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RESEARCH PAPER

The histamine H_4 receptor is functionally expressed on neurons in the mammalian CNS

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Background and purpose: The histamine H_4 receptor is the most recently identified of the G protein-coupled histamine receptor family and binds several neuroactive drugs, including amitriptyline and clozapine. So far, H_4 receptors have been found only on haematopoietic cells, highlighting its importance in inflammatory conditions. Here we investigated the possibility that H_4 receptors may be expressed in both the human and mouse CNS.
Methods: Immunological and pharmacological studies were performed using a novel anti-H_4 receptor antibody in both human and mouse brains, and electrophysiological techniques in the mouse brain respectively. Pharmacological tools, selective for the H_4 receptor and patch clamp electrophysiology, were utilized to confirm functional properties of the H_4 receptor in layer IV of the mouse somatosensory cortex.
Results: Histamine H_4 receptors were prominently expressed in distinct deep laminae, particularly layer VI, in the human cortex, and mouse thalamus, hippocampal CA4 stratum lucidum and layer IV of the cerebral cortex. In layer IV of the mouse somatosensory cortex, the H_4 receptor agonist 4-methyl histamine (20 $\mu mol \cdot L^{-1}$) directly hyperpolarized neurons, an effect that was blocked by the selective H_4 receptor antagonist JNJ 10191584, and promoted outwardly rectifying currents in these cells. Monosynaptic thalamocortical CNQX-sensitive excitatory postsynaptic potentials were not altered by 4-methyl histamine (20 $\mu mol \cdot L^{-1}$) suggesting that H_4 receptors did not act as hetero-receptors on thalamocortical glutamatergic terminals.
Conclusions and implications: This is the first demonstration that histamine H_4 receptors are functionally expressed on neurons, which has major implications for the therapeutic potential of these receptors in neurology and psychiatry.
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Keywords: histamine H_4 receptor; human; mouse; brain; neurons; hyperpolarization; JNJ 10191584

Abbreviations: CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; EPSP, excitatory postsynaptic potential; JNJ 10191584, (1-[(5-chloro-1H-benzimidazol-2-yl)carbonyl]-4-methylpip erazine maleate)

Introduction

The histamine H_3 and H_4 receptors are two closely related and recently discovered members of the histamine receptor family, both targets for the new-generation of 'anti-histamine' drugs (de Esch *et al.*, 2005). The H_3 receptor is a presynaptic auto- and hetero-receptor, reported to be abundantly expressed in the CNS of different mammalian species (Chazot *et al.*, 2001; Pillot *et al.*, 2002; Cannon *et al.*, 2006), including human (Coge *et al.*, 2001a).

The human histamine H_4 receptor (hH_4 receptor) is the most recently discovered member of the G protein-coupled receptor subfamily of histamine receptors (Nakamura *et al.*, 2000; Oda *et al.*, 2000; Liu *et al.*, 2001; Morse *et al.*, 2001; Nguyen *et al.*, 2001; Zhu *et al.*, 2001). The H_4 receptor is predominantly expressed in haematopoietic cells and is suggested to play a role in inflammation (O'Reilly *et al.*, 2002; Hofstra *et al.*, 2003; Dijkstra *et al.*, 2007; 2008; Bäumer *et al.*, 2008) and allergy (Dunford *et al.*, 2006). The H_4 receptor has also been linked with rheumatoid arthritis (Ikawa *et al.*, 2005), colon cancer (Cianchi *et al.*, 2005; Varga *et al.*, 2005) and breast cancer (Maslinska *et al.*, 2006). Our recent data

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have suggested that the H_4 receptor is not exclusively expressed on haematopoietic cells (Grandi *et al*., 2008) and, consequently, this receptor has potential as a new drug target in a number of therapeutic areas. Several early studies could not detect H_4 receptors in the brain (e.g. Nakamura *et al*., 2000), but others have detected mRNA for these receptors in discrete brain regions (Coge *et al*., 2001b; Liu *et al*., 2001), but these authors do speculate whether the relatively sparse CNS labelling may reflect immune cell infiltration of the CNS. Here, we use our previously validated anti-hH_4 receptor antibody (van Rijn *et al*., 2006; Dijkstra *et al*., 2007) combined with patch clamp electrophysiology and selective pharmacological probes to locate human and rodent H_4 receptor protein in the CNS and address the important hypothesis that the H_4 receptor protein is functionally expressed on neurons in the brain.

This study demonstrates, for the first time, the topological and functional expression of H_4 receptors in mammalian brain, allowing neuroscientists and neurologists to profile its roles (in the healthy and diseased brains) and appraise its potential for future drug development strategies.

Methods

Antibodies

Anti-hH_4 receptor 374-390 antibodies were produced and validated for detecting both human and rodent H_4 receptors in the School of Biological and Biomedical Sciences, Durham University (van Rijn *et al*., 2006; 2008; Dijkstra *et al*., 2007; 2008; Bäumer *et al*., 2008; Grandi *et al*., 2008; Morini *et al*., 2008). The human 374-390 peptide sequence shares over 50% homology with rodent sequences.

Immunological studies

Immunoblotting and immunohistochemistry were performed using brains from 6 week C3H/HeJ mice as described in our previous studies (Chazot *et al*., 2001; Cannon *et al*., 2006; van Rijn *et al*., 2006; Dijkstra *et al*., 2007; Grandi *et al*., 2008). The normal human post-mortem cortex material for the immunoblots was obtained with full ethical approval under the UK Newcastle and Tyneside LREC 2002/295.

Immunoblotting. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out using 7.5% (w/v) polyacrylamide slab gels under reducing conditions. Samples of membranes (20–50 μg protein) were prepared using a chloroform/methanol method of protein precipitation, and immunoblotting was performed as previously described (Chazot *et al*., 2001; Bakker *et al*., 2006; van Rijn *et al*., 2006). Blots were probed with our validated rabbit anti-hH_4 receptor 374-390 antibodies at 1 $\mu g \cdot mL^{-1}$, in the absence and presence of 50-fold excess 374-390 peptide (van Rijn *et al*., 2006; Dijkstra *et al*., 2007; 2008). Horseradish peroxidase-conjugated goat anti-rabbit antibodies (1:2000) were used as secondary antibodies (Little Chalfont, Buckinghamshire, England).

Immunohistochemistry. The human brain tissue for the immunohistochemistry study was obtained from the Neurological Foundation of New Zealand Human Brain Bank (Department of Anatomy with Radiology, University of Auckland). The University of Auckland Human Participants Ethics Committee approved the protocols used in these studies and all tissue was obtained with full consent of the families. Brain tissue was obtained from three neurologically normal cases, with an average age of 63 years (range 53–77 years), with no history of neurological disease and no evidence of neuropathology. The cases had a post-mortem interval between 16 and 23 h after death (mean post-mortem interval 18.6 h). For the immunohistochemistry studies, the human brains were processed as previously described (Waldvogel *et al*., 2006). In brief, the human brains were fixed by perfusion through the basilar and internal carotid arteries, first with phosphate-buffered saline (PBS) with 1% (w/v) sodium nitrite, followed by 15% (v/v) formalin in 0.1 $mol \cdot L^{-1}$ phosphate buffer, pH 7.4. After perfusion, blocks from the basal ganglia were carefully dissected out and kept in the same fixative for 24 h. The tissue blocks were cryoprotected in 20% (w/v) sucrose in 0.1 $mol \cdot L^{-1}$ phosphate buffer with 0.1% (w/v) sodium azide for 2–3 days, and then in 30% (w/v) sucrose in 0.1 $mol \cdot L^{-1}$ phosphate buffer with 0.1% (w/v) sodium azide for a further 2–3 days. The blocks were sectioned on a freezing microtome (50 μm) and the sections were stored in PBS with 0.1% (w/v) sodium azide.

Adjacent series of sections were selected and processed free-floating in tissue culture wells using standard immunohistochemistry procedures. Sections were washed in PBS and 0.2% (v/v) Triton-X (PBS-triton) and pretreated for antigen retrieval using standard protocols (Waldvogel *et al*., 2004) before being processed. Briefly, sections for antigen retrieval were transferred to six-well tissue culture plates and incubated overnight in 0.1 $mol \cdot L^{-1}$ sodium citrate buffer, pH 4.5, transferred to 10 mL of fresh sodium citrate buffer solution, microwaved in a 650 W microwave oven for 30 s and allowed to cool before washing (3×15 min) in PBS-triton. The sections were then incubated for 20 min in 50% (v/v) methanol and 1% (v/v) H_2O_2, washed (3×15 min) in PBS-triton, and incubated in primary antibodies for 2–3 days on a shaker at 4°C. The primary antibodies were washed off (3×15 min, PBS-triton) and the sections incubated overnight in biotinylated sheep anti-rabbit at 1:500 (secondary antibody, Chemicon, USA). The secondary antibodies were washed off (3×15 min, PBS-triton), the sections incubated for 4 h at room temperature in ExtrAvidin™, 1:1000 (Sigma) and reacted in 0.05% (w/v) 3,3-diaminobenzidine tetrahydrochloride (Sigma) and 0.01% (v/v) H_2O_2 in 0.1 $mol \cdot L^{-1}$ phosphate buffer, pH 7.4, for 15–30 min to produce a brown reaction product. The sections were washed in PBS, mounted on gelatine chrom-alum-coated slides, rinsed in distilled water, dehydrated through a graded alcohol series to xylene, and coverslipped with DPX (BDH, Poole, England, UK).

Control sections were routinely processed to determine non-specific staining using the same immunohistochemical procedures detailed above, except that the primary antibody was omitted from the procedure. In addition, some sections were Nissl-stained with cresyl violet according to standard techniques.

For mouse studies, in brief, perfusion fixation was performed with 4% (w/v) paraformaldehyde (PFA)/PBS pH 7.4.

Cardiac perfusion fixation in the mice was performed initially with PBS/0.01% (w/v) sodium nitrite, followed by ice-cold 4% (v/v) PFA/PBS pH 7.4, and brains dissected. The brains were then incubated overnight in ice-cold 4% (w/v) PFA/PBS pH 7.4, prior to sequential PBS/20% and 30% (w/v) sucrose/PBS pH 7.4 for 2 days as described above. Following sucrose infiltration, the samples were frozen in iso-pentane at –70°C for 1 min and sectioned in a cryostat at –26°C (20–30 μm sections). The immunohistochemistry for the mouse brain slices was performed as described in Chazot *et al.* (2001). Slices were probed with the rabbit anti-hH_4 receptor 374-390 antibodies at 1 $\mu g \cdot mL^{-1}$, in the absence and presence of 50-fold excess 374-390 peptide (van Rijn *et al.*, 2006; Dijkstra *et al.*, 2007; 2008).

Electrophysiological and pharmacological studies

The 4- to 6-week-old male C3H/HeJ mice were given a lethal dose of pentobarbital (120 $mg \cdot kg^{-1}$; i.p.), according to University of Otago animal welfare protocol ET27/07. Brains were rapidly dissected out into ice-cold sucrose artificial CSF (aCSF) of the following composition (in $mmol \cdot L^{-1}$): 248 sucrose, 3 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 10 glucose (saturated with 95% O_2, 5% CO_2). Thalamocortical slices containing the somatosensory cortex were prepared as described by Agmon and Connors (1991). Briefly, using a vibratome (VT1000S; Leica, Ora, Italy) 200–300 $\mu mol \cdot L^{-1}$ thick slices were cut at 50° to the coronal plane, rotated through the caudo-rostral axis and placed in a holding chamber at 35°C in aCSF of the following composition (in $mmol \cdot L^{-1}$): 124 NaCl, 3 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 1 sodium ascorbate, 3 sodium pyruvate (bubbled with 95% O_2, 5% CO_2) for 30 min and then allowed to return to room temperature. Slices were incubated under these conditions for at least 30 min before recording began. Slices were placed in a custom-made recording chamber on the stage of a differential interference contrast microscope (E600FM DIC; Nikon, Tokyo, Japan) and perfused with room temperature (20–24°C) aCSF (Lees *et al.*, 2006; Payne *et al.*, 2006).

Whole-cell patch clamp recordings were made using 2–5 MΩ thin-walled electrodes filled with the following solution (in $mmol \cdot L^{-1}$): 120 potassium gluconate, 10 EGTA, 10 HEPES, 1 $CaCl_2$, 2 $MgCl_2$, 4 phosphocreatine, 2 Na_2ATP, 0.2 NaGTP. During voltage clamp experiments, series resistance was regularly monitored and, if it rose above 30 MΩ or changed by more than 20%, the cell was excluded from further analysis. Series resistance was compensated by 70–80%. Neurons were designated as pyramidal cells, fast-spiking interneurons or low-threshold interneurons using standard morphological and electrophysiological characteristics (Kawaguchi and Kubota, 1993; Povysheva *et al.*, 2006). 4-Methylhistamine (4-MH), mepyramine maleate and cimetidine (Tocris, UK) were dissolved in aCSF, and JNJ 10191584 (Tocris, UK) was dissolved in DMSO. All solutions were made freshly each day. The final constant concentration of 1:10 000 DMSO was also present during control and wash phases.

Data analysis

Results are shown as means ± SEM and were analysed by Student's *t*-test or one-way ANOVA with Dunnett's *post hoc* test, as appropriate. *P*-values less than 0.05 were taken to show significant differences between means. Data analysis assumed normality and used Prism 4 software from Graphpad (CA, USA).

Results

Immunological studies

We used established immunoblotting and immunohistochemistry approaches (van Rijn *et al.*, 2006; Dijkstra *et al.*, 2007; Morini *et al.*, 2008) to seek evidence that the H_4 receptor protein is expressed in both the human and mouse brains. On immunoblots, a specific major protein species (approximately M_r 75 000) was detected in human and mouse brain samples, which is coincident with the N-glycosylated dimeric species detected in human lymphocytes and recombinant hH_4 receptors expressed in HEK 293 cells (Figure 1, lanes 1 and 2; van Rijn *et al.* (2006; 2008). These immunoreactive species were greatly suppressed by prior incubation with the respective oligopeptide (Figure 1, lanes 3 and 4).

In order to investigate the anatomical topography of the expression of H_4 receptors, immunohistochemical studies were performed using human cortical and mouse whole brain slices. Clear punctate labelling was observed decorating the cell bodies and processes of neurons within sections of healthy human insular cortex in multiple deep laminae (Figure 2), particularly prominent in layer VI, which may indicate a new signalling role for H_4 receptors in grey matter.

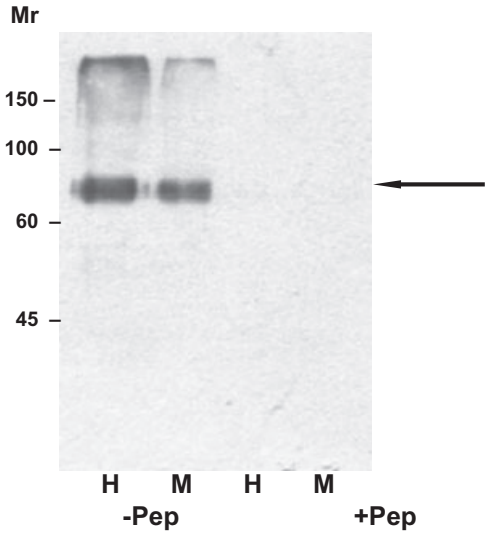

Figure 1 Immunological evidence for the presence of histamine H_4 (hH_4) receptors in the human and mouse brains. Immunoblotting studies were performed using brains from 6 week C3H/HeJ mice and post-mortem tissue from normal human brain. Human (lane 1) and mouse (lane 2) cortex membranes were applied to 7.5% (w/v) sodium dodecyl sulphate polyacrylamide gel electrophoresis gels under reducing conditions, subjected to immunoblotting and probed with rabbit anti-hH_4 receptor 374-390 antibodies at 1 $\mu g \cdot mL^{-1}$, in the absence (lanes 1 and 2) and presence of 50-fold excess 374-390 peptide (lanes 3 and 4). A coincident immunoreactive species (M_r 75 000) was detected, which corresponds well with both the recombinant dimeric hH_4 receptor and native dimeric species detected in human lymphocytes (van Rijn *et al.*, 2006; 2008).

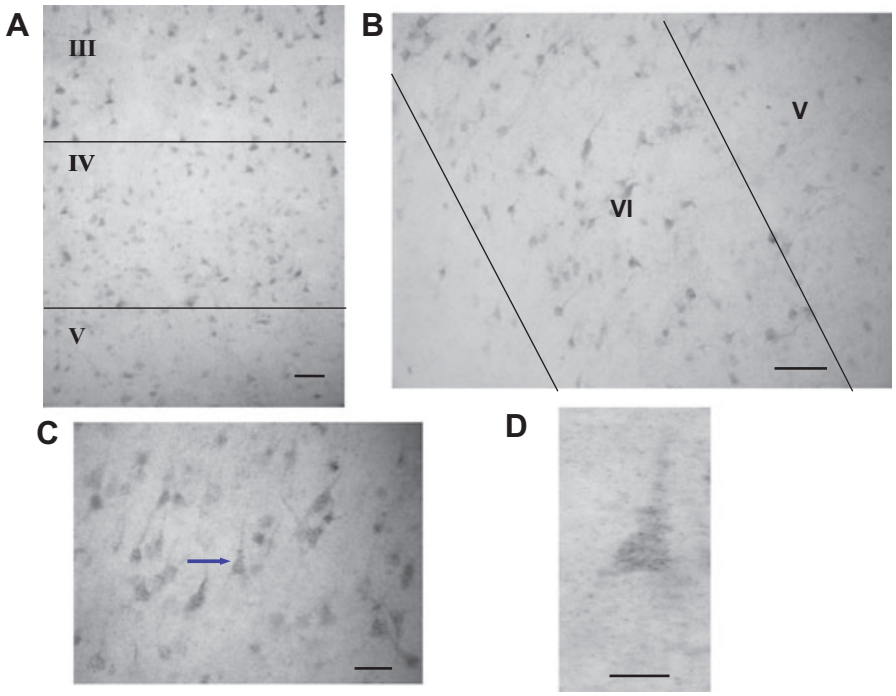

Figure 2 Immunological evidence for the presence of histamine H_4 receptors in the human cortex. Fixed post-mortem normal human brain slices (described in *Methods* section and Waldvogel *et al.*, 2006) were permeabilized and probed with rabbit anti-hH_4 receptor 374-390 antibodies at 1 $\mu g \cdot mL^{-1}$. (A) Insular cortex layers III–V lateral to the basal ganglia (scale bar = 50 μm). (B) Insular cortex layers V–VI lateral to the basal ganglia (scale bar = 50 μm). (C) and (D) Magnified layer VI immunoreactive human cortical cell showing punctate decoration of both cell soma and processes (scale bar = 100 μm).

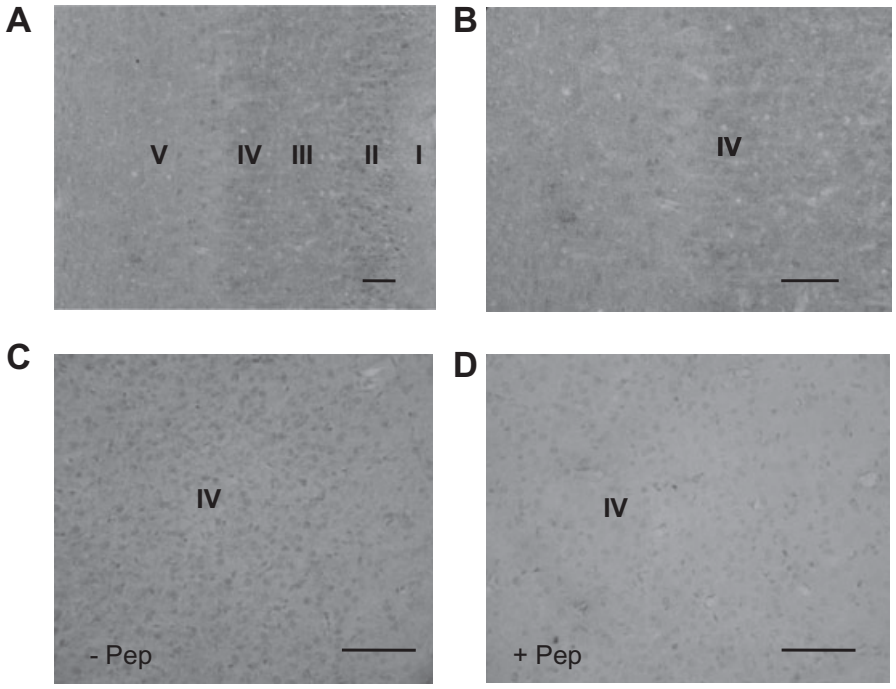

Figure 3 Immunological evidence for the presence of histamine H_4 receptors in the mouse cortex. Perfusion-fixed C3H mouse horizontal brain slices were permeabilized and subjected to immunohistochemical analysis as described in Chazot *et al.* (2001), probed with rabbit anti-hH4 receptor 374-390 antibodies at 1 $\mu g \cdot mL^{-1}$. Images focusing on layer IV cerebral cortex, (A) magnification ×100, scale bar = 100 μm; (B) magnification ×200, scale bar = 50 μm; (C) anti-hH_4 receptor 374-390 antibodies (magnification ×400; scale bar = 50 μm); (D) anti-hH_4 receptor 374-390 antibodies at 1 $\mu g \cdot mL^{-1}$ pretreated with 50-fold excess peptide hH_4 receptor 374-390 (magnification ×400; scale bar = 50 μm), magnification ×200, scale bar = 50 μm.

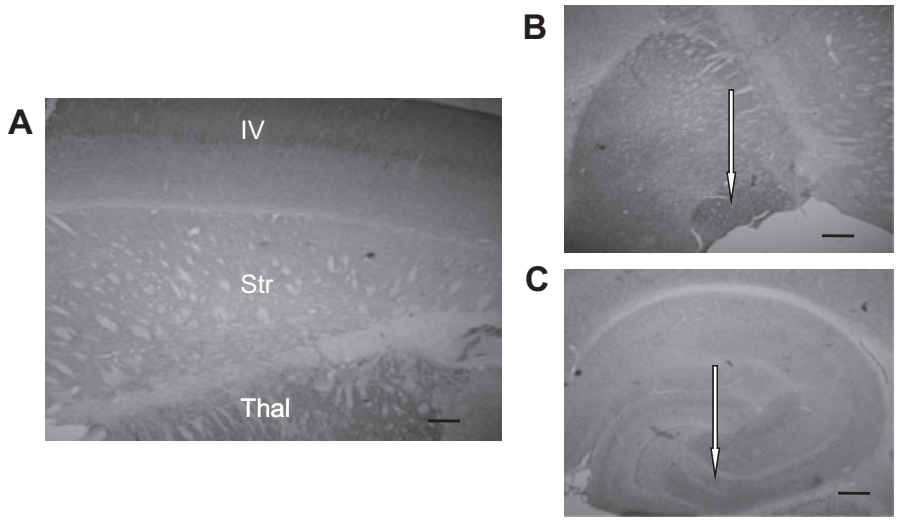

Figure 4 Immunological evidence for the presence of histamine H_4 receptors in the mouse thalamus and hippocampal formation. Perfusion-fixed 4–6 week C3H mouse horizontal brain slices were permeabilized and subjected to immunohistochemical analysis as described in Chazot *et al.* (2001), probed with rabbit anti-hH4 receptor 374-390 antibodies at 1 $\mu g \cdot mL^{-1}$. Images focusing on: (A) layer IV cerebral cortex, striatum and thalamus, scale bar = 150 μm; (B) thalamus (strong labelling of posterior pole) and striatum, scale bar = 200 μm; (C) hippocampal formation CA4 (prominent labelling)/dentate gyrus, scale bar = 200 μm.

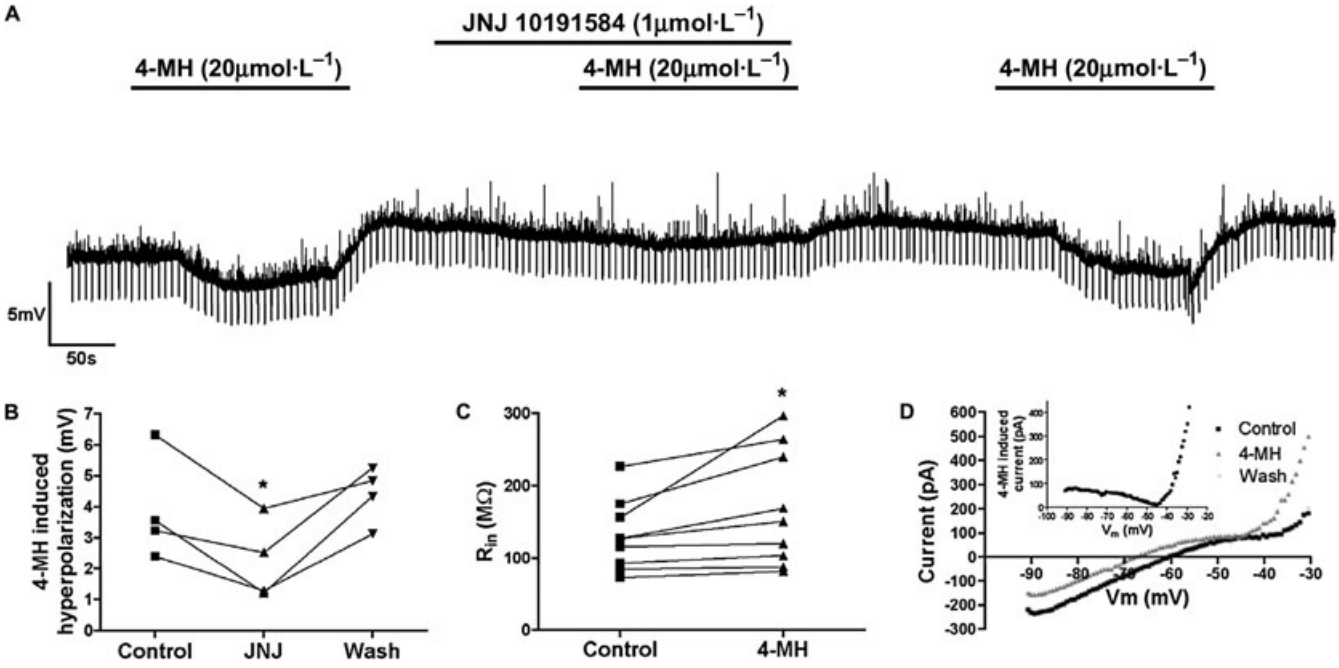

Figure 5 The histamine H_4 receptor was functionally active in the mouse layer IV somatosensory cortex: activation of H_4 receptors produced a hyperpolarizing response in layer IV somatosensory cortex neurons of 4- to 6-week-old C3H mice by closing an ion channel. (A) A representative trace showing the effect of the H_4 receptor agonist 4-methylhistamine (4-MH, 20 $\mu mol \cdot L^{-1}$) on resting membrane potential, and the partial block of this by the selective H_4 receptor antagonist JNJ 10191584 (JNJ, 1 $\mu mol \cdot L^{-1}$). (B) JNJ significantly reduced the hyperpolarizing effect of 4-MH (control, -3.9 ± 0.8 mV; JNJ, -2.2 ± 0.6 mV; wash, -4.4 ± 0.4 mV; control vs. JNJ $P < 0.05$, $n = 4$, Dunnett's test). (C) Under voltage clamp, 4-MH produced a significant increase in input resistance (control, 131 ± 16.2 MΩ; 4-MH, 168.3 ± 26.8 MΩ, $P < 0.05$, $n = 9$, paired *t*-test). (D) A slow voltage ramp revealed that the hyperpolarizing effect of 4-MH was due to modulation of a largely voltage-independent current, and that at depolarized potentials, 4-MH enhanced an outward current. $^*P < 0.05$.

In the mouse forebrain, a distinct unique expression pattern of anti-H_4 receptor immunoreactivity was revealed, with notable prominent expression in the thalamus (particularly in the posterior nuclei), layer IV of the cerebral cortex, the entorhinal cortex and stratum lucidum of the CA4 (Figures 3 and 4, Table 1). In contrast, very low expression was seen in the striatum and the remaining subfields of the hippocampus (Figure 4C). All positive immunoreactivity was greatly suppressed by pre-absorption with oligopeptide (e.g. Figure 3D for cortical labelling).

Table 1 Comparison of histamine H_3 and H_4 receptor expression in the mouse brain

Brain structure	*Expression levels*	
	H_4	*H_3*
Cerebral cortex		
Lamina I	+	(+)
II	++	++
III	+	+
IV	+++	++
V	++	+++
VI	+	+
Hippocampal formation		
CA1	+	(+)
CA2	+	(+)
CA3/4	++	+
Dentate gyrus	+	+
Entorhinal cortex	+++	+++
Cerebellum		
Granule cell layer	+++	+
Purkinje cell layer	(+)	+++
Molecular cell layer	+	(+)
Thalamus	+++	+
Striatum	(+)	+++

Qualitative summary of the relative levels of histamine H_3 and H_4 receptors in selected C3H mouse brain structures: +++ high, ++ moderate, + low overall intensity of staining, (+) light diffuse or scattered profiles. Data from immunohistochemical experiments using anti-H_3 and anti-H_4 receptor antibodies (Chazot *et al.*, 2001; Cannon *et al.*, 2006; van Rijn *et al.*, 2006).

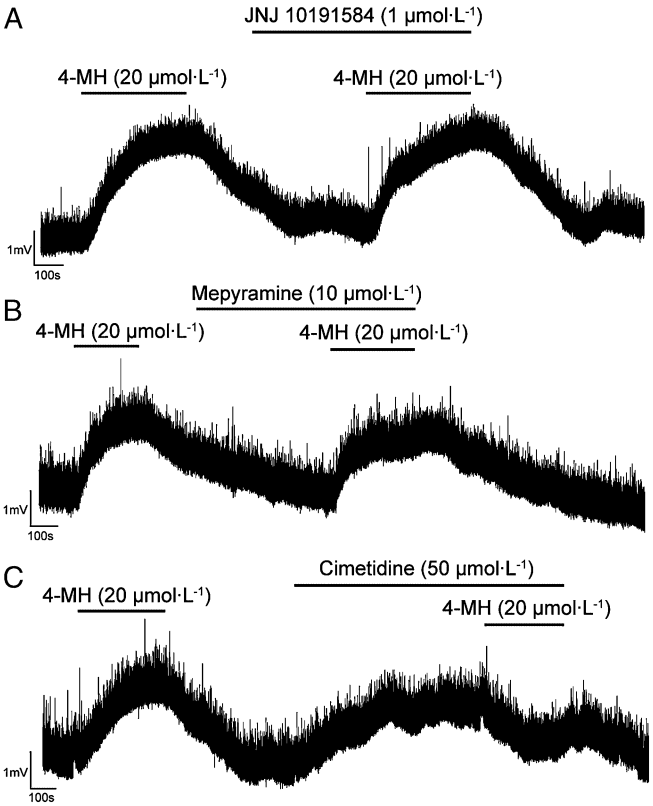

Figure 6 4-Methylhistamine (4-MH) induced a depolarization in a small number of cells via the histamine H_2 receptor. In three out of 24 cells, 4-MH (20 μmol·L^{-1}) produced a depolarizing response. (A) A representative trace showing the lack of effect of the H_4 receptor antagonist JNJ 10191584 (1 μmol·L^{-1}). (B) The H_1 receptor antagonist mepyramine (10 μmol·L^{-1}) had no effect on the depolarizing response induced by 4-MH (20 μmol·L^{-1}). (C) Cimetidine (50 μmol·L^{-1}) blocked the depolarizing response induced by 4-MH (20 μmol·L^{-1}) and revealed a small hyperpolarizing response.

Functional electrophysiological and pharmacological studies

To evaluate whether the presence of H_4 receptors in the most heavily labelled neurons was of functional significance, we performed an electrophysiological study using recently developed hH_4 receptor selective compounds (Jablonowski *et al.*, 2003; Lim *et al.*, 2005).

Whole cell voltage and current clamp recordings were taken from layer IV somatosensory cortex cells in adult mouse brain slices. In the presence of tetrodotoxin (500 nmol·L^{-1}) to block multiquantal neurotransmitter release, application of the H_4 receptor agonist 4-MH (20 μmol·L^{-1}) produced a significant reversible hyperpolarization in the majority of the neurons tested (17/24; control, −64.8 ± 1.0 mV; 4-MH, −68.9 ± 1.0 mV, $P < 0.0001$, paired *t*-test; Figure 5A). A small number of neurons were unresponsive (4/24) or responded with a ~4 mV depolarization (3/24). There was no apparent correlation between the nature of the response and whether the neuron was a pyramidal cell, a fast-spiking or low-threshold spiking interneuron. The hyperpolarizing response was significantly and reversibly reduced by the highly selective H_4 receptor antagonist, JNJ 10191584 (1 μmol·L^{-1}; control, −3.9 ± 0.8 mV; JNJ, −2.2 ± 0.6 mV; wash, −4.4 ± 0.4 mV; control vs. JNJ $P < 0.05$, $n = 4$, Dunnett's test; Figure 5B). The depolarizing response was mediated by histamine H_2 receptors as it was blocked by the selective H_2 receptor antagonist cimetidine (50 μmol·L^{-1}), and was unaffected by the H_1 receptor antagonist mepyramine (10 μmol·L^{-1}) or the H_4 receptor antagonist JNJ 10191584 (1 μmol·L^{-1}). Indeed, cimetidine revealed a small, presumably H_4 receptor-mediated, hyperpolarizing response that was previously masked by the H_2 receptor-mediated depolarization (Figure 6). Under voltage clamp (V_h −70 mV), 4-MH (20 μmol·L^{-1}) induced an apparent outward current and a significant increase in input resistance (control, 131 ± 16.2 MΩ; 4-MH, 168.3 ± 26.8 MΩ, $P < 0.05$, $n = 9$, paired *t*-test; Figure 5C). A slow voltage ramp (7.5 mV·s^{-1}) demonstrated that 4-MH (20 μmol·L^{-1}) reduced a largely voltage-independent current between −90 and −45 mV, but at potentials more depolarized than this the H_4 receptor agonist markedly enhanced an outward voltage-gated current (Figure 5D).

Because the layer IV cortical neurons, which are the major site for the termination of thalamocortical fibres (Herkenham, 1980), were selectively labelled for H_4 receptors, it was possible that some of the H_4 receptor immunoreactivity was due to H_4 receptors expressed on thalamocortical terminals. To address this possibility we evoked monosynaptic thalamocortical excitatory postsynaptic potentials by stimulating the ventrobasal thalamus. However, the amplitude of excitatory postsynaptic potentials, sensitive to CNQX, was not significantly altered by 4-MH (20 μmol·L^{-1}) (control, 112 ± 5.2% of baseline; 4-MH, 116 ± 17% of baseline, $P > 0.05$, $n = 4$, paired *t*-test; Figure 7).

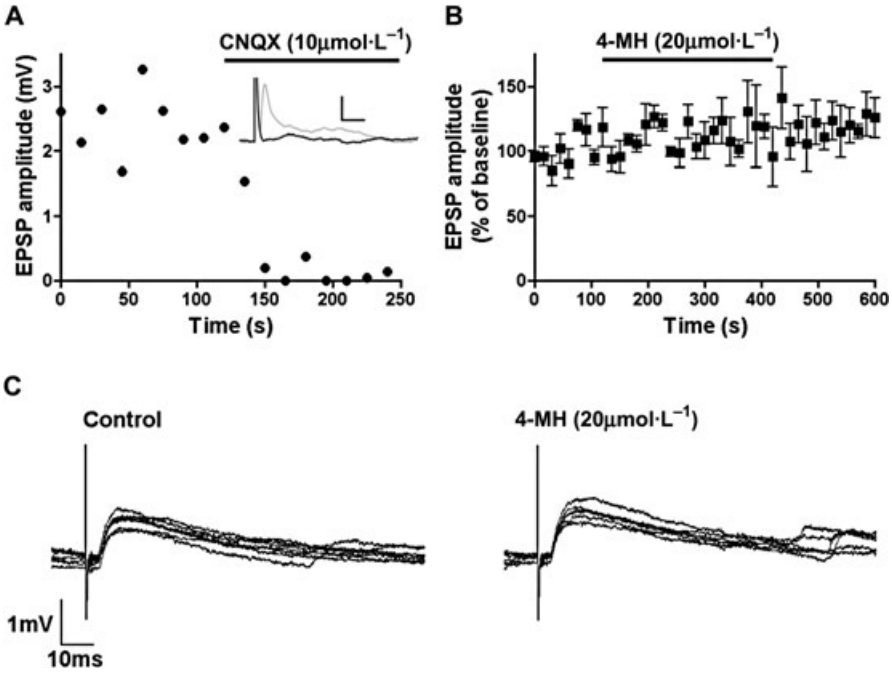

Figure 7 Activation of histamine H_4 receptors did not modulate thalamocortical synaptic traffic. (A) Corticothalamic excitatory postsynaptic potentials (EPSPs) in the 4- to 6-week-old C3H mouse were CNQX-sensitive (averaged trace in insert, control in grey, CNQX in black, scale bars 1 mV, 10 ms). (B) The amplitude of thalamicocortical EPSPs were not modulated by 4-MH (20 μmol·L^{-1}; $n = 4$). (C) Representative overlays of thalamocortical EPSPs before and after the application of 4-MH.

Discussion

Based on the selectivity of our antibodies (van Rijn *et al.*, 2006; 2008; Dijkstra *et al.*, 2007; 2008; Bäumer *et al.*, 2008; Grandi *et al.*, 2008; Morini *et al.*, 2008)) and pharmacological probes (Terzioglu *et al.*, 2004; Varga *et al.*, 2005; Venable *et al.*, 2005), these findings provide strong evidence that the H_4 receptor was both expressed, and was functionally active, on neurons in the mammalian CNS. The unique expression pattern indicates that the H_4 receptor may have an important role to play in the central histaminergic system in addition to the other well-described members of the histamine receptor family (Table 1, Brown *et al.*, 2001; Haas *et al.*, 2008). The presence of labelling in the cerebral cortex in both the human and mouse brains contrasts with the apparent lack of the corresponding mRNA in the human cerebral cortex (Coge *et al.*, 2001b) and implies that, in the human cortex, expression of H_4 receptors is on fibres emanating from other brain regions (most likely the hippocampus or thalamus). Our electrophysiological data demonstrated that 4-MH (20 μmol·L^{-1}) induced a ~4 mV hyperpolarization in layer IV neurons, and that this effect was blocked by the highly selective H_4 receptor antagonist JNJ 10191584. At this concentration, 4-MH is likely to act as an agonist at histamine H_2 receptors (Lim *et al.*, 2005; Breunig *et al.*, 2007) and potentially also at H_3 receptors (Lim *et al.*, 2005). Therefore, it is not surprising that in three out of 24 neurons tested, 4-MH (20 μmol·L^{-1}) produced a H_2 receptor-mediated depolarization. It is unlikely that the hyperpolarization can be attributed to 4-MH acting at other histamine receptors, as histamine H_3 receptor ligands have no effect on the resting potential of cortical neurons and H_1 receptor agonists are known to depolarize cortical neurons (W.M. Connelly, unpubl. obs.; Reiner and Kamondi, 1994). The pattern of expression of H_4 receptors shows some similarities to the H_3 receptors, such as overlap in entorhinal cortex and deep laminae of the cortex, but contrasts particularly in the relatively high expression in the thalamus (Chazot *et al.*, 2001; Pillot *et al.*, 2002). Recent published *in vivo* pharmacological studies would concur with a role for H_4 receptors in the CNS, in control of pain transmission at the level of the spinal cord (Cowart *et al.*, 2008; Strakhova *et al.*, 2009). Indeed, we also have preliminary evidence for H_4 receptor expression in the rodent spinal cord and dorsal root ganglia, which yields an anatomical framework for this proposed nociceptive role, and for the potential of H_4 receptors as a novel analgesic therapeutic target (not shown).

Our results indicate that activation of H_4 receptors directly hyperpolarized cortical neurons but concurrently enhanced the input resistance of the cells. Such actions are opposed to those of activated H_1 receptors, which depolarize cortical neurons by closing leak potassium currents (Reiner & Kamondi, 1994). The mechanism for this inhibitory response and the contributory ion channels and second messengers will be the focus of future studies in both the Otago and Durham laboratories. It is interesting to note that H_4 receptors are much more sensitive sensors for histamine than H_1 receptors (affinites of 1–50 nmol·L^{-1} vs. 2–10 μmol·L^{-1}; Liu *et al.*, 2001; Nguyen *et al.*, 2001; Zhu *et al.*, 2001; Booth *et al.*, 2002; Seifert Wenzel-Seifert *et al.*, 2003; Haas *et al.*, 2008). This may indicate that H_4 receptors are able to sense the low level of histamine present during sleep, while the higher concentration of histamine during wakefulness may allow the depolarizing influence of H_1 receptors to predominate (Strecker *et al.*, 2002; Chu *et al.*, 2004). It would be interesting to explore the

systems and behavioural effects of co-activation of H_1 and H_4 receptors by histamine on neuronal excitability. The growing availability of brain-permeant selective agonists and antagonists for H_4 receptors will help unravel the roles for the new receptor and its potential as a therapeutic target.

Our study suggests that H_3 receptors (already established as a predominately presynaptic species regulating the release of histamine and a range of other transmitters) and H_4 receptors subserve distinct and contrasting roles in the mammalian brain. Whether alternative splicing in H_4 receptors has functional relevance in the brain, as indicated for the H_3 receptor (Bakker *et al*., 2006; van Rijn *et al*., 2006), remains to be seen. It is fascinating to reflect that many of the drugs that bind to hH_4 receptors in the low micromolar range, such as amitriptyline and clozapine, are frequently used as neurological or psychiatric disease modifiers (Nguyen *et al*., 2001). Furthermore, these results demonstrate the urgent need to re-evaluate previously published studies in the CNS, which utilized first-generation ligands, which are now known to bind both H_3 and H_4 receptors, including thioperamide and clobenpropit (Lim *et al*., 2005). Revealing the functional presence of this receptor in the CNS for the first time will allow a broader neuropharmacological community to characterize roles for this G protein-coupled receptor, appraise its involvement in brain diseases (and drug action) and to evaluate its potential as a target for new drugs particularly in neurological diseases, notably inflammatory pain disorders and dementias where inflammation (the potential of hH_4 receptors in immunology and inflammation is already established) plays a prominent role.

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