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Histamine regulates activities of neurons in the ventrolateral preoptic nucleus

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The neurons responsible for the onset of sleep are thought to be located in the ventrolateral preoptic nucleus (VLPO), which receives a dense histaminergic innervation from the tuberomammillary nucleus (TMN). Yet, the role of histamine in the VLPO remains unclear. Here we report that microinjection of histamine into the VLPO increases the motor activity of rats. Moreover, a bath application of histamine to acute brain slices inhibits the majority of VLPO neurons, which are also inhibited by noradrenaline. Histamine hyperpolarizes the membrane potential and lowers the firing rate. These effects are associated with an increase in the frequency but not in the amplitude of spontaneous $GABA_A$ receptor-mediated inhibitory postsynaptic currents, and are blocked by gabazine or tetrodotoxin, indicating an indirect action. Conversely, on the noradrenaline-excited VLPO neurons, histamine depolarizes the membrane potential and increases the firing rate via activation of H_1 and H_2 subtype histamine receptors. Moreover, histamine-induced depolarization persists in the presence of gabazine or tetrodotoxin, indicating a direct action. Based on these findings, we propose that in the VLPO, noradrenaline-inhibited neurons may normally be under the inhibitory control of noradrenaline-excited neurons. By facilitating the inhibitory control of the noradrenaline-excited neurons, histamine may inhibit the noradrenaline-inhibited neurons, resulting in excitation of histamine-releasing neurons in the TMN through disinhibition. This effect of histamine in the VLPO may contribute to the maintenance of wakefulness.

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Abbreviations AHP, afterhyperpolarization; AMT, amthamine; BET, betahistine; eIPSCs, evoked inhibitory postsynaptic currents; GABA, γ-aminobutyric acid; $GABA_A$Rs, A-type GABA receptors; $GABA_B$Rs, B-type GABA receptors; NA, noradrenaline; sIPSCs, spontaneous inhibitory postsynaptic currents; SNr, substantia nigra pars reticulata; TMN, tuberomammillary nucleus; TTX, tetrodotoxin; VLPO, ventrolateral preoptic nucleus.

Introduction

Histamine, an aminergic neurotransmitter, plays an important role in the regulation of several pathological/physiological processes including sleep/wakefulness, hormonal secretion, cardiovascular control, thermoregulation, food intake and memory formation (Yanai & Tashiro, 2007). The evidence that histamine is important for consciousness and cognitive function is supported by the fact that histamine levels in human cerebrospinal fluid are lower in narcoleptics (Nishino *et al.* 2009). Thus, reduced histamine transmission could be responsible for some of the excessive daytime sleepiness in narcolepsy. Moreover, knockout mice lacking the histidine decarboxylase gene have impaired cortical activation (Anaclet *et al.* 2009) and narcoleptic canines experience continuous firing of putative histamine neurons during cataplexy (John *et al.* 2004). In the mammalian brain, the histamine-releasing neurons are located exclusively in the tuberomamillary nucleus (TMN) of the hypothalamus, from where they project to practically all brain regions, with the ventral areas (ventrolateral preoptic nucleus (VLPO) in hypothalamus, basal forebrain, amygdala) receiving a particularly strong innervation (Brown *et al.* 2001). A previous *in vitro* study has revealed two major types of neurons in the VLPO: the majority (69%) are inhibited by noradrenaline (NA) and the minority are excited by NA (Gallopin *et al.* 2000). The NA-inhibited neurons containing γ-aminobutyric acid (GABA) are sleep-promoting neurons and project to the histamine-releasing neurons in the TMN (Szymusiak, 1995; Sherin *et al.* 1996, 1998; Szymusiak *et al.* 1998;

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Gallopin *et al.* 2000). When excited, these neurons release GABA into the TMN, thus inhibiting the activity of this arousal-producing nucleus and reducing the release of histamine to elicit the onset of sleep. Conversely, the maintenance of wakefulness requires that the VLPO neurons are inhibited by arousal systems. Interestingly, previous studies found that the NA-inhibited neurons are unaffected by histamine (Gallopin *et al.* 2000, 2004). This finding is puzzling, given the important role of histamine and VLPO neurons in the regulation of wake–sleep. The objective of the current study is to re-evaluate the effects of histamine on VLPO neurons.

Methods

We have read Drummond's article (Drummond, 2009), and our experiments comply with the policies and regulations. The experiments were carried out according to the guidelines set by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

Locomotion assessment

A group ($n = 10$) of Sprague–Dawley rats (200–220 g at the start of the experiments) were housed individually in a climate-controlled room on a 12 h light/dark cycle (light off at 5 pm).

VLPO cannulation surgery. Under ketamine/xylazine anaesthesia (80 mg/5 mg kg^{-1}, I.P.), rats were stereotaxically implanted with a stainless steel double guide cannula (23 gauge; Plastics One, Roanoke, VA, USA) 1 mm above the VLPO (AP −0.24 mm; ML ±1.0 mm; DV −8.0 mm from the skull surface) according to Paxinos and Watson (Paxinos & Watson, 2007). A guide cannula was fixed to the skull surface with dental acrylic (Geristore kit, Denmat Inc.) and four stainless steel anchorage screws. When not in use, a 33-gauge stainless steel dummy cannula was inserted into the guide to keep it clean and prevent occlusion.

Microinjections. Rats were allowed to recover for at least 7 days after surgery in a light-controlled room with *ad libitum* access to food and water before being assigned randomly to receive microinjection of either histamine or saline. All injections (0.2 μl/side) were done through the guide cannula over 1 min using a micro-infusion pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA).

Locomotion monitoring. Wakefulness in the rats was evaluated by locomotive activity, which was measured in each rat using an automated Truscan photobeam apparatus (Coulbourn Instruments, Whitehall, PA, USA). Data were collected over a 3 min period followed by analysis using Truscan 2.0 software (Coulbourn Instruments).

Histology. At the end of the experiments, rats were overdosed with pentobarbital and decapitated and coronal brain slices (30 μm) were prepared using a Zeiss Microm HM 550 Cryostat. Slides were examined under the microscope to confirm the accuracy of cannula tip placement in the VLPO area. Data were used only when the correct placement of the probes had been microscopically confirmed on cresyl violet-stained brain sections.

Slice preparation

Given that the histaminergic fibres reach an adult-like appearance about 2 weeks after birth (Haas *et al.* 2008), electrophysiological experiments were done on slices retrieved from postnatal Sprague–Dawley rats of 17–26 days.

The hypothalamic slices were prepared as described previously (Li *et al.* 2009*b*). Briefly, Sprague–Dawley rats were anaesthetized with ketamine/xylazine (80 mg/5 mg kg^{-1}, I.P.) and then killed by decapitation. VLPO was identified according to the stereotaxic coordinates (Paxinos & Watson, 2007). Coronal midbrain slices (250 μm thick) containing VLPO were cut using a Compresstome VF-200 slicer (Precisionary Instruments Inc., Greenville, NC, USA) in ice-cold glycerol-based artificial cerebrospinal fluid (GACSF) containing (in mM): 250 glycerol, 1.6 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose, and saturated with 95% O_2–5% CO_2 (carbogen) (Ye *et al.* 2006). Slices were allowed to recover for at least 1 h in a holding chamber at room temperature (22–24°C) in carbogen-saturated regular artificial cerebrospinal fluid (ACSF), which has the same composition as GACSF, except that glycerol was replaced by 125 mM NaCl.

Electrophysiological recordings

Electrical signals were obtained in a conventional whole-cell configuration with an Axon 200B amplifier (Molecular Devices Co., Union City, CA, USA), a Digidata 1440A A/D converter (Molecular Devices Co.) and pCLAMP 10.2 software (Molecular Devices Co.). Data were filtered at 1 kHz and sampled at 5 kHz. The patch electrodes had a resistance of 4–6 MΩ when filled with the pipette solution containing (in mM): 135 CsF, 5 KCl, 2 $MgCl_2$, 10 Hepes, 2 MgATP, 0.2 GTP (for voltage clamp). The theoretical Cl^- equilibrium potential (E_{Cl}) calculated from the Nernst equation should be −70.9 mV (extra- and intracellular Cl^- concentrations in our recording solutions were 133.8 and 9 mM, respectively). For current clamp recordings, CsF in the above pipette solution was replaced

with potassium gluconate. The pH was adjusted to 7.2 with Tris base. A single coronal slice was transferred into a 0.4 ml recording chamber, where it was held down by a platinum ring. Warm carbogenated ACSF flowed through the bath ($\sim$2 ml min^{-1}). VLPO neurons were identified under visual guidance using infrared-differential interference contrast (IR-DIC) video microscopy with a $\times$40 water immersion objective. The image was detected with an IR-sensitive CCD camera and displayed on a monitor. Neurons were voltage clamped at 0 mV to record γ-aminobutyric acid type A ($GABA_A$) receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) or evoked inhibitory postsynaptic currents (eIPSCs). To evoke monosynaptic IPSCs (eIPSCs), a nichrome wire bipolar stimulating electrode was placed 100–200 μm away from the recording site in the VLPO. The series resistance (15–30 MΩ) or input resistance (300–500 MΩ) was monitored throughout the whole cell recording, and data were discarded if the resistance changed by more than 20%. All these recordings were made at 32°C, maintained by an automatic temperature controller (Warner Instruments, Hamden, CT, USA).

Chemicals and applications

The chemicals, including histamine, betahistine, pyrilamine, amthamine, ranitidine, gabazine, bicuculline, CGP52432, tetrodotoxin, noradrenaline and other standard chemicals were purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA). All drugs were diluted in fresh ACSF to the final concentration immediately before the experiment.

Statistical analysis

The electrophysiological signals were analysed with Clampfit 10.2 (Molecular Devices Co.); sIPSCs, eIPSCs and spontaneous action potentials were screened automatically, checked visually, and accepted or rejected according to their rise and decay times. The frequency and amplitude of all events, during and after drug application, were compared with the mean of the values observed during the initial control period. Cumulative probability plots of the incidence of various interevent intervals and amplitudes, recorded under different conditions from the same neuron, were subjected to the Kolmogorov-Smirnov (K-S) test. For other plots, data obtained over a 1–2 min period at the peak of a drug response were compared with the average values of frequency (or amplitude) of the sIPSCs and the spontaneous action potentials recorded during the initial control period (4–5 min).

Average values are expressed as the mean $\pm$ S.E.M., with n equal to the number of cells studied. Statistical significance of results was assessed using Student's t test. Statistical analysis of concentration–response data was performed using the non-linear curve-fitting program ALLFIT (DeLean *et al.* 1978), which uses an ANOVA procedure. Values reported for concentration–response analysis are those obtained by fitting the data to the equation:

$$Y = E_{\max} / [1 + (EC_{50}/X)^n]$$

where X and Y are concentration and response, respectively; $E_{\max}$ is the maximal response, EC_{50} is the concentration yielding 50% of maximal effect (EC_{50} for activation, IC_{50} for inhibition), and n is the Hill coefficient.

Results

Administration of histamine into the VLPO induces wakefulness

To determine whether histamine has an effect on wakefulness of rats, histamine (100 μM/0.2 μl) or saline of equal volume was injected into the VLPO (see Fig. 1*A* for cannula placement). As illustrated in Fig. 1*C*, rats treated with histamine ($n = 8$) demonstrated sustained movement activity. The two-way ANOVA conducted on the locomotion counts obtained in the first 30 min post-injection revealed significant effects of treatment ($F_{(1,130)} = 24.82$, $P < 0.001$) and a significant treatment–time interaction ($F_{(9,130)} = 2.07$, $P < 0.05$), but no time significance ($F_{(9,130)} = 1.63$, $P = 0.11$). The *post hoc* test indicated that the animals which received intra-VLPO injection of histamine maintained elevated locomotion during the 18–30 min time point, when the locomotion began to decrease in the control group which received intra-VLPO saline. In the current study, we implanted cannulae in 10 rats, and in 8 of them the cannulae were in the VLPO. In the other two rats histamine slightly increased locomotion but the change was not significant (data not shown), but a conclusion should not be made based on two rats. In a future study, we will determine the effects of histamine injected outside of the VLPO.

Identification of the VLPO neurons

Electrophysiological experiments were conducted in acute brain slices. We found two major cell types within the VLPO: the majority were triangular and multipolar in shape (Fig. 2*Aa*), hyperpolarized by a brief application (30 s) of noradrenaline (NA, 100 μM) (Fig. 2*Ab*), and characterized by a low-threshold spike (Fig. 2*Ac*). Conversely, the minority were fusiform and bipolar in shape (Fig. 2*Ba*), depolarized by NA (Fig. 2*Bb*), and had no low-threshold spike (Fig. 2*Bc*). These are in

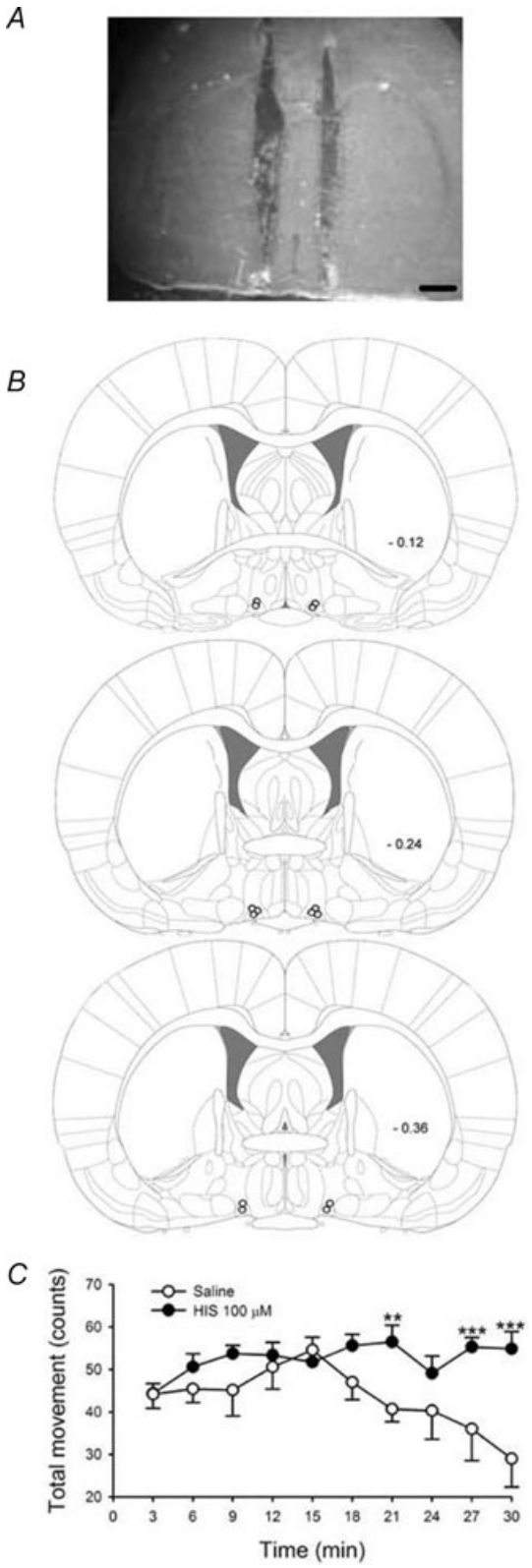

Figure 1. Administration of histamine into the VLPO induces wakefulness
A, photomicrograph of a representative brain slice with (via a double guide cannulae) injection sites within the VLPO. Scale bar: 1 mm. *B*, schematic drawings of coronal sections of the rat brain showing the cannulae placements. Black circles indicate injecting placement for animals included in data analysis. Acceptable VLPO placements were those corresponding to −0.12 to −0.36 mm posterior to bregma according to the atlas of Paxinos and Watson (Paxinos & Watson, 2007). *C*, intra-VLPO injection of histamine increases locomotion. Data are illustrated as group mean locomotor activity counts obtained during the 30 min following the drug injection. Symbols indicate significant differences as revealed by *post hoc* Student-Newman-Keuls comparisons following two-way ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$; histamine compared to saline ($n = 8$).

line with previous reports (Gallopin *et al.* 2000; Li *et al.* 2009*a*). In keeping with a previous report (Gallopin *et al.* 2000), the basic membrane parameters, including the action potential thresholds, did not differ between the two cell types. Specifically, the action potential thresholds were -41.7 ± 0.5 mV (from -39.1 to -48.2 mV, $n = 12$) in the NA-inhibited neurons, and were -42.6 ± 0.7 mV (from -38.5 to -49.7 mV, $n = 9$) in the NA-excited neurons, respectively.

Histamine depresses NA-inhibited neurons in the VLPO

Next, we tested the effects of histamine on NA-inhibited neurons in acute brain slices of rats. Bath application of histamine (100 μM, 6 min) hyperpolarized the membrane potential and decreased the firing rate (Fig. 3*Aa* and *b*). Histamine (100 μM, 6 min) suppressed 19 of the 20 cells tested; however, it excited one. On average, histamine (100 μM, 6 min) hyperpolarized the membrane potential by 3.7 ± 0.3 mV (from -47.1 ± 2.0 mV to -50.8 ± 2.2 mV, $n = 19$, $P < 0.01$) and decreased the firing rate by $41.8 \pm 8.8\%$ (from 6.0 ± 0.4 to 3.3 ± 0.6 Hz, $n = 19$, $P < 0.01$, Fig. 3*Aa* and *b*). Remarkably, histamine did not significantly alter the mean amplitude and the kinetics of the action potential (58.8 ± 6.8 mV in control *vs.* 59.0 ± 7.2 mV in histamine, $P > 0.5$, Fig. 3*Ac*), suggesting that histamine suppresses NA-inhibited VLPO neurons indirectly. Intriguingly, histamine inhibition increased with its application duration, requiring a minimum of 0.5 min to have an appreciable effect, and reached a plateau at ∼5–6 min. Therefore, we selected a 6 min perfusion time for the rest of the experiments.

We then tested the effect of pyrilamine, the selective antagonist of the H_1 receptor and ranitidine, the selective antagonist of the H_2 receptor. The application of the mixture containing pyrilamine (30 μM) and ranitidine (10 μM) slightly but not significantly changes the membrane potential (by 0.9 ± 0.1 mV, $n = 5$, $P > 0.05$) and the firing rate (by $4.9 \pm 0.5\%$, from 7.0 ± 0.7 to 7.4 ± 0.4 Hz, $n = 8$, $P > 0.05$, Fig. 3*Ba* and *b*). When the response to pyrilamine and ranitidine had stabilized, the addition of histamine (100 μM, for 6 min) failed to significantly alter either the firing rate (by

1.4 ± 0.2%, to 6.9 ± 0.8 Hz, $n = 5$, $P > 0.2$), or the membrane potential (by 0.5 ± 0.1%, from −46.5 ± 2.8 to −46.3 ± 2.5 mV, $n = 5$, $P > 0.2$), indicating that histamine-induced inhibition is mediated by H_1 and H_2 receptors.

The VLPO neurons contain the inhibitory neurotransmitter GABA which activates $GABA_A$ receptors ($GABA_A$Rs, Sherin *et al.* 1998; Szymusiak *et al.* 1998; Gaus *et al.* 2002; Matsuo *et al.* 2003). To determine a role of $GABA_A$Rs in the VLPO, we tested the effects of the specific $GABA_A$R antagonist gabazine. Bath application of gabazine (10 μM) significantly increased the firing rate of NA-inhibited neurons (by 19.3 ± 2.2%, from 5.8 ± 0.9 to 7.2 ± 0.6 Hz, $n = 5$, $P < 0.01$, Fig. 3*Ca* and *b*), indicating that these neurons are normally under GABAergic inhibitory control. When the effects of gabazine reached a plateau, application of histamine (100 μM, 6 min in the presence of gabazine) reduced the firing rate only by 15.8 ± 1.8% (to 6.0 ± 0.6 Hz, $n = 5$, $P < 0.05$, Fig. 3*Ca* and *b*) which is significantly smaller than that by histamine alone (41.8%, $P < 0.01$, Fig. 3*D*). Furthermore, an additional application of CGP52432 (1 μM), a specific $GABA_B$ receptor antagonist, increased the firing rate by 15.9 + 2.0% (to 7.2 ± 1.0 Hz, $n = 5$, $P < 0.05$, Fig. 3*Ca* and *b*) but did not significantly alter the shape of the action potential (data not shown). This result suggests that histamine-induced inhibition is mediated at least in part by increasing GABA transmission through $GABA_A$Rs and $GABA_B$Rs on NA-inhibited neurons. We next examined the effects of histamine in the presence of tetrodotoxin (TTX), which blocks action potentials. In the presence of 0.5 μM TTX, histamine (100 μM, 6 min) failed to induce any appreciable change in the membrane potential (−50.8 ± 1.8 mV in control *vs.* −51.0 ± 1.9 mV in histamine, $n = 5$, $P > 0.5$, Fig. 3*E*). These results suggest that histamine suppresses NA-inhibited VLPO neurons indirectly.

Histamine increases the probability of release of GABA onto NA-inhibited neurons

In searching for the mechanisms which are responsible for histamine inhibition of NA-inhibited VLPO neurons, we tested the effect of histamine on the inhibitory postsynaptic currents (IPSCs). IPSCs were recorded from NA-inhibited neurons in the presence of DL-2-amino-5-phosphonopentanoic acid (AP5; 50 μM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX; 20 μM) at a holding potential of 0 mV. These IPSCs were eliminated by bicuculline (10 μM), indicating that they were mediated by $GABA_A$Rs (Fig. 4*A*). Histamine (100 μM) increased the frequency of spontaneous IPSCs (sIPSCs, by 53.7 ± 4.9%, from 0.5 ± 0.1 to 1.1 ± 0.1 Hz, $n = 6$, $P < 0.01$, Fig. 4*Aa* and *b*) without significantly changing the mean amplitude (by 4.5 ± 1.2%, from 20.1 ± 1.9 pA in control *vs.* 20.3 ± 2.0 pA in histamine, $n = 6$, $P > 0.5$, Fig. 4*Aa* and *c*) and the kinetics of sIPSCs (Fig. 4*Ac*).

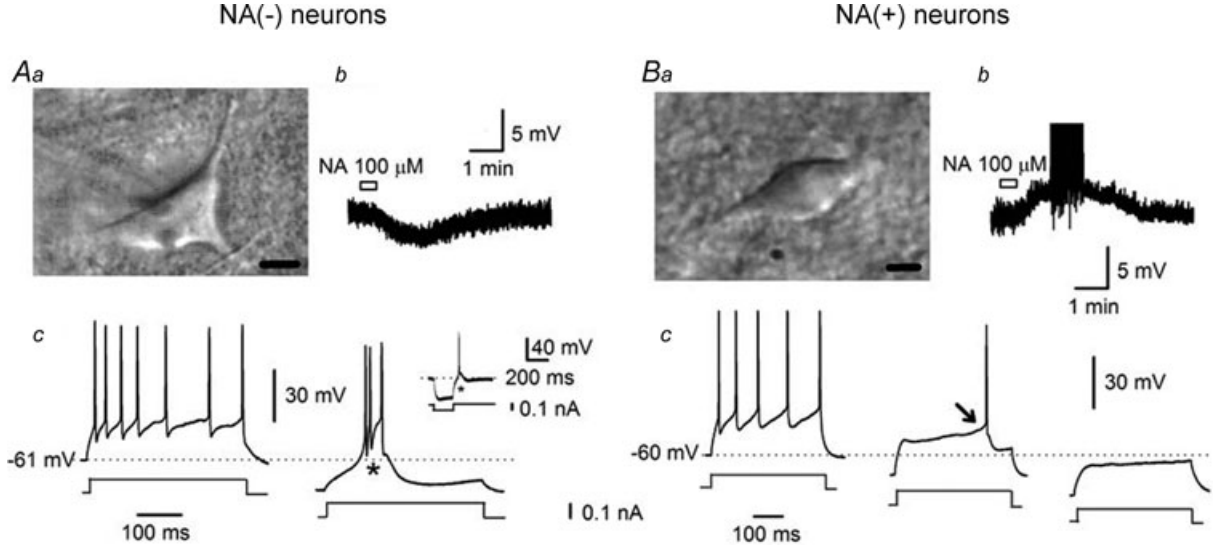

Figure 2. Characterization of two types of neurons in the VLPO slice
A multipolar neuron (*Aa*) and a fusiform and bipolar neuron (*Ba*), scale bar 5 μm. Noradrenaline (NA, 100 μM) induced hyperpolarization of the multipolar neuron (NA(−) neuron, *Ab*) but depolarization and firings in the fusiform neuron (NA(+) neuron, *Bb*), for clarity, the action potentials were truncated. *Ac*, the NA(−) neurons are characterized by a low threshold spike (LTS, asterisks, right panel, and inset) when depolarized from a hyperpolarized level. *Bc*, non-LTS cells do not have low-threshold spikes when they are depolarized from any level of membrane hyperpolarization. They usually show a slowing of the voltage response towards the first action potential (arrow).

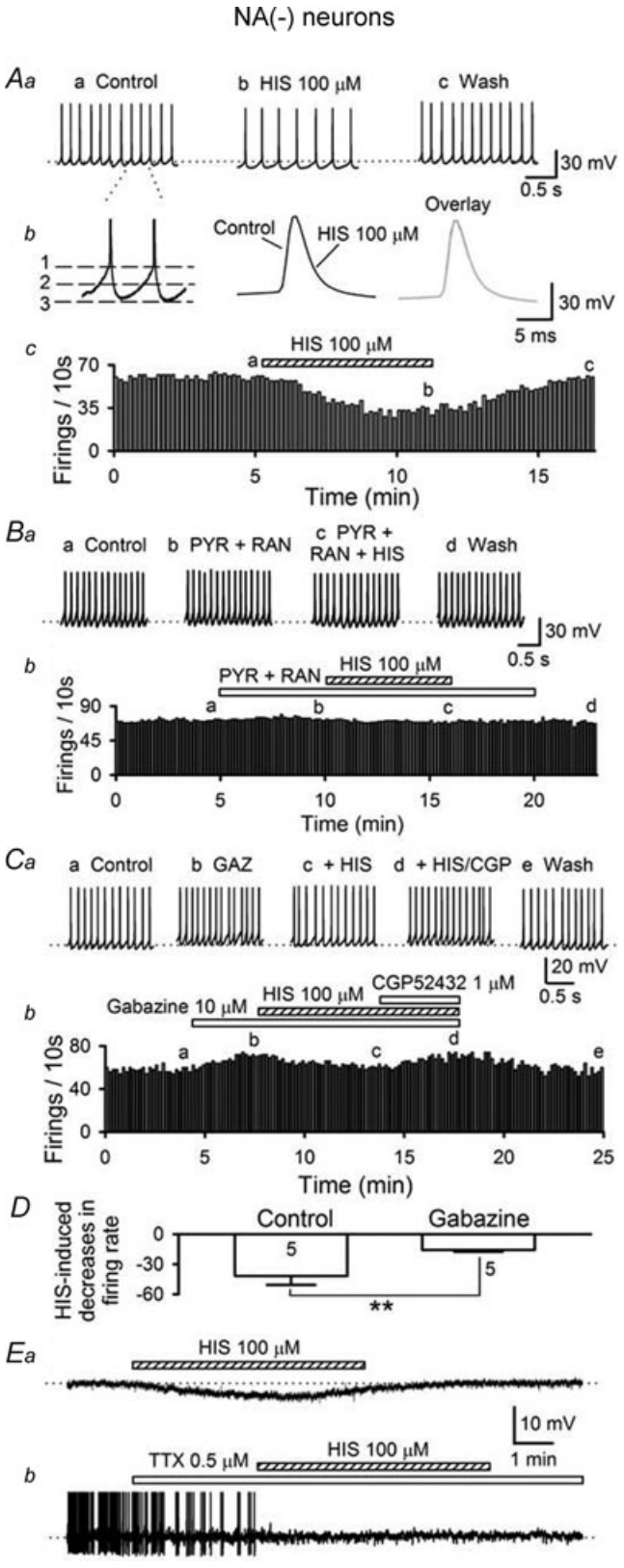

Figure 3. Histamine inhibits the NA-inhibited VLPO neurons
Aa, spontaneous firings obtained at times indicated by lowercase letters in *Ac*. The dotted line in this and in the following figures indicates the resting membrane potential under control conditions. *Ab*, left panel, a section from *Aa* shows the spike threshold (dashed line 1), the afterhyperpolarization (dashed line 3) and the estimated resting potential (dashed line 2). Right panel, averaged traces ($n = 300$–700 spikes) show histamine (100 μM, 6 min) did not induce appreciable change in the amplitude and the kinetics of the spikes. *Ac,* time course of histamine-induced decrease in spontaneous firing rate. *Ba,* spontaneous firings obtained at times indicated by lowercase letters in *Bb*. *Bb,* time course of change in spontaneous firing rate. *Ca,* spontaneous firings obtained at times indicated by lowercase letters in *Cb*. *Cb,* time course of change in spontaneous firing rate. *D,* summary (mean ± S.E.M., $n = 5$) of histamine-induced inhibition of the firing rate in the absence (Control) and presence of 10 μM gabazine. $^{**}P < 0.01$, by paired *t* test, histamine *vs.* gabazine plus histamine. *E*, sample traces show that histamine induces membrane hyperpolarization in the absence (*Ea*), but not in presence of 0.5 μM TTX (*Eb*).

These were further illustrated by cumulative plots of the incidence of various intervals and amplitudes between successive sIPSCs (Fig. 4*B*). These results suggest that histamine may increase the probability of GABA release. This notion is further supported by the observation that histamine (100 μM, 6 min) selectively enhanced the amplitude of the first IPSC ($IPSC_1$) elicited by the first stimulation of the paired pulses (at an interval of 50 ms) but not the second IPSC ($IPSC_2$), thus suppressing paired pulse facilitation (Fig. 4*Ca*, inset). Manipulations that increase transmitter release generally result in a shift of paired pulse facilitation toward depression (Mennerick & Zorumski, 1995; Bonci & Williams, 1997; Ye *et al.* 2004). Interestingly, as shown in Fig. 4*Aa*(d), bicuculline alone causes a change in the holding conductance, indicating block of GABAergic inhibitory tone. Histamine causes the opposite effect, suggesting that histamine increases the release of GABA which activates both the phasic and tonic $GABA_A$Rs on NA-inhibited neurons. This hypothesis is supported by the observation that bicuculline (10 μM) completely blocked the evoked IPSCs (eIPSCs) in the presence of histamine (100 μM, Fig. 4*C*).

Histamine directly excites NA-excited neurons in the VLPO

The results described above suggest that histamine may inhibit NA-inhibited VLPO neurons indirectly through an increase in the probability of GABA release onto NA-inhibited neurons. In addition to NA-inhibited neurons, about 30% of the neurons are excited by NA (Gallopin *et al.* 2000). Like the former, most of these neurons contain galanin (Matsuo *et al.* 2003), may contain GABA, and may release GABA onto the NA-inhibited neurons in the VLPO. Thus, histamine may inhibit NA-inhibited neurons through excitation of NA-excited VLPO neurons. To test this idea, we examined the effects of histamine on NA-excited VLPO neurons. Under current clamp conditions, bath application of

histamine (100 μM, 6 min) reversibly induced membrane depolarization in 42 out of 46 of the NA-excited neurons tested (Fig. 5*A*–*C*). Histamine (100 μM, 6 min) inhibited three neurons and had no effect on one (not illustrated). Histamine facilitated the firing rates of 21 neurons, which had ongoing discharges, and induced firing in 11 of 17 neurons that were silent before the application of histamine (not illustrated). As shown in Fig. 5*Aa*–*c* and summarized in Fig. 5*B* and *C*, histamine (100 μM, 6 min) increased the firing rate by $41.7 \pm 3.5\%$ (from 6.8 ± 1.2 to 11.9 ± 2.7 Hz, $n = 7$, $P < 0.01$), reduced the peak amplitude by $75.3 \pm 6.8\%$ (from 61.8 ± 3.6 to 15.4 ± 1.1 mV, $n = 7$, $P < 0.001$), increased the half-width of action potential by $32.7 \pm 1.9\%$ (from 0.99 ± 0.08 to 1.48 ± 0.09 ms, $n = 7$, $P < 0.01$), and reduced the amplitude of afterhyperpolarization (AHP) by $82.8 \pm 6.5\%$ (from -17.2 ± 1.8 to -3.0 ± 0.4 mV, $n = 7$, $P < 0.001$). Histamine-induced depolarization depended on its concentration. A fit of the concentration–response curve obtained an EC_{50} of 31.0 ± 5.5 μM and Hill coefficient of 0.9 (Fig. 5*C*). Histamine (100 μM, 6 min) induced an inward current of 22.4 ± 2.8 pA ($n = 7$) under voltage clamp conditions (not illustrated).

We next compared the effects of histamine in the absence and presence of gabazine. Bath application of gabazine (10 μM) significantly increased the firing rates of NA-excited neurons by $21.6 \pm 2.3\%$ (from 4.5 ± 0.4 to 5.7 ± 1.0 Hz, $n = 5$, $P < 0.01$, Fig. 5*Da* and *b*), indicating that these neurons were under GABAergic inhibitory control under normal physiological conditions. Intriguingly, when the effects of gabazine stabilized, the application of the mixture containing gabazine plus histamine produced a significantly greater facilitation of the firing rate compared to that by histamine alone. Specifically, in five NA-excited neurons tested, in the presence of 10 μM gabazine, 100 μM histamine increased the firing rate robustly (by $49.9 \pm 4.5\%$, to 10.9 ± 0.7 Hz, $n = 5$, $P < 0.01$, Fig. 5*D*), which was significantly greater than that by histamine alone ($n = 5$, $P < 0.05$, Fig. 5*E*). As shown in Fig. 5*F*, bath application of 100 μM histamine (6 min) in the presence of TTX depolarized the membrane of the NA-excited VLPO neurons, indicating that histamine-induced excitation is attributable to the direct actions on postsynaptic histamine receptors. Similar records were obtained from an additional three cells (by 6.5 ± 0.7 mV, $n = 4$).

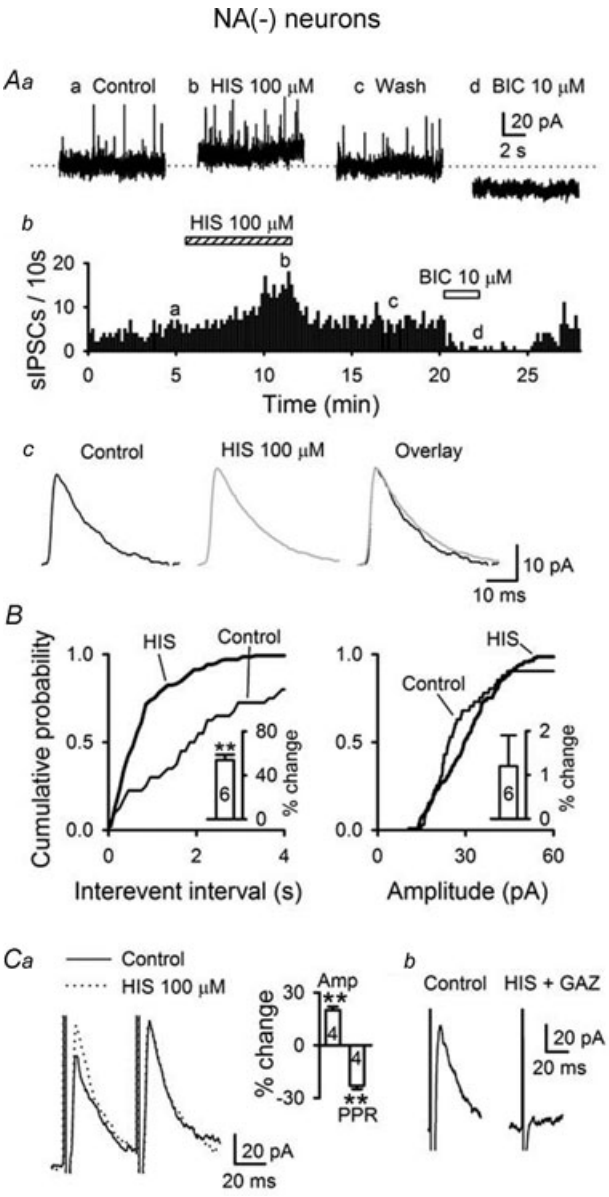

Figure 4. Histamine enhances the probability of GABA release onto NA-inhibited VLPO neurons
Aa, GABAergic sIPSCs recorded before, during and after the application of 100 μM histamine and were completely blocked by 10 μM bicuculline. *Ab*, time course of histamine-induced increase in sIPSC frequency. *Ac*, averaged traces show only minor change in amplitude, overlay of normalized traces indicates no significant changes in sIPSC kinetics in the presence of 100 μM histamine. *B*, for the same data, cumulative probability plots and insets show increase in sIPSC frequency (left panel; K-S test, $P < 0.01$) but not amplitude (right panel; $P > 0.5$). Insets are summaries of increases in sIPSC frequency (left) and amplitude (right) induced by histamine ($n = 6$). *Ca*, IPSCs evoked by pulse stimulation within VLPO and recorded from a NA-inhibited neuron, which was enhanced by histamine. Inset: histamine increases the amplitude of $IPSC_1$ and depresses the paired pulse ratio (PPR). *Cb*, eIPSC was completely blocked by 10 μM bicuculline even in the presence of 100 μM histamine.

The action of histamine is mediated by H_1 and H_2 receptors in NA-excited neurons

Previous studies have shown that histamine activates three subtypes of receptors: H_1, H_2 and H_3. H_1 and H_2 receptors

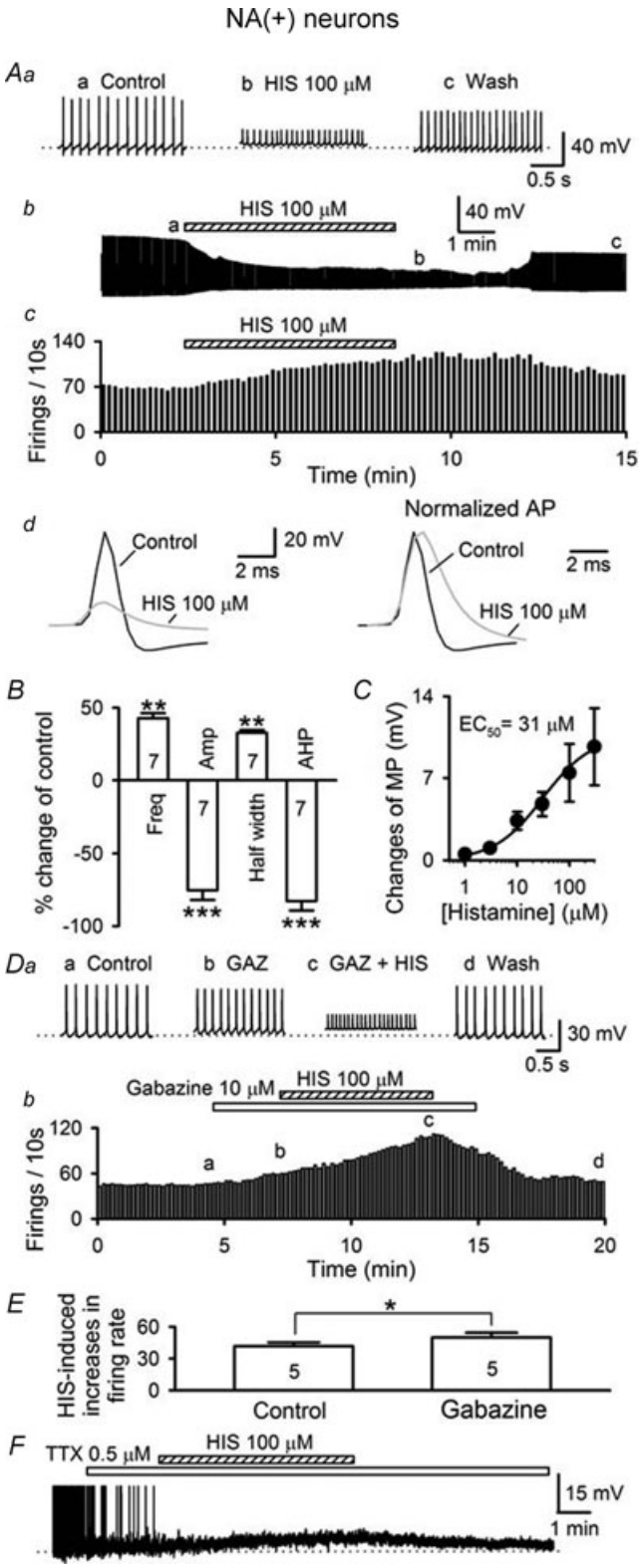

Figure 5. Histamine excites the NA-excited VLPO neurons
Aa, original traces of spontaneous action potential obtained at times indicated by lowercase letters in *Ab*. *Ab*, a typical continuous record from a NA-excited neuron showing histamine-induced change in membrane potential and concomitant action potential firing. *Ac*, time course of histamine-induced increase in firing rate (the same cell as *Ab*). *Ad*, averaged traces show histamine-induced robust changes in amplitude (left panel) and kinetics (right panel) of the action potentials. *B*, relative changes in the parameters of action potentials induced by 100 μM histamine from seven neurons. *C*, histamine-induced depolarization depended on its concentration. Error bars not visible are smaller than the size of the symbols. The curve shown is the best fit of the data to the equation described in Methods. Fitting the data to this equation yielded the EC_{50} value of 31.0 ± 5.5 μM and the Hill coefficient of 0.9. *Da*, histamine-induced increase in firing rate was greater in the presence of gabazine (10 μM). Spontaneous firings obtained at times indicated by lowercase letters in *Db*. *Db*, time course of histamine-induced increase in firing rate in the presence of gabazine (10 μM). *E*, relative increases in firing rate induced by histamine in the absence (Control) and presence of gabazine. $^*P < 0.05$ by paired *t* test for histamine *vs.* gabazine plus histamine data. *F*, a sample trace showing that histamine still induced depolarization in the presence of 0.5 μM TTX. Abbreviations: Freq, frequency; Amp, amplitude; AHP, afterhyperpolarization.

are mainly postsynaptically located, whereas H_3 receptors are exclusively presynaptically located (Brown *et al.* 2001; Haas *et al.* 2008). High densities of H_1 receptors are found in the hypothalamus. Activation of H_1 (and H_2) receptors causes depolarization (Brown *et al.* 2001). To determine which type(s) of receptors mediate the effects of histamine, we examined the effects of the selective H_1 receptor antagonist pyrilamine (30 μM) and the selective H_2 receptor antagonist ranitidine (10 μM), first separately, then in combination. When applied separately, these agents did not significantly alter the firing of NA-excited neurons. However, these agents significantly reduced the effects of histamine. Specifically, in the presence of pyrilamine (30 μM), histamine (100 μM, 6 min) increased the firing rate by $27.8 \pm 2.3\%$ ($n = 6$, $P < 0.01$, Fig. 6*A*) and depolarized the membrane potential by 4.4 ± 0.3 mV ($n = 6$, $P < 0.01$), which was significantly smaller than that induced by histamine alone ($P < 0.01$, Fig. 6*D*). Similarly, in the presence of ranitidine (10 μM), histamine (100 μM) increased the firing rate by $20.7 \pm 1.9\%$ ($n = 6$, $P < 0.01$, Fig. 6*B*) and depolarized the membrane potential by 3.2 ± 0.3 mV ($n = 6$, $P < 0.01$), which was significantly smaller than that induced by histamine alone ($P < 0.01$, Fig. 6*D*). The application of the mixture containing pyrilamine (30 μM) and ranitidine (10 μM) induced a small but significant change in membrane potential (-1.9 ± 0.2 mV, $n = 8$, $P < 0.05$) and a decrease in firing rate (by $11.7 \pm 0.9\%$, from 5.9 ± 0.6 to 5.2 ± 0.5 Hz, $n = 8$, $P < 0.05$, Fig. 6*C*), indicating that NA-excited neurons are normally under the tonic excitation of histamine via the activation of H_1 and H_2 receptors. When the response to pyrilamine and ranitidine had stabilized, the application of the cocktail containing pyrilamine (30 μM), ranitidine (10 μM) and histamine (100 μM) altered neither the firing rate (by $1.8 \pm 0.2\%$, to 6.0 ± 0.6 Hz, $n = 8$, $P > 0.2$), nor the membrane potential (by $1.3 \pm 0.1\%$, from -45.7 ± 2.7 to -45.1 ± 2.4 mV, $n = 8$, $P > 0.2$, Fig. 6*D*), indicating

that the excitatory effect of histamine is mediated by both H_1 and H_2 receptors.

If histamine-induced excitation of NA-excited VLPO neurons is mediated by H_1 and H_2 histamine receptors, the agonists of these receptors should mimic the effects of histamine. As expected, bath application of the selective H_1 receptor agonist betahistine (BET, 100 μM) and the selective H_2 receptor agonist amthamine (AMT, 10 μM) reversibly depolarized the membrane potential and increased the firing rate (Fig. 6*E*–*G*), suggesting that both H_1 and H_2 receptors exist in NA-excited neurons.

Histamine and the agonists of H_1 and H_2 receptors raise the frequency of sIPSCs on NA-excited neurons

As mentioned in Fig. 5*D*–*E*, histamine facilitation of NA-excited neurons was greater in the presence of

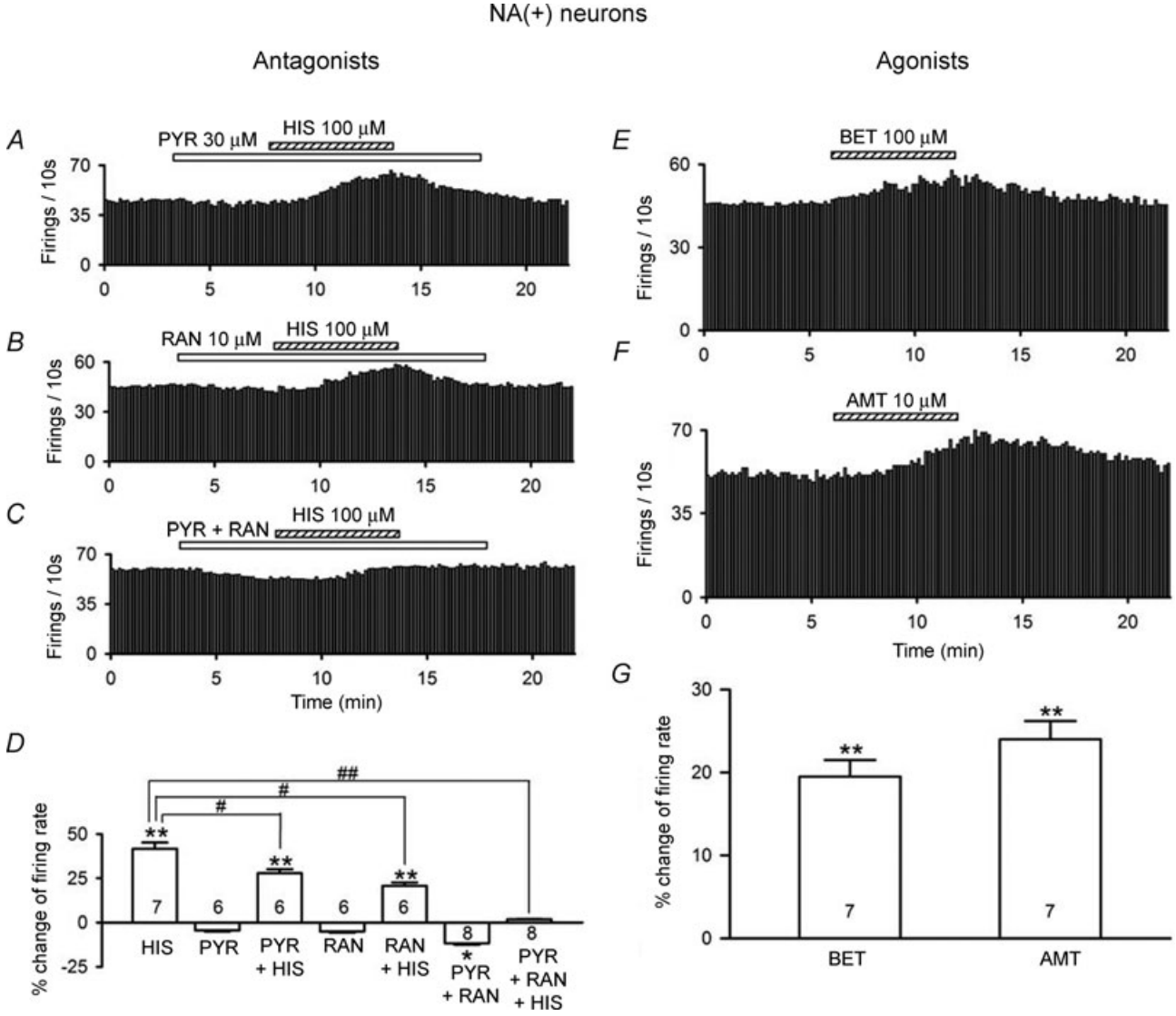

Figure 6. Histamine excites the NA-excited VLPO neurons through the H_1 and H_2 receptors
A–D, histamine-induced excitation of NA-excited neurons are blocked by the selective antagonists of H_1 and H_2 receptors. *A*, time course of histamine-induced increase in firing rate in the presence of the H_1 antagonist pyrilamine (30 μM). *B*, time course of histamine-induced increase in firing rate in the presence of the H_2 antagonist ranitidine (10 μM). *C*, time course of histamine-induced increase in firing rate in the presence of pyrilamine (30 μM) + ranitidine (10 μM). *D*, relative changes induced in firing rate of the NA-excited neurons under a variety of situations. $^*P < 0.05$, $^{**}P < 0.01$; compared to baseline firing rate. $\#P < 0.05$, $\#\#P < 0.01$; compared to firing rate in 100 μM histamine. *E–G*, histamine-induced excitation is mimicked by the selective agonists of H_1 and H_2 receptors. *E*, time course of betahistine (BET, 100 μM)-induced increase in firing rate. *F*, time course of amthamine (AMT, 10 μM)-induced increase in firing rate. Abbreviations: HIS, histamine; PYR, pyrilamine; RAN, ranitidine; BET, betahistine; AMT, amthamine.

gabazine, indicating that the effect of histamine was attenuated by $GABA_A$Rs. To determine the mechanisms underlying this observation, we recorded sIPSCs from voltage-clamped NA-excited neurons in the presence of AP5 (50 μM) and DNQX (20 μM). Under these experimental conditions, all events were eliminated by bicuculline (10 μM), indicating that they were mediated by $GABA_A$Rs (data not shown). Intriguingly, the application of histamine (100 μM) robustly increased sIPSC frequency (by $74.6 \pm 7.8\%$, from 0.44 ± 0.06 to 1.73 ± 0.12 Hz, $n = 7$, $P < 0.001$, Fig. 7*Aa–c*, inset in 7*B*); conversely, histamine did not significantly change the mean amplitude and the kinetics of the sIPSCs (Fig. 7*Ad* and *B*). This was further illustrated by cumulative plots of the incidence of various intervals and amplitudes between successive sIPSCs (Fig. 7*B*). These results indicate that histamine increases the probability of GABA release onto NA-excited VLPO neurons.

Similarly, bath application of the H_1 agonist betahistine (BET, 100 μM) increased sIPSC frequency (by $15.8 \pm 1.1\%$, from 2.18 ± 0.05 to 2.59 ± 0.05 Hz, $n = 7$, $P < 0.05$, Fig. 7*Ca–c* and *Ea*); bath application of H_2 agonist amthamine (AMT, 10 μM) increased sIPSC frequency (by $46.1 \pm 4.9\%$, from 0.48 ± 0.05 to 0.89 ± 0.07 Hz, $n = 8$, $P < 0.01$, Fig. 7*Da–c* and *Eb*). However, these two agents did not significantly change the mean amplitude and the kinetics of sIPSCs.

Discussion

Our study provides the first solid behavioural and electrophysiological evidence that histamine regulates the excitability of VLPO neurons and that functional histamine receptors exist in the VLPO.

Administration of histamine into the VLPO induces wakefulness

We showed that perfusion of histamine into the VLPO increases motor activity of rats, indicating increased wakefulness. Our result is in general agreement with a previous cat study, which shows that microinjections of histamine in the preoptic-anterior hypothalamus increases wakefulness (Lin *et al.* 1994).

Histamine suppresses NA-inhibited neurons in the VLPO

Previous studies reported that a brief application of histamine (100 μM for 15 s) has no effect on NA-inhibited VLPO neurons (Gallopin *et al.* 2000, 2004). However, we found that when the duration of histamine application was extended to more than 30 s, an appreciable effect on NA-inhibited VLPO neurons was observed. While the mechanisms by which histamine requires a longer time to elicit an effect warrant further investigation, the following factors may contribute to this issue. (1) Histamine receptors are G-protein mediated and their response may involve various second messenger pathways (Haas & Panula, 2003). (2) Histamine may indirectly inhibit NA-inhibited neurons, caused by postsynaptic actions on another neuron in the network, since histamine inhibition was attenuated or blocked by gabazine (10 μM) or TTX (0.5 μM). (3) A previous microdialysis study has found that the extracellular levels of histamine in the preoptic/anterior hypothalamic area are relatively high, between 0.245 and 1.155 pg μl^{-1} (equal to $\sim$22–104 μM) (Strecker *et al.* 2002), and the final concentration reaching the cell under recording in the slice after 15 s application of histamine (100 μM) may be only a fraction of 100 μM, and thus may not change the extracellular concentration of histamine much. Moreover, the longer time for histamine to react may reflect the time it takes for histamine to accumulate in the slice; finally, it is worth noting that in several previous electrophysiological studies in slice preparations, the application time of histamine is usually $\geq$1 min (Whyment *et al.* 2006; Zhou *et al.* 2006; Zhang *et al.* 2008).

Histamine depolarizes the membrane potentials and increases discharges of NA-excited neurons

NA-excited neurons in the VLPO have been neglected by most researchers in the field. To our surprise, histamine induced an inward current, membrane depolarization, and facilitation of spontaneous firing in NA-excited VLPO neurons. These effects are accompanied by changes in the action potential parameters (amplitude, width and AHP). A membrane depolarization can potentially increase the excitability of these neurons by bringing the membrane potential closer to the action potential threshold and increasing the responsiveness of the neuron to depolarizing stimuli (Hardwick *et al.* 2006). Histamine-induced changes in the action potential parameters are most likely to be caused by Na^+ channel inactivation due to membrane depolarization, since this can result in reduced activation of K^+ channels (both voltage-gated and Ca^{2+}-gated) during action potentials. These results indicate a direct excitation of postsynaptic histamine receptors. This possibility is further supported by the observation that histamine-induced membrane depolarization persists in the presence of gabazine or TTX.

Compare the mechanisms of NA and histamine on VLPO neurons

The specific mechanisms of NA effects on VLPO neurons are unclear. However, NA is known to act on adrenoceptors, which belong to the G protein-coupled receptor

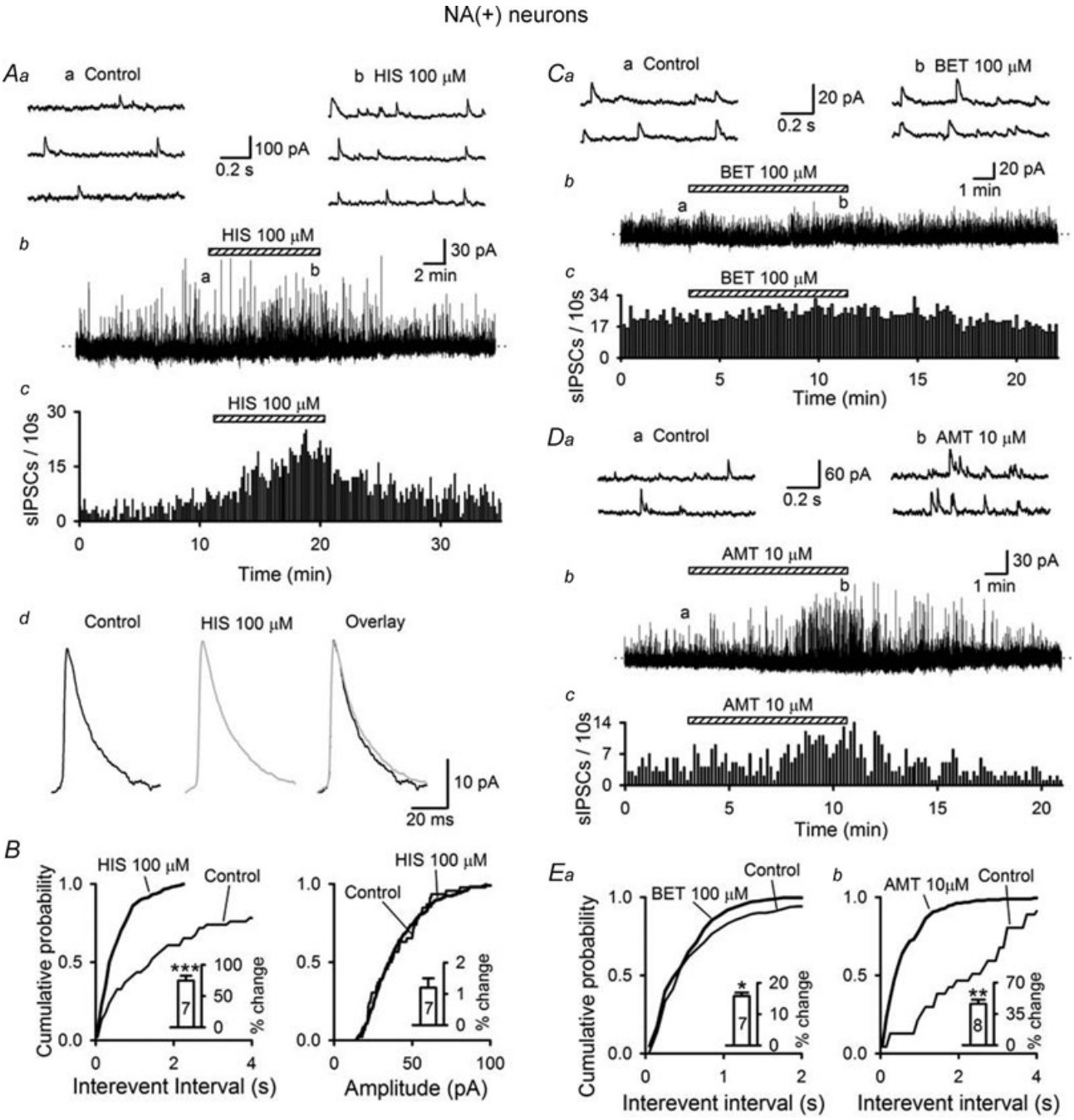

Figure 7. Histamine and the agonists of H_1 and H_2 receptors increase sIPSC frequency in NA-excited neurons

Histamine (100 μM) robustly increased sIPSC frequency shown in expanded traces (*Aa*), original trace (*Ab*) and time course (*Ac*). *Ad*, averaged traces show histamine (100 μM) did not induce a significant change in amplitude and kinetics of the sIPSCs. *B*, for the same data, cumulative probability plots show an increase in sIPSC frequency (left panel; K-S test, $P < 0.001$) but not in amplitude (right panel; $P > 0.5$). Insets are summaries of increases in sIPSC frequency (left) and amplitude (right) induced by histamine ($n = 7$). The H_1 agonist betahistine (100 μM) increased sIPSC frequency shown in expanded traces (*Ca*), original trace (*Cb*) and time course (*Cc*). The H_2 agonist amthamine (10 μM) increased sIPSC frequency shown in expanded traces (*Da*), original trace (*Db*) and time course (*Dc*). For the same data, cumulative probability plots show an increase in sIPSC frequency with 100 μM betahistine (K-S test, $P < 0.05$) (*Ea*) or 10 μM amthamine ($P < 0.01$) (*Eb*), respectively. Insets are summaries of increases in sIPSC frequency with betahistine ($n = 7$) or amthamine ($n = 8$), respectively.

superfamily. Previous studies found that activation of α1 adrenoceptors in a variety of brain regions (e.g. cerebral cortex, lateral geniculate nucleus, supraoptic nucleus, dorsal raphe and dorsal motor nucleus of the vagus) causes a slow depolarization, thereby increasing cell excitability. This effect occurs through the inhibition of a non-voltage-dependent K^+ current (I_{KL}) that is present under resting conditions and contributes to the normal 'leak' of ions through the membrane. In contrast, α2 receptors exert inhibitory (hyperpolarizing) effects because of activating an inward-rectifying K^+ current (I_{KG}). α2 receptors also block Ca^{2+} currents in some cells (Nicoll *et al.* 1990; Feldman *et al.* 1997). Based on these observations, we speculate that NA acts on α1 adrenoceptors of NA-excited neurons and on α2 adrenoceptors of NA-inhibited neurons in the VLPO. In addition, in mechanically isolated preparations, NA inhibited the release of GABA onto multipolar VLPO neurons but not to the bipolar neurons (Matsuo *et al.* 2003). Conversely, in our current study of brain slices, histamine increases the release of GABA onto both multipolar and bipolar VLPO neurons, although the increase to the former is probably an indirect one.

Histamine typically excites neurons by depolarization and a subsequent increase in firing frequency (Brown *et al.* 2001). While activation of H_1 histamine receptors may increase neuronal excitability by blocking a leak K^+ conductance, activation of H_2 receptors may increase neuronal excitability by inhibiting Ca^{2+}-activated K^+ channels that mediate AHP. Conversely, activation of H_3 receptors, the inhibitory autoreceptor on histamine neurons, may inhibit neuronal excitability, Ca^{2+} influx, and the release of histamine and several other transmitters (Brown *et al.* 2001; Zhou *et al.* 2006, 2007; Haas *et al.* 2008).

Our data suggest that histamine increases the firing rates of NA-excited VLPO neurons directly by activating the H_1 and H_2 receptors. This is supported by the observation that histamine's excitatory effect was mimicked by the H_1 and H_2 receptor agonists and abolished by the H_1 and H_2 receptor antagonists. Our data are in general agreement with a previous cat study showing that preoptic histaminergic innervation is involved in sleep–wake control and that the action might be mediated via both H_1 and H_2 receptors (Lin *et al.* 1994). Furthermore, the inhibition (a small hyperpolarization and decreased firing rate) induced by the H_1 and H_2 receptor antagonists indicates that histamine receptors are tonically activated under normal physiological conditions.

Histamine raises the frequency of sIPSCs in NA-excited neurons

In the current study, we found that histamine excites NA-excited neurons. Interestingly, histamine also increases the frequency of sIPSCs of NA-excited neurons. A previous study on substantia nigra pars reticulata (SNr) reported a similar observation: histamine excites SNr neurons, which is associated with an increase in sIPSCs (Zhou *et al.* 2006). These researchers interpreted their data to indicate that SNr neurons are GABAergic projection neurons, which innervate each other by their relatively sparse intranigral axonal collaterals (Deniau *et al.* 1982; Mailly *et al.* 2003). Analogous to SNr neurons, NA-excited neurons may also be GABAergic and may innervate each other as well. Thus, histamine excites NA-excited neurons which may lead to increased GABA release through their collaterals. This latter effect may attenuate the excitatory effect of histamine. This idea is

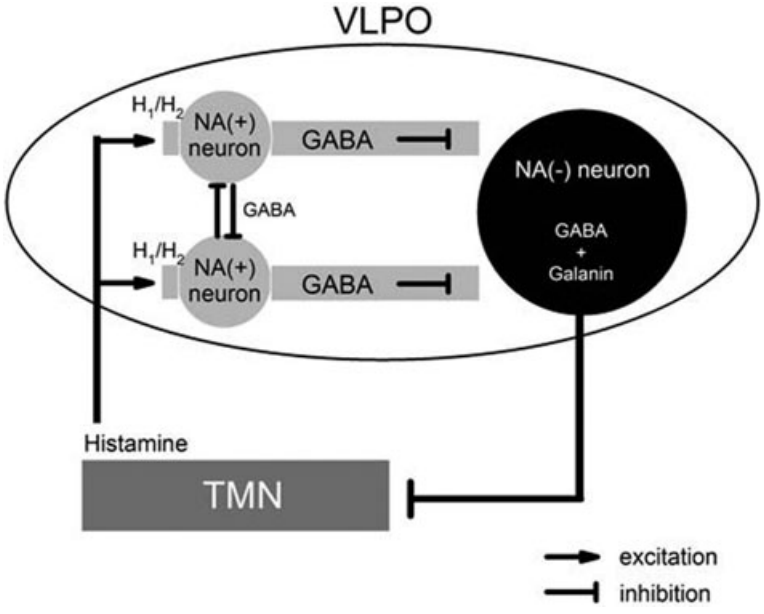

Figure 8. Our working model on how histamine in the VLPO contributes to the maintenance of wakefulness
Histamine excites NA-excited (NA(+)) neurons and increases their probability of releasing GABA onto the NA-inhibited (NA(−)) neurons, thus inhibiting the activity of these sleep-promoting neurons. This will result in disinhibition of the TMN neurons, and maintain the wakefulness of the animal.

supported by the observation that the excitatory effect of histamine was stronger in the presence of gabazine.

Functional implications

Our present study indicates that histamine directly excites NA-excited VLPO neurons. Consistent with a previous report (Matsuo *et al.* 2003), our data show that the NA-excited VLPO neurons have $GABA_AR$-mediated IPSCs, indicating that they contain functional $GABA_ARs$. Moreover, there is no report of the presence of non-GABA neurons in the VLPO, and we therefore propose that the NA-excited VLPO neurons are GABA-containing neurons. As mentioned in the Introduction, the NA-inhibited VLPO neurons are known to contain GABA (Szymusiak, 1995; Sherin *et al.* 1996, 1998; Szymusiak *et al.* 1998; Gallopin *et al.* 2000). When excited, these NA-inhibited VLPO neurons release GABA into the TMN, thus inhibiting the activity of this arousal-producing nucleus and reducing the release of histamine to induce the onset of sleep. However, when awake, the NA-inhibited VLPO neurons are probably normally under the inhibitory control of the NA-excited neurons. Histamine may excite these NA-excited VLPO neurons by acting on the H_1 and H_2 receptors in the cells, leading to the inhibition of the NA-inhibited VLPO neurons. As a result, the histamine-releasing neurons in the TMN are excited through disinhibition. This effect of histamine may be crucial for maintaining normal wakefulness. This possibility is supported by our *in vivo* observations and those of others that administration of histamine into the preoptic-anterior hypothalamus induces wakefulness (Lin *et al.* 1994). A model is proposed (Fig. 8) in which wake- and sleep-promoting neurons inhibit each other; this reciprocal relationship is similar to a type of circuit that is called a 'flip-flop' circuit by electrical engineers, and results in stable wakefulness and sleep (Saper *et al.* 2001).

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Author contributions

Y.-W.L., J.L. and J.-H.Y. designed the study. Y.-W.L. performed the electrophysiological experiments, analysed and interpreted the data, drafted the article and revised it. J.L. performed the *in vivo* experiments, analysed and interpreted the data, drafted the article and revised it. J.-H.Y. designed, analysed and interpreted data, drafted the article, revised it, and finally approved the version to be published. All authors read and approved the final manuscript. The experiments were done in the Department of Anesthesiology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School.

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