Supporting Information

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SI Materials and Methods

Ethical Approval. All procedures in handling and treating the animals were approved by The Rockefeller University's Animal Care and Use Committee in accordance with the Animal Welfare Act and the Department of Health and Human Services Guide for the Care and Use of Laboratory Animals.

Materials. Histamine and its agonists (H1: betahistidine; H2: dimaprit; and H3: imetit) were introduced into the picospritzer pipette at 10 mM in Artificial CerebroSpinal Fluid (ACSF, see *Electrophysiology*). Antagonists (H1: mepyramine; H2: cimetidine; and H3: thioperamide) were dissolved in regular ACSF for bath perfusion. E2 and testosterone were dissolved initially in 100% ethanol and then diluted in distilled water to 1 mM as stock solution and finally in ACSF to 10 or 50 nM before use. All chemicals and test agents for electrophysiology were purchased from Sigma.

TaqMan assays (H1, H2, H3, ERα, and ERβ, which all include the corresponding primers), TaqMan Universal PCR Master Mix, RNase-free water, RNase inhibitor, and high capacity cDNA reverse transcription kits were purchased from Applied Biosystems.

Electrophysiology. ***Brain slices preparation.*** Twelve- to 30-d-old intact Sprague-Dawley female rats were used. The age range was chosen to guarantee successful biophysical recordings from VMN neurons, and females have been shown to be responsive to estrogen, as monitored behaviorally (1). Hypothalamic slices containing VMN were prepared as described previously (2, 3). Briefly, rats were deeply anesthetized by urethane [160 mg/100 g body weight (B.W.)] and decapitated to remove their brain. Following the removal of the pia mater from the ventral surface, the hypothalamus was blocked out and placed on the cutting stage of a Vibratome (model 1000 Plus; Vibratome). Thin (300 μm) coronal slices containing the VMN were cut and collected based on anatomical landmarks. For the blocking and slicing, ice-cold sucrose ACSF was used, which was composed of (mM) sucrose, 210; KCl, 3.5; $CaCl_2$, 1; $MgCl_2$, 4; NaH_2PO_4, 1.25; dextrose, 10; and oxygenated with 95% O_2/5% CO_2. Slices were then stored in rACSF at room temperature for at least 1 h before recording. The rACSF is composed of (mM) NaCl, 126; KCl, 5; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgCl_2$, 2; $NaHCO_3$, 26; dextrose, 10; and oxygenated with 95% O_2/5% CO_2.

Electrophysiological recordings. One slice was placed on the bottom of the recording chamber fixed to the stage of an upright microscope (BX50WI; Olympus) and superfused with ACSF at 2 mL/min at room temperature. The slice was examined with infrared differential interference contrast (IR-DIC) optics. The ventrolateral portion of the VMN (vlVMN) was located under low magnification (40×) before switching to higher magnification (400×). Then, cells were visually selected and recorded using Axoclamp 200A initially and Multiclamp 700B later (Axon Instruments). In separate studies (4), we have used voltage clamp to investigate HA actions and found that, comparing to current clamp mode, it was ineffective and could underestimate the proportion of the responsive neurons and the extent of responses. Therefore, in the present study, current-clamp mode was used exclusively for recording. Clampex software was used for recording and Clampfit (both v9.2; Axon Instruments) for data analyses. The extracellular solution was ACSF. Recording pipettes were pulled from Borosilicate glass pipettes (G8515OT-4; Warner Instruments) with a Narishige PP-830 puller and then filled with internal solution which was composed of (mM) K-gluconate, 140; EGTA, 5; $MgCl_2$, 2; $NaHCO_3$, 0.6; hepes, 10; Mg-ATP, 2; Na_2-ATP, 2; $CaCl_2$,1; Na-GTP, 0.3; and sucrose, 8.3. Electrode resistances ranged from 2 to 5 mΩ. Potentials were recorded with respect to the Ag/AgCl reference electrode placed near the outflow of the chamber. Once a patch was obtained, membrane test protocol was performed to check following criteria: Access resistance (Ra) ≤15 mΩ, leak current ≤30 pA and membrane voltage (Vm) to be equal or more negative than −45 mV. When those criteria were met, the recording was switched to current clamp. If a sudden and unexpected change in membrane potential occurred, the electrical properties mentioned above were checked again and if not met the recording was terminated.

Application of test agents. Test agents were applied in two ways: stimulatory agents (HA and its agonists, 10 mM in ejecting pipette) were applied with a picospritzer (Spritzer-8; Cornerstone) and modulatory agents (HA antagonists and E2) were applied by switching bath perfusion medium (bath application). Picospritzer has the following advantages over bath application: (*i*) because its application duration is very brief (fractions of a second to 2 s versus 2–3 min with bath application) and applied only locally near the recorded neurons, the ejected agent can be washed away quickly. This results in brief response durations (a few seconds versus several minutes by bath application) and allows for more frequent application; (*ii*) less desensitization effect, obviously as the consequence of (*i*); and (*iii*) more importantly, it is more effective and capable of evoking more and wider variety of responses. However, the concentration of the test agent that is reaching the cell is unknown when using this method.

There are two major concerns for using a picospritzer: mechanical disturbance and dose–response. To assess the possibility that the evoked response may be due to or contaminated by mechanical disturbances caused by the ejection, a vehicle (ACSF) instead of test agonists ejected at distances that would be effective for agonists did not evoke any response in any of the six neurons tested. Further advance of the ejecting pipette toward the neuron with the intention to create artifacts failed to evoke any artifact in any of the six neurons even when large, visible mechanical disturbances were evoked (Movie S1). Therefore, any response evoked by an ejected agonist is specific to the agonist and is not contaminated by any movement artifact. This conclusion is further supported by the facts that ejection of different agonists evoked different response patterns (Fig. 1 and Fig. S1) that were modulated differentially by antagonists (Figs. 2 and 3) and E2 (Fig. 4 and Table 1).

The concentration of the test agent that is reaching the cell is unknown when using a picospritzer, thus preventing the establishment of a dose–response curve. However, the amount of drug reaching the cell, or the neuron's response, is dependent on the relative position of the picosprizer pipette. When very close to the recorded neuron, ejection of HA or its agonists could cause responses exceeding ±50 mV. To avoid the "ceiling effect" and obtain modulable responses, the location of the ejecting pipette was adjusted to evoke a submaximal response that would not exceed +10 mV in depolarizations or go beyond −5 mV in hyperpolarizations.

Experimental procedure. When a patch was obtained, a membrane test was performed to measure the cell capacitance, the access resistance, and the membrane resistance. After having recorded those values, the recording mode was switched from voltage clamp to current clamp with a holding membrane voltage set to VM = −65 mV. In current clamp, the stimulation pipette was brought to the neuron and tests of drug ejection (time = 1 s, pressure = 2.5×10^5 Pa, concentration of the agonist inside the pipette = 10 mM) were

performed until a significant response (cf. criterion below) was obtained or until drug ejection caused mechanical disturbance (in which case recording was terminated).

The response of a cell to histamine or an agonist was validated if a series of a minimum of three stimuli gave an identical response to the drug. Once the response to histamine or an agonist was clearly established in a cell, antagonists or E2 were applied for 10 min during which the stimulations continued at the same interval.

Criterion of an effect. When the ejection of an agonist evoked a time-locked, measurable change in membrane potential (Vm) consistently for at least three repetitions, the Vm changes were considered as effects of the ejected agonist. As explained above (cf. application of test agents), the position of the ejecting pipette was adjusted to maximize the response up to +10 mV or down to −5 mV.

Any change in responses caused by these agents was regarded as an effective modulation if it was beyond ±20% of the pretreatment control.

Single Cell RT-qPCR. ***Collection of single cells.*** To collect cells, pipettes with a tip diameter of approximately 3.5 μm were pulled from Borosilicate glass (G8515OT-4; Warner Instruments) with a Narishige PP-830 puller and filled with 15 μL of RNase-free water (Ambion). To avoid contamination, pulled pipettes were kept in a cleaned closed container until use. Cells from vlVMN were aspirated under visual control by applying gentle negative pressure. The suction was stopped when 2/3 of the cell was in the pipette so as to prevent debris or neighboring cells from being collected as well. The pipette with the cell was then removed and its tip broken into a 0.2-mL PCR tube (Molecular BioProducts) where its content was released and immediately frozen with dry ice.

Twenty-four cells in one slice per animal were collected. The advantage of collecting all of the cells on the same slice is that it allows after RT-qPCR to map which of the collected cells express what type of receptor and detect whether or not cells containing certain receptors form expression patterns. For negative controls, rACSF extracted close to the slice was collected rather than cell debris because debris can still contain mRNA from recently dead cells. As expected, rACSF did not result in any amplification.

RT-qPCR. When 24 cells were collected from a slice, mRNA was reverse-transcribed (RT reaction) into cDNA (1 h at 37 °C, Programmable Thermal Controller PTC-100; MJ Research) following the manufacturer's protocol (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems): 11.55 μL of RNase-free water, 2.2 μL of 10× TaqMan RT Buffer, 2.0 μL of dNTPs mixture, 0.5 μL of random hexamers, 0.2 μL of RNase inhibitor, 0.55 μL MultiScribe reverse transcriptase (1×, 5.5 mM, 500 μM each dNTP, 2.5 μM, 0.36 unit/μL, 1.25 unit/μL; final concentrations, respectively), and 5 μL of mRNA.

RT was followed by quantitative PCR on a Prism 7700 (Applied Biosystems) using the following TaqMan gene expression assays: Rn00566691_s1 for H1R, Rn00564216_s1 for H2R, Rn00585276_m1 for H3R, Rn00562166_m1 for ERα (5), and Rn00562610_m1 for ERβ (6). Conditions for the cycles followed the manufacturer's protocol for TaqMan assays: PCR initial activation step, 2 min at 50 °C followed by 10 min at 95 °C; two-step cycling, 15 s at 94 °C followed by 1 min at 60 °C. Number of cycles: 55. Total volume of PCR was 20 μL, and it contained 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μL TaqMan Gene Expression Assay, 4 μL RNase-free H_2O, and 5 μL of template cDNA. Results were analyzed by using Prism 7000 SDS software (Applied Biosystems) and Matlab 2007b (Mathworks).

Although the sequence of the primers is not given by the manufacturer (proprietary information), the following is available: H1R assay targets NM_017018.1 at exon 1, H2R assay targets NM_012965.3 at exon 1, H3R assay targets NM_053506.1 at the junction between exons 1 and 2, ERα assay targets NM_012689.1 at the junction between exons 1 and 2 and ERβ assay targets NM_012754.1 at the junction between exons 5 and 6.

Every collected sample was tested for false positives by looking at the amplification of products other than cDNA (e.g., genomic DNA). In each sample, a control group of cells was used for NO-RT reaction where normal RT reaction was run with MultiScribe reverse transcriptase being replaced by an equal volume of RNase-free water. In 79 NO-RT reactions only one produced amplification before cycle 40, suggesting that the false positive occurrence was at most 2%. Consequently, gene expression for a particular target gene was considered to have occurred if the amplification plot crossed the cycle threshold at or before 40 cycles.

Statistics. Match-paired, two-tailed *t* test was used to test the significance of the effects of E2 on histamine agonists (Table 1) and the significance of the effects of histamine receptors antagonists (Figs. 2–4).

1. Kow L-M, Bogun M, Zhang Q, Pfaff DW (2007) Hormonal induction of lordosis and ear wiggling in rat pups: gender and age differences. *Endocrine* 32:287–296.
2. Kow L-M, Easton A, Pfaff DW (2005) Acute estrogen potentiates excitatory responses of neurons in rat hypothalamic ventromedial nucleus. *Brain Res* 1043:124–131.
3. Kow L-M, Devidze N, Pataky S, Shibuya I, Pfaff DW (2006) Acute estradiol application increases inward and decreases outward whole-cell currents of neurons in rat hypothalamic ventromedial nucleus. *Brain Res* 1116:1–11.
4. Wu W, et al. (2008) Network analysis of temporal effects of intermittent and sustained hypoxia on rat lungs. *Physiol Genomics* 36:24–34.
5. Wiik A, Hellsten Y, Berthelson P, Lundholm L, Fischer H, Jansson E (2009) Activation of estrogen response elements is mediated both via estrogen and muscle contractions in rat skeletal muscle myotubes. *Am J Physiol Cell Physiol* 296:C215–C220.

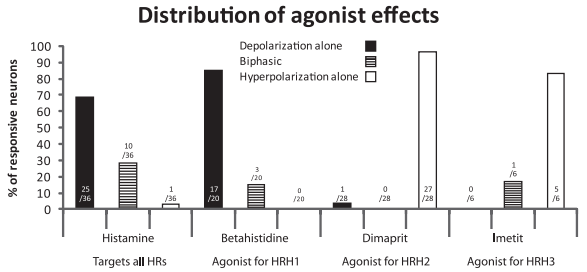
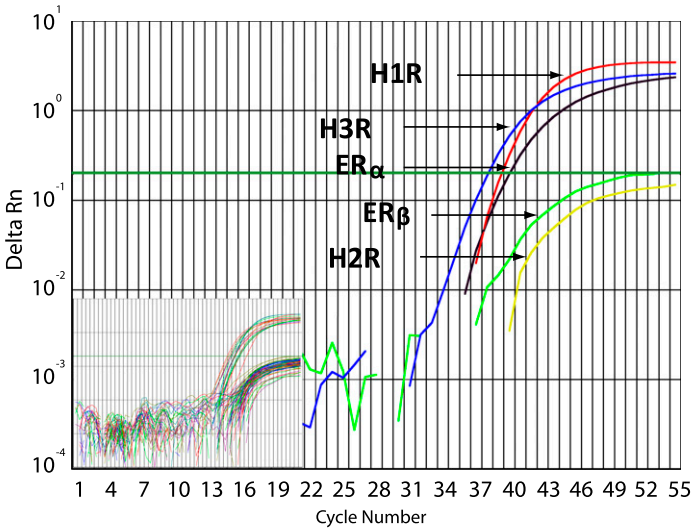


Fig. S1. Percentage distribution of biphasic, depolarizing, and hyperpolarizing responses evoked by histamine receptor agonists. The numbers inside or above the bars represent the number of responsive neurons/total number tested. Cells that did not respond to agonists were not included. HA and BH have very similar distribution profiles, being highest in depolarization, intermediate in biphasic response, and almost null in hyperpolarization alone. In contrast, dimaprit and imetit evoked almost exclusively hyperpolarization (32/34) and nearly never depolarization or biphasic response (1/34, each).

A



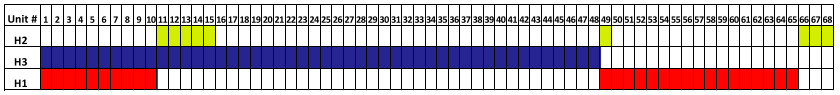
B

Cells positive for each type of receptor and extents of various coexpressions

Receptors	H1		H2		H3		ERα		ERβ	
	N	%	N	%	N	%	N	%	N	%
Positive cells/Total	27/97	28	9/97	9	48/97	49	51/97	53	0/15	0
Co-localized with:										
H1	--	--	1/9	11	10/48	21	10/51	20	--	--
H2	1/27	4	--	--	5/48	10	2/51	4	--	--
H3	10/27	37	5/9	56	--	--	16/51	31	--	--
ERα	10/27	37	2/9	22	16/48	33	--	--	--	--

C

Individual cells expressing histamine receptors



D

Individual cells expressing ERα

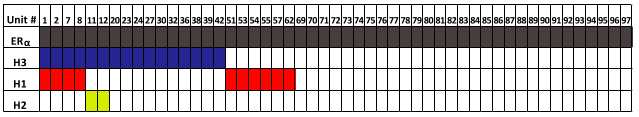
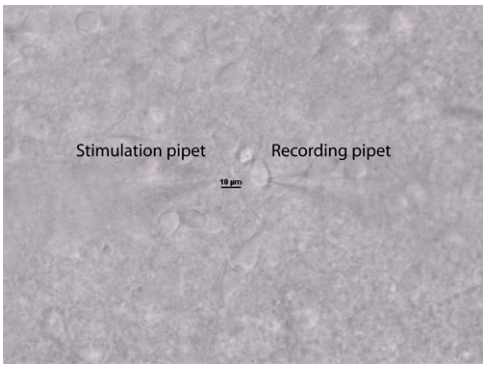


Fig. S2. Single-cell RT-qPCR detection of the expressions of histaminergic receptor subtype and estrogen receptor subtype genes in VMN cells. (*A*) An example of the results of one PCR assay showing a cell expressing H1, H3, and ERα, but not H2 or ERβ genes (threshold for amplification = 40 cycles; *SI Materials and Methods*). (*Inset*) amplification plot for the five tested genes of a 24 neurons sample collected from the same VMN slice. (*B–D*) receptor coexpression is represented for the 97 cells (of 250 assayed) that were positive for at least one receptor. (*B*) Numerical values that are represented in *C* and *D*.



Movie S1. To assess the possibility that the evoked response may be due to or contaminated by mechanical disturbances caused by the ejection, a vehicle (ACSF) instead of test agonists ejected at distances that would be effective for agonists did not evoke any response in any of the six neurons tested. Further advance of the ejecting pipette toward the neuron with the intention to create artifacts failed to evoke any artifact in any of the six neurons even when large, visible mechanical disturbances were evoked. This video shows one of such intentionally created mechanical disturbances, with the stimulation pipette on the left and the recording pipette on the right. Movie recorded using Clampex v9.2 (Axon Instruments) and edited with Any Video Converter Version 3.0.5.

Movie S1