemicals

Bicuculline methiodide and thioperamide malate were purchased from Sigma (UK). All other reagents and solvents were of HPLC grade or the highest grade available (Sigma, UK).

Results

After 120 min of equilibration following the insertion of the dialysing membranes, histamine was released spontaneously at a stable rate from all brain regions investigated. After this 120-min equilibration period, fractions were collected at 15-min intervals. Histological analysis confirmed that the probes were located in the correct areas, with no signs of unusual tissue damage or bleeding (Fig. 1).

Effects of local perfusion of thioperamide or bicuculline into the TMN on histamine release from the TMN and NBM

After collection of four, 15-min baseline samples, thioperamide or bicuculline were infused locally into the TMN, and histamine release

was monitored from both the TMN and NBM using a double-probe microdialysis protocol. As shown in Fig. 2A and B, 60-min perfusion with 300 nM thioperamide induced a significant increase in histamine release from both the NBM (ANOVA, $F_{11,36} = 10.861$, $P < 0.0001$) and the TMN (ANOVA, $F_{11,36} = 18.574$, $P < 0.0001$). Thioperamide at 300 nM is a selective antagonist/inverse agonist of the H_3 -R (Arrang *et al.*, 1987; Morisset *et al.*, 2000). In the TMN the maximal increase, about $118 \pm 14\%$, was achieved in the fourth 15-min fraction collected after onset of the TMN perfusion with thioperamide, and histamine release was restored to control levels during subsequent TMN perfusion with control medium. Histamine spontaneous release from TMN averaged 0.037 ± 0.004 pmol/15 min ($n = 4$). All histaminergic neurons are localized in the TMN and, supposedly, histamine is released by short projections in the posterior hypothalamus, where histaminergic neurons have extensive axonal arborizations (Inagaki *et al.*, 1988; Panula *et al.*, 1989a). Histamine release also increased in the NBM, when thioperamide was infused into the TMN, and it reached a maximal level of approximately $103 \pm 15\%$. Histamine output tended to remain elevated for the duration of thioperamide application to the TMN, and then returned slowly to baseline values after TMN perfusion with thioperamide was complete. Histamine spontaneous release from NBM averaged 0.041 ± 0.005 pmol/15 min ($n = 4$). A significant increase of histamine release from the TMN was also observed during local perfusion for 75 min with $10 \mu\text{M}$ bicuculline, a GABA_A-R antagonist (Bowery *et al.*, 1984; ANOVA, $F_{11,48} = 15.778$, $P < 0.0001$; Fig. 2D). The maximal increase was $102 \pm 8\%$ ($n = 5$). Conversely, in the NBM, histamine spontaneous release was not modified by TMN perfusion with bicuculline (Fig. 2C). The mean spontaneous release in this set

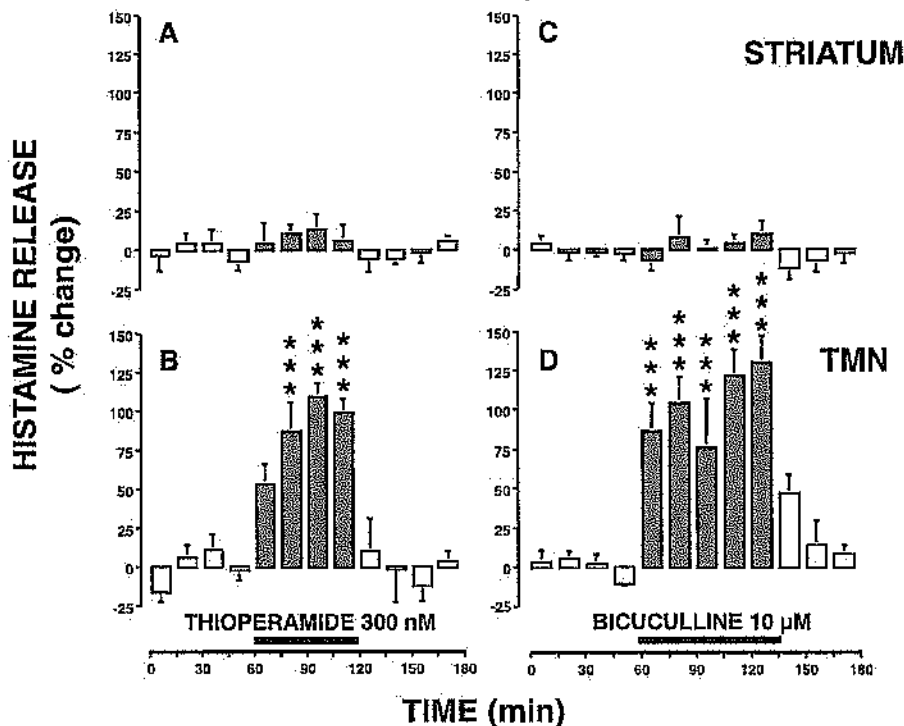


FIG. 3. Influence of thioperamide or bicuculline administration into the tuberomammillary nucleus (TMN) on histamine release from the TMN and the dorsal striatum of freely moving rats. Histamine was measured and calculated as described in Fig. 2. (A and B) TMN was perfused with 300 nM thioperamide, and histamine release was measured from the dorsal striatum (A) and the TMN (B). (C and D) Bicuculline, at a concentration of $10 \mu\text{M}$, was infused into the TMN, and histamine release was measured in the dorsal striatum (C) and TMN (D). Bars indicate the period of drug application. Shown are means \pm SEM of five (A and B) and six (C and D) experiments. *** $P < 0.001$ vs. last sample before drug treatment (ANOVA and Bonferroni's test).

of experiments was 0.100 ± 0.008 pmol/15 min in the TMN, and 0.100 ± 0.012 pmol/15 min in the NBM ($n = 5$).

Perfusion of thioperamide or bicuculline into the TMN did not modify spontaneous release of histamine from the dorsal striatum

Perfusion of the TMN with either 300 nM thioperamide (Fig. 3B) or 10 μ M bicuculline (Fig. 3D) increased significantly histamine release from the TMN (ANOVA, $F_{11,48} = 12$, $P < 0.0001$, and $F_{11,60} = 12.673$, $P < 0.0001$, respectively), with comparable magnitude and time course to studies shown in Fig. 2. Thioperamide increased histamine release significantly, up to $109 \pm 9\%$, and bicuculline up to $130 \pm 17\%$ of respective basal levels. The mean histamine spontaneous release from the TMN was 0.041 ± 0.005 pmol/15 min in the experiments with thioperamide ($n = 5$), and 0.046 ± 0.004 pmol/15 min in those with bicuculline ($n = 6$). Histamine levels returned to basal values during washout of the compounds. However, neither thioperamide nor bicuculline, infused into the TMN, produced any change in the release of histamine from the dorsal striatum (Fig. 3A and C). Histamine spontaneous release from the dorsal striatum was 0.044 ± 0.004 pmol/15 min in the experiments with thioperamide ($n = 5$), and 0.049 ± 0.004 pmol/15 min in those with bicuculline ($n = 6$).

Effects of local perfusion of thioperamide or bicuculline into the TMN on histamine release from the TMN and NAcc

When thioperamide (300 nM) was added to the TMN-perfusing medium for 60 min, histamine release from the TMN increased

significantly by a maximum of $109 \pm 11\%$ (ANOVA, $F_{11,84} = 22.357$, $P < 0.0001$), but was not significantly changed in the NAcc (Fig. 4A and B). Increased histamine levels persisted during perfusion with thioperamide, after which basal histamine levels were quickly attained. The mean spontaneous release of histamine was 0.043 ± 0.004 pmol/15 min in the TMN, and 0.037 ± 0.004 pmol/15 min in the NAcc ($n = 8$). Conversely, perfusion of the TMN for 75 min with 10 μ M bicuculline increased significantly the spontaneous release of histamine not only from the TMN (ANOVA, $F_{11,60} = 27.589$, $P < 0.0001$; Fig. 4D), but also from the NAcc (ANOVA, $F_{11,60} = 13.445$, $P < 0.0001$; Fig. 4C). Histamine maximal increases were, respectively, $99 \pm 5\%$ in the TMN, and $123 \pm 8\%$ in the NAcc. Histamine output returned slowly to baseline values after TMN perfusion with bicuculline was complete. Histamine spontaneous release averaged 0.044 ± 0.003 pmol/15 min from the TMN, and 0.035 ± 0.002 pmol/15 min from the NAcc ($n = 6$).

Local perfusion of thioperamide or bicuculline into the TMN increased histamine spontaneous release from both the TMN and the prefrontal cortex

Application of either thioperamide (300 nM) or bicuculline (10 μ M) in the TMN elicited a significant increase of histamine release from both the TMN (ANOVA, $F_{11,48} = 8.266$, $P < 0.0001$, and $F_{11,48} = 17.559$, $P < 0.0001$, respectively) and the prefrontal cortex (ANOVA, $F_{11,48} = 8.387$, $P < 0.0001$, and $F_{11,48} = 11.395$, $P < 0.0001$, respectively). In the TMN, thioperamide and bicuculline produced maximal increases of $110 \pm 13\%$ (Fig. 5B) and $106 \pm 12\%$ (Fig. 5D), respectively. The mean spontaneous release of histamine from the TMN was

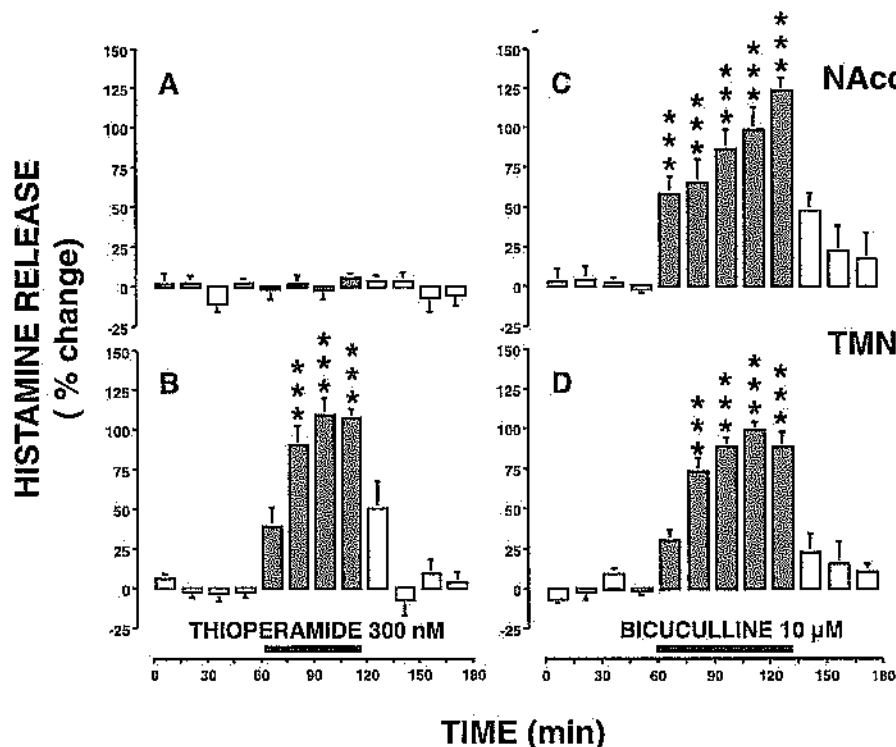


FIG. 4. Influence of thioperamide or bicuculline administration into the tuberomammillary nucleus (TMN) on histamine release from the TMN and the nucleus accumbens (NAcc) of freely moving rats. Histamine was measured and calculated as described in Fig. 2. (A and B) TMN was perfused with 300 nM thioperamide, and histamine release was measured from the NAcc (A) and the TMN (B). (C and D) Bicuculline, at a concentration of 10 μ M, was infused into the TMN, and histamine release was measured in the NAcc (C) and TMN (D). Bars indicate the period of drug application. Shown are means \pm SEM of eight (A and B) and six (C and D) experiments. *** $P < 0.001$ vs. last sample before drug treatment (ANOVA and Bonferroni's test).

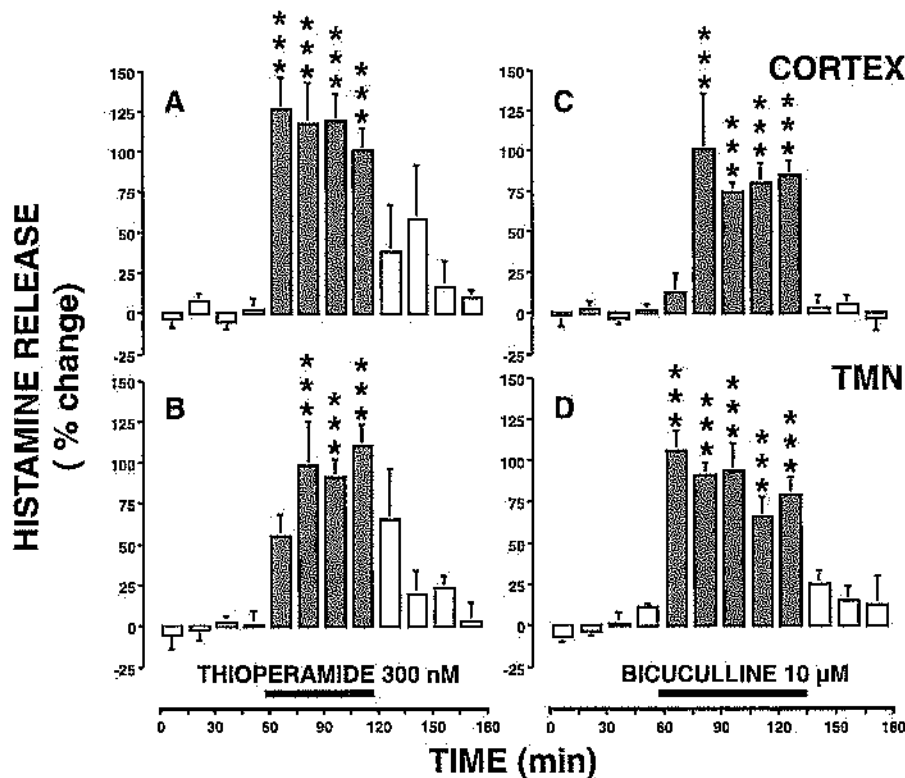


FIG. 5. Influence of thioperamide or bicuculline administration into the tuberomammillary nucleus (TMN) on histamine release from the TMN and the prefrontal cortex of freely moving rats. Histamine was measured and calculated as described in Fig. 2. (A and B) TMN was perfused with 300 nM thioperamide, and histamine release was measured from the prefrontal cortex (A) and the TMN (B). (C and D) Bicuculline, at a concentration of 10 μ M, was infused into the TMN, and histamine release was measured in the prefrontal cortex (C) and TMN (D). Bars indicate the period of drug application. Shown are means \pm SEM of five (A and B) and five (C and D) experiments. *** P < 0.001 vs. last sample before drug treatment (ANOVA and Bonferroni's test).

0.042 \pm 0.004 pmol/15 min in the experiments with thioperamide (n = 5), and 0.046 \pm 0.005 pmol/15 min in those with bicuculline (n = 5). In the prefrontal cortex, thioperamide and bicuculline produced maximal increases of 136 \pm 30% (Fig. 5A) and 101 \pm 34% (Fig. 5D), respectively. The mean spontaneous release of histamine from the prefrontal cortex was 0.042 \pm 0.003 pmol/15 min in the experiments with thioperamide (n = 5) and 0.033 \pm 0.004 pmol/15 min in those with bicuculline (n = 5).

Single-probe experiments: local perfusion with thioperamide increased histamine spontaneous release from the NBM and the prefrontal cortex, but neither from the dorsal striatum nor the NAcc

To explore the local effects of H₃-R blockade in the histaminergic projection areas, each rat was implanted in the NBM, dorsal striatum, NAcc or prefrontal cortex with a single probe used to simultaneously administer thioperamide and monitor local changes in histamine release. A 60-min application of thioperamide (300 nM) in the NBM elicited a significant, transient increase of histamine release (ANOVA, $F_{11,108}$ = 21.199, P < 0.0001) with a maximal value of 130 \pm 10% (Fig. 6A). The mean spontaneous release of histamine from the NBM was 0.050 \pm 0.005 pmol/15 min (n = 10). A similar effect was observed when the prefrontal cortex was perfused with thioperamide. When this drug was added to the medium perfusing the prefrontal cortex at the concentration of 300 nM for 60 min, histamine spontaneous release increased significantly (ANOVA, $F_{11,24}$ = 3.962, P < 0.0001), with a maximum of 87 \pm 8%. The mean average

spontaneous release was 0.040 \pm 0.004 pmol/15 min (n = 3). Histamine spontaneous release returned to baseline levels after perfusion with thioperamide was complete (Fig. 6C). Conversely, perfusion of the NAcc (Fig. 6B) or the dorsal striatum (Fig. 6D) for 60 min with 300 nM thioperamide had no significant effect on spontaneous histamine release. The changes in spontaneous histamine release were always within the range of variability (approx. 20%) observed between individual 15-min collection periods during perfusion with control medium. The mean spontaneous release of histamine was 0.037 \pm 0.003 pmol/15 min from the NAcc (n = 5) and 0.045 \pm 0.009 pmol/15 min from the dorsal striatum (n = 4).

Unilateral, local perfusion of thioperamide into the TMN increased histamine spontaneous release from the TMN and the time spent in wakefulness of freely moving rats

Under baseline conditions during microdialysis experiments about 90% of the rats were quiescent or sleeping during the entire period prior to drug treatment. To understand the profile of wake behaviour in thioperamide-treated rats, we performed a set of experiments specifically aimed at establishing the total time spent in the awake state for 2 h, that is under baseline conditions (TMN-perfusion with control medium) for the first 60 min and during unilateral TMN-perfusion with thioperamide (300 nM) for the following 60 min. Simultaneously, we determined the level of histamine release from the TMN. Experiments were performed between 10.00 and 12.00 h to reduce the influence of circadian phases. As shown in Fig. 7, perfusion of the TMN with 300 nM thioperamide increased signif-

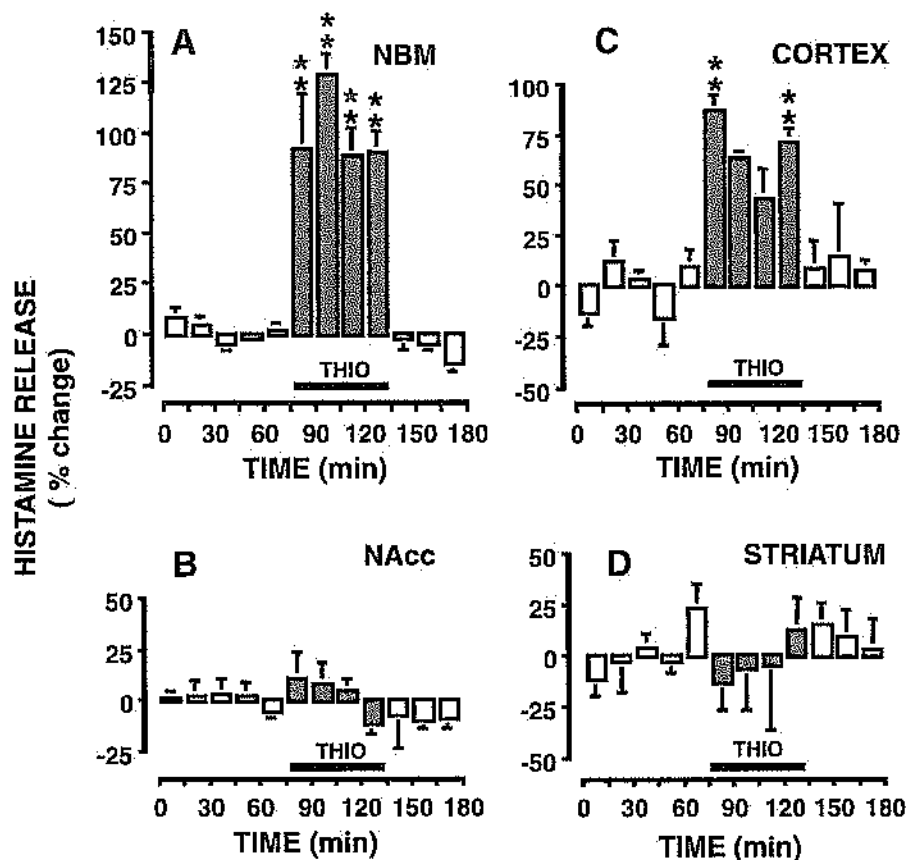


FIG. 6. Effects of local perfusion with thioperamide on histamine release from the nucleus basalis magnocellularis (NBM), the nucleus accumbens (NAcc), the prefrontal cortex and the dorsal striatum of freely moving rats: single-probe experiments. Each rat was implanted in the NBM (A), NAcc (B), prefrontal cortex (C) or dorsal striatum (D) with a single probe used to simultaneously administer thioperamide locally and monitor changes in histamine release. Control values of spontaneous release were calculated for each experiment by averaging the mean of five initially collected 15-min samples. The different regions were perfused with 300 nM thioperamide for 60 min. Bars indicate the period of drug application. Shown are means \pm SEM of 10 (A), five (B), three (C) and four (D) experiments. $**P < 0.01$ vs. last sample before drug treatment (ANOVA and Bonferroni's test).

icantly TMN histamine release from 265 ± 31 fmol/60 min to 530 ± 46 fmol/60 min ($n = 7$, unpaired t -test, $P < 0.0005$), and the time spent in wakefulness from 78 ± 20 to 290 ± 96 s ($n = 7$, unpaired t -test, $P < 0.05$).

H₃-R immunostaining in the posterior hypothalamus

The pattern of H₃-R immunostaining in coronal sections of rat brains was comparable to that previously reported (Chazot *et al.*, 2001; Cannon *et al.*, 2007). The distribution of H₃-Rs on histaminergic neurons in the posterior hypothalamus was examined by performing double-immunofluorescence labelling of hypothalamic slices with a combination of anti-H₃-R and anti-HDC antibodies, the latter to identify histaminergic neurons. In the E2-E3 subdivisions of the TMN, HDC-immunoreactive neurons are densely packed and H₃-R immunostaining is relatively dense. All HDC-positive cells displayed H₃-R-immunoreactive product (Fig. 8).

Discussion

All histaminergic axons originate from the TMN to innervate several brain regions, including the hypothalamus, NBM, NAcc, striatum and prefrontal cortex (Inagaki *et al.*, 1988; Panula *et al.*, 1989a). These

features are consistent with histamine functional influence over a host of physiological processes, such as sleep-wake cycle, appetite, nociception, cognition and emotion (Brown *et al.*, 2001; Haas & Panula, 2003; Passani *et al.*, 2004). However, histaminergic neurons have been regarded as a single functional unit acting as a regulatory network for whole-brain activity (Wada *et al.*, 1991). Here we demonstrate that responses to H₃- or GABA_A-R antagonists differentiate histaminergic neurons according to their projection areas. This is the first evidence that they are organized into functionally distinct circuits impinging on different brain regions, and displaying selective control mechanisms. The histaminergic neurons fire at a rate that can vary, depending on the behavioural state, and the TM neurons are under the feedback control of the H₃ autoreceptor (Haas & Panula, 2003). Ca²⁺, K⁺ and Na⁺ membrane conductances maintain a stable, pacemaker-like firing pattern intrinsic to histaminergic neurons (Haas & Reiner, 1988; Stevens & Haas, 1996). H₃-Rs on neuronal somata provide a tonic inhibition, as the H₃-R antagonist thioperamide enhances neuronal firing (Haas & Panula, 2003) by modulating inward Ca²⁺ currents (Reiner, 1987; Stevens *et al.*, 2001). H₃-Rs are also present on presynaptic terminals (varicosities) of histaminergic axons, where they restrict histamine synthesis and release (Arrang *et al.*, 1983, 1985). Therefore, blockade of somatic and presynaptic H₃-autoreceptors converge in augmenting histamine levels in the synaptic cleft.

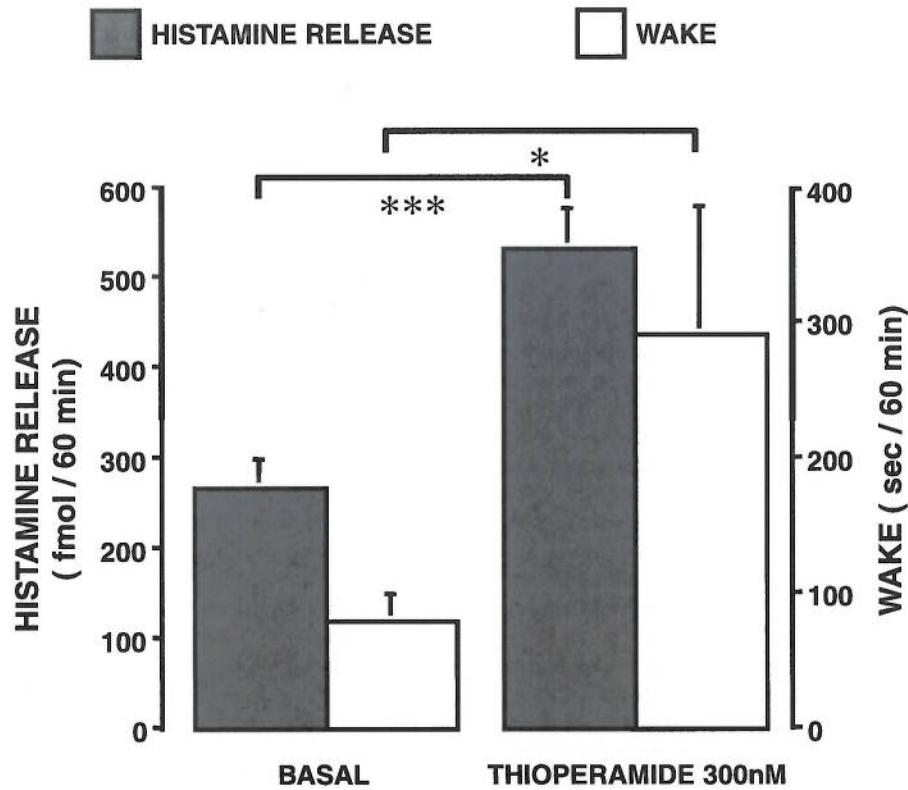


FIG. 7. Influence of thioperamide administration into the TMN on histamine release from the TMN and time spent in wakefulness. Experiments were performed between 10.00 and 12.00 h. Histamine levels were measured in the absence and presence of thioperamide. Rats, implanted with a single probe in the TMN, were initially perfused with control medium for 60 min (basal), then with a medium containing thioperamide (300 nM) for an additional 60 min. Simultaneously, the waking time of each animal was measured by experienced individuals, blind to the experimental protocol. Shown are means \pm SEM of seven rats. * $P < 0.05$; *** $P < 0.001$ (unpaired t -test).

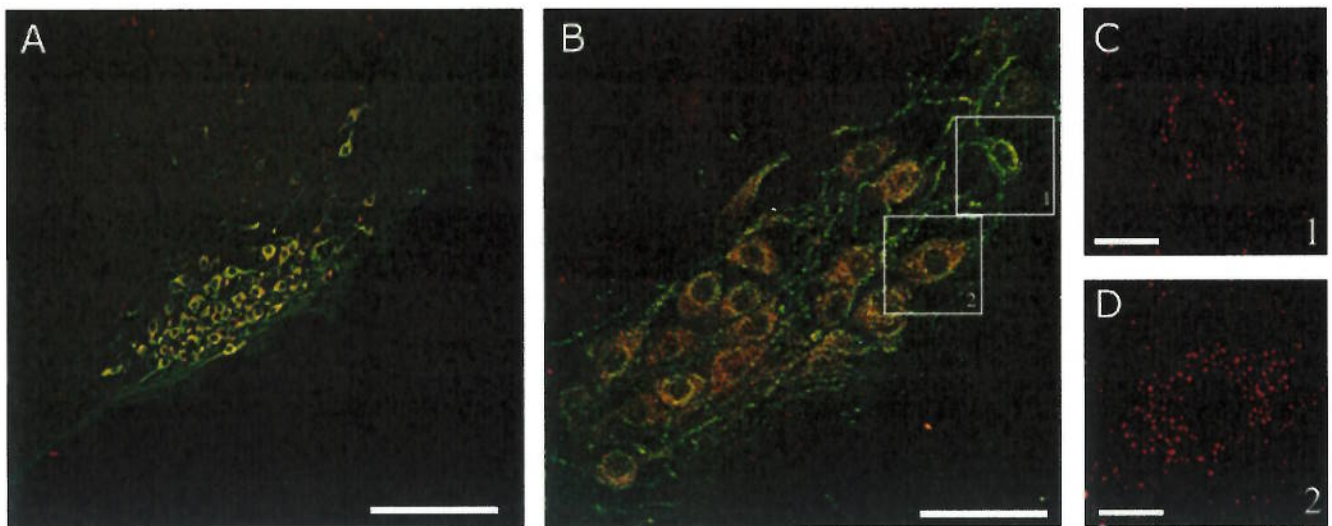


FIG. 8. Distribution of H_3 -R on TMN histaminergic neurons. (A and B) HDC-positive cells in the E2–E3 subdivision of the TMN showing H_3 -R fluorescence; (C and D) high-resolution z-projections of H_3 -R expression in the HDC-positive neurons selected in (B). Observations were repeated in three rats with similar results. Calibration bars: 160 μ m (A); 40 μ m (B); 8 μ m (C and D).

Double-probe microdialysis in freely moving animals provides a powerful means for defining the dynamics regulating histamine release in discrete brain regions. TMN histamine is probably released from

short projections that form extensive axonal arborizations in the posterior hypothalamus. Because in the present study thioperamide was applied locally to the TMN, histamine increase was the

consequence of blocking both somatic and presynaptic H_3 -autoreceptors, although the participation of only one component cannot be excluded.

Following thioperamide administration into the TMN, histamine release was significantly increased in the NBM and prefrontal cortex. This was likely due to discharge potentiation of histaminergic neurons sending afferents to these regions. Analogously, stimulation of the TMN with prostaglandin- E_2 (Huang *et al.*, 2003), orexin-A (Huang *et al.*, 2001) or endocannabinoids (Cenni *et al.*, 2006) increased histamine release from discrete brain regions, as a result of histaminergic projection activation. The NBM and cortex receive a very abundant histaminergic innervation (Panula *et al.*, 1989b), and display both high H_3 -R binding and gene transcripts (Pillot *et al.*, 2002). Accordingly, single-probe microdialysis experiments showed that administration of thioperamide in the NBM or prefrontal cortex augmented significantly histamine release within these regions, an effect that can be explained by blockade of local H_3 -autoreceptors.

It is generally assumed that all histaminergic neurons express H_3 -Rs, and response to H_3 -R ligands is a criterion for their identification *in vitro*. Surprisingly, during TMN perfusion with thioperamide histamine levels remained stable in the dorsal striatum and NAcc, despite the fact that these brain areas receive histaminergic innervation (Panula *et al.*, 1989b). Under the same experimental conditions, another H_3 -R antagonist, GSK189254 (Medhurst *et al.*, 2007), fully mimicked thioperamide actions (Giannoni *et al.*, 2007). Our results indicate that histamine neurons projecting to the striatum and NAcc are insensitive to thioperamide. Although the perfusion site was close to the ventral, and far from the dorsal and rostral TMN, spatial segregation due to probe localization does not explain the lack of response. In fact, retrograde tracing with dye injections into the striatum or prefrontal cortex showed that most histaminergic somata are within the medial part of the ventral TMN (Köhler *et al.*, 1985). This proximity suggests that histaminergic somata projecting to the striatum and prefrontal cortex had the same exposure to thioperamide, but were not affected in the same way. Moreover, bicuculline administered into the TMN augmented significantly histamine release from the NAcc. Under the same experimental conditions, TMN perfusion with cannabinoid receptor 1 agonists increased histamine release from the dorsal striatum (Cenni *et al.*, 2006). Noticeably, also the local perfusion of the striatum or NAcc with thioperamide did not modify histamine release, thus indicating that the whole somatodendritic domain of histaminergic neurons projecting to these regions is insensitive to H_3 -R antagonists. Accordingly, lesion experiments indicate that the vast majority of H_3 -Rs in the NAcc and striatum are not associated with histaminergic fibres (Cumming *et al.*, 1991; Pollard *et al.*, 1993), which is consistent with high densities of mRNAs levels in the same regions (Chazot *et al.*, 2001; Pillot *et al.*, 2002; Cannon *et al.*, 2007), suggesting a postsynaptic localization of these receptors.

Several H_3 -R isoforms have been described, including 6-transmembrane-domain isoforms lacking functional response to H_3 -R ligands in recombinant cells (Bakker *et al.*, 2006). All HDC-positive cell bodies exhibited H_3 -R immunoreactivity, but the antibodies available do not discriminate between isoforms (Chazot *et al.*, 2001). Hence, *in vivo* insensitivity to thioperamide may depend on high expression of particular isoforms.

GABAergic inputs from the ventrolateral preoptic nucleus innervate histaminergic neurons (Ericson, 1991; Sherin *et al.*, 1998). GABA $_A$ -R activation inhibits directly histaminergic cells firing rate (Yang & Hattori, 1997; Stevens *et al.*, 1999), whereas inhibition increases significantly TMN histamine release (Cenni *et al.*, 2006). In the

present study, bicuculline is ineffective on TMN neurons projecting to the NBM, as shown previously (Cenni *et al.*, 2006), and striatum. Conversely, TMN perfusion with bicuculline significantly increased histamine release from the NAcc and prefrontal cortex. These differences are consistent with previous findings. Functional heterogeneity of GABAergic synaptic potentials among histaminergic neurons was found following stimulation of the diagonal band of Broca, antero-lateral hypothalamus or lateral preoptic area (Yang & Hattori, 1997); histaminergic neurons display different degrees of GABA sensitivities depending on GABA $_A$ -R subunit expressions (Sergéeva *et al.*, 2002, 2005). The novel finding here is that sensitivity to bicuculline relates to TMN neurons heterogeneity with respect to projection fields.

Unilateral, intra-TMN administration of thioperamide elicited a significant increase of TMN histamine release, but only a moderate, although significant, waking effect. Thioperamide increased release of histamine from the prefrontal cortex and NBM, two brain regions with prominent roles in histamine waking action (Chu *et al.*, 2004; Ramesh *et al.*, 2004). However, a recent report showed that only a relatively low level of cumulative wake activity for thioperamide was linearly correlated with up to 80% of the receptor occupancy (Le *et al.*, 2008). In contrast, an abrupt break from linearity and a robust increase of waking activity was observed at doses that produce greater than 80% occupancy (Le *et al.*, 2008). Our results suggest a relatively small increase of waking activity that may be consistent with the low level of receptor occupancy achievable with unilateral administration of the drug.

In conclusion, the present data suggest that histaminergic neurons are not uniform, and that they establish functionally distinct pathways according to their terminal projections, that are sensitive to selective pharmacological manipulations, and related to independent functions. Further studies are required to understand the full implications of such functional heterogeneity of histaminergic neurons, especially after systemic administration of H_3 -R antagonists, given their potential for treating a number of important human diseases (Esbenshade *et al.*, 2008).

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Abbreviations

FCS, foetal calf serum; GABA, γ -aminobutyric acid; GABA $_A$ -R, GABA $_A$ receptor; H_3 -R, H_3 -receptor; HDC, histidine decarboxylase; HPLC, high-performance liquid chromatography; NAcc, nucleus accumbens; NBM, nucleus basalis magnocellularis; PB, phosphate buffer; TBS, Tris-buffered saline; TMN, tuberomammillary nuclei.

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