

Excitation of histaminergic tuberomamillary neurons by thyrotropin-releasing hormone

Régis Parmentier^{1,2}, Sergej Kolbaev², Boris P. Klyuch², David Vandael², Jian-Sheng Lin¹, Oliver Selbach², Helmut L. Haas², Olga A. Sergeeva^{2*}

¹ *Physiologie intégrée du système d'éveil INSERM : U628, IFR19, Université Claude Bernard - Lyon 1, Université Claude Bernard 8, Avenue Rockefeller 69373 LYON CEDEX,FR*

² *Department of neurophysiology Department of neurophysiology, Heinrich-Heine-University, D40001 Duesseldorf,DE*

* Correspondence should be addressed to: Olga Sergeeva <olga.sergeeva@uni-duesseldorf.de >

Abstract

The histaminergic tuberomamillary nucleus (TMN) controls arousal and attention and the firing of TMN neurons is state-dependent, active during waking, silent during sleep. Thyrotropin-releasing hormone (TRH) promotes arousal and combats sleepiness associated with narcolepsy. Single-cell RT-PCR (scRT-PCR) demonstrated variable expression of the two known TRH receptors in the majority of TMN neurons. TRH increased the firing rate of most (ca 70%) TMN neurons. This excitation was abolished in the presence of the TRH receptor antagonist chlordiazepoxide (50 μ M). In the presence of tetrodotoxin TRH depolarized TMN neurons without changing their input resistance. This effect reversed at the potential typical for nonselective cation channels. The potassium channel blockers barium and cesium did not influence the TRH-induced depolarization. TRH effects were antagonized by inhibitors of the Na⁺/Ca²⁺ exchanger, KB-R7943 and benzamil. The frequency of spontaneous inhibitory GABAergic postsynaptic potentials was either increased (TTX-insensitive) or decreased (TTX-sensitive GABA release sites) by TRH, indicating a heterogeneous modulation of GABAergic inputs by TRH. Montirelin (TRH analogue, 1 mg/kg ip) induced waking in wild type mice but not in histidine decarboxylase knockout mice lacking histamine. Inhibition of histamine synthesis by (S)- α -fluoromethylhistidine blocked the arousal effect of montirelin in WT mice. We conclude, that direct excitation of rodent TMN neurons by TRH is receptor-mediated and demands activation of nonselective cation channels as well as electrogenic Na⁺/Ca²⁺ exchange. Our findings indicate a key role of histamine in TRH-induced arousal.

MESH Keywords Action Potentials ; physiology ; Animals ; Calcium Channels ; metabolism ; physiology ; Cations, Divalent ; metabolism ; Histamine ; deficiency ; physiology ; Hypothalamic Area, Lateral ; metabolism ; physiology ; Inhibitory Postsynaptic Potentials ; physiology ; Male ; Mice ; Mice, Knockout ; Neurons ; metabolism ; physiology ; Rats ; Rats, Wistar ; Receptors, Thyrotropin-Releasing Hormone ; agonists ; antagonists & inhibitors ; physiology ; Sleep Stages ; physiology ; Sodium Channels ; metabolism ; physiology ; Thyrotropin-Releasing Hormone ; physiology

Author Keywords posterior hypothalamus ; thyrotropin releasing hormone ; montirelin ; H3-receptors ; intracellular recording ; sleep EEG

Introduction

The tripeptide thyrotropin-releasing hormone (TRH) was the first identified hypothalamic releasing factor which influences peripheral hormone levels through thyroid-stimulating hormone (TSH) release. Beyond neuroendocrine function, two TRH receptors, TRH and the TRH degrading enzyme (TRH-DE) are expressed in many brain regions, where they can modulate neuronal activity, suggesting a role of TRH as a neurotransmitter or neuromodulator {Gershengorn & Osman 1996 };{Heuer, Schafer, et al. 2000 }. Clinical and experimental reports demonstrated a role of TRH in the modulation of locomotion, cognition, mood and sleep. Biologically stable TRH analogues such as CG3703 (montirelin) and TA0910 increase wakefulness and decrease sleep time in narcoleptic canines {Nishino, Arrigoni, et al. 1997 };{Riehl, Honda, et al. 2000 }. TRH analogues are also known as antiepileptics in animal seizure models {Nillni & Sevarino 1999 } and in clinical use {Kubek & Garg 2002 }. While the antiepileptic function of TRH can be explained by its excitatory action on hippocampal interneurons {Deng, Porter, et al. 2006 };{Atzori & Nistri 1996 } cellular mechanisms underlying the promotion of arousal are not fully elucidated. Broberger and McCormick {Broberger & McCormick 2005 } demonstrated a depolarization of perigeniculate and thalamocortical cells in the lateral geniculate nucleus by TRH, and a transformation of perigeniculate neurons from bursting to the tonic, single-spike mode of action potential generation. These actions shift the behavioral state from sleep to waking. However, the wake-promoting potential of TRH involves other structures and neurons too. The posterior hypothalamus with the histaminergic and orexinergic (hypocretinergic) neurons has a crucial function for the waking state and TRH provides excitatory drive in this location {Hara, Xie, et al. 2007 };{Sergeeva, Parmentier, et al. 2007 }. The histaminergic tuberomamillary nucleus (TMN) plays a prominent role in sleep-waking regulation {Haas & Panula 2003 }; Haas et al., 2008). In freely moving animals, histaminergic neurons discharge tonically and specifically during waking {Steininger, Alam, et al. 1999 };{Vanni-Mercier, Gigout, et al. 2003 };{Takahashi, Lin, et al. 2006 }. Enhancing histaminergic transmission promotes wakefulness {Lin, Sakai, et al. 1988 };{Monti, Jantos, et al. 1991 }. Finally, abolition of histamine synthesis in knock-out mice affects the cortical EEG during all sleep-wake states and causes behavioral deficits indicating a key role in the maintenance of an awake state, notably in the presence of behavioural challenges {Parmentier, Ohtsu, et al. 2002 }. Some histaminergic neurons show TRH-immunoreactivity {Airaksinen, Alanen, et al. 1992 } and express TRH receptors {Gotoh, Fukagawa, et al.

2007 }, whereas release of TRH from neurosecretory nerve endings in the mediobasal hypothalamus is stimulated by histamine through H2 receptors {Charli, Joseph-Bravo, et al. 1978 }. The histaminergic system has been made responsible to some extent for the effects of TRH on the regulation of feeding: TRH-induced suppression of feeding after food deprivation was missing in H1 (histamine receptor-1) knock-out mice and in histamine-depleted rats {Gotoh, Fukagawa, et al. 2007 }.

The aim of the present study was the elucidation of molecular and electrophysiological actions of TRH on histaminergic neurons and of the role of histamine in TRH-induced arousal. We describe the expression of TRH receptors and demonstrate, that similar to the orexin- and serotonin-mediated excitation {Eriksson, Sergeeva, et al. 2001 };{Eriksson, Stevens, et al. 2001 }, TRH-receptor mediated excitation of TMN neurons demands activation of a $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Parts of this work have been presented in abstract form {Sergeeva, Parmentier, et al. 2007 }.

Materials and methods

Slice preparation

Coronal brain slices from the posterior hypothalamus about 400 μm thick containing the TMN were prepared from 21–28 days old male Wistar rats and 5–10 week-old mice (129/Sv strain). Animal experiments were conducted according to German law and the local guidelines (Bezirksregierung Duesseldorf). All efforts were made to minimize the number of animals and their suffering. The animals were quickly decapitated and the brains transferred to ice-cold modified artificial cerebrospinal fluid (ACSF), saturated with carbogen (95% O_2 /5% CO_2), in which NaCl had been replaced by 207 mM sucrose. In this solution slices were cut with a vibroslicer (Campden Instruments, U.K.) and placed into ACSF containing (in mM): NaCl 124, KCl 3.7, CaCl_2 2.0, MgSO_4 1.3, NaH_2PO_4 1.24, NaHCO_3 25.6, D-glucose 10, phenol red 0.01%, bubbled with carbogen (pH 7.4) for at least 1 hour at room temperature and then transferred to the recording chamber at 32°C, where they were constantly perfused with the same ACSF at a flow rate of 1–2 ml/min.

Slice-electrophysiology

Extracellular recordings were obtained using glass microelectrodes filled with ACSF (resistance 4–8 M Ω). According to Ericson et al {Ericson, Watanabe, et al. 1987 } the TMN is subdivided into three subgroups: a diffuse part (neurons are scattered within the lateral hypothalamic area) and two compact (nucleus-like) parts: the ventral TMN (neurons situated at the ventral surface of the brain) and the medial TMN (dense neuronal groups on each side of the mamillary recess of the third ventricle). Neurons were recorded in the ventral, most dense part of TMN, which was visually identified under a dissecting microscope. Signals were recorded using an Axoclamp 2B amplifier and a Digidata 1200 interface board (Axon Instruments, USA), filtered between 0.5–10 kHz, sampled at 20 kHz and analyzed with pClamp8 software (Axon Instruments, USA). The frequency of extracellular action potentials was determined online in bins of 15 s duration. Intracellular recordings from TM neurons were obtained using sharp glass microelectrodes filled with 3 M KCl (if not mentioned otherwise) with resistances of 80–110 M Ω . Biocytin (Sigma, Deisenhofen, 1%) was added to the electrode solution. We used the following electrophysiological criteria to identify TM neurons. They exhibit a regular, spontaneous firing rate (typically 2 – 6 Hz) and no burst firing at a resting membrane potential of approximately -50 mV, a broad action potential with a Ca^{2+} shoulder, and a long after-hyperpolarization. Finally, an inward current is activated during a large hyperpolarizing step and a transient outward K^+ current is activated after removing the hyperpolarization (See Fig. 4). For the voltage ramp experiments K^+ acetate 4M intracellular solution was used and CdCl_2 (10 μM), D-AP5 (50 μM), CNQX (20 μM), TTX (1 μM), bicuculline methiodide (10 μM) added to the bath solution. Neurons were filled with biocytin at the end of experiments by 200 ms-long anodal (depolarizing) current pulses (frequency 1 Hz) for at least 20 minutes. Slices were fixed after recording over night in 4% paraformaldehyde (prepared in 0.1 M phosphate buffer saline (PBS), pH 7.4) and cryoprotected in PBS with 20% sucrose, than cryosectioned at 40 μm thickness and mounted on gelatin-coated slides, dried and stained according to the immunofluorescence staining protocol. The sections were first washed in PBS with 0.25% TritonX-100 (PBS-T) for 5 min and then preincubated with 2% normal goat serum in PBS-T for 30 min at room temperature. This solution was also used to dilute primary guinea pig polyclonal antibody to HDC (histidine decarboxylase, Acris, Bad Nauheim, Germany) to 1:600. This antibody was applied to the sections for 12–16 hr at 4°C. After washing, sections were incubated with Alexa Fluor 488-labeled goat-anti-guinea pig IgG (1:500; Molecular Probes, Eugene, OR) to reveal HDC immunoreactivity and Texas Red-streptavidin (1:200, Molecular Probes) to stain biocytin-filled neurons, for 90 min at room temperature.

Whole cell patch-clamp recordings of GABAergic spontaneous inhibitory postsynaptic currents (sIPSCs) were made from ventral TMN neurons in coronal rat brain slices. Cells were visually identified and approached with the help of infrared differential interference contrast (IR-DIC). Voltage clamp (at -70 mV) recordings of sIPSCs were done either at room temperature (22–24°C) or at $32 \pm 0.5^\circ\text{C}$ with a flow rate of 2–2.5 ml/min in the presence of AMPA and NMDA receptor blockers: D-AP5 (100 μM) and DNQX (10 μM), using an EPC9 patch-clamp amplifier (Heka Elektronik, Germany). The patch pipette solution contained (mM) 135 KCl, 1 CaCl_2 2 MgCl_2 , 1(10⁻²) EGTA, 10 HEPES, 2 Na2ATP, 0.5 Na2GTP (pH 7.2 adjusted with KOH). Histaminergic neurons were identified by presence of the inwardly rectifying current activated by hyperpolarization (I_h) {Kamondi & Reiner 1991 } and the transient outward current (I_A) {Greene, Haas, et al. 1990 } (See Fig. 4).

Spontaneous IPSCs were analysed with MiniAnalysis 4.2 (Synaptosoft, Leonia, NJ, USA): Peak amplitude, the 10–90% rise time, T_{dec} (exponential decay time constant in a 100 ms window from the time of peak) and frequency of sIPSCs were calculated. All events were visually inspected before analysis in order to exclude obvious artefacts. Cumulative inter-event intervals, kinetic (T_{dec}) and amplitudes were compared between control (before TRH application) versus a period of recording with TRH using the Kolmogorov-Smirnov 2 sample test in every cell. Each of three testing periods lasted 180–360 s. The non-parametrical Wilcoxon test was used for comparison between groups. The significance level was set at $p < 0.05$.

Single-cell RT-PCR

Acutely isolated hypothalamic neurons were prepared from the brains of 22–28 days old male Wistar rats ($n = 6$) or 21–60 days old male 129/Sv mice ($n = 4$). Transverse slices containing the TM region were cut and incubated for 1 hour in a solution containing (mM): NaCl 125, KCl 3.7, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO_4 1.3, $NaHCO_3$ 23, D-glucose 10, phenol red 0.01%, bubbled with carbogen (pH 7.4). TMN was dissected from the slice and incubated with papain in crude form (0.3 – 0.5 mg/ml) for 30 min at 37°C. After rinsing the tissue was placed in a small volume of recording solution with the following composition (in mM): NaCl 150, KCl 3.7, $CaCl_2$ 2.0, $MgCl_2$ 2.0, HEPES 10, pH adjusted to 7.4 with NaOH. Cells were separated by gentle pipetting and placed in the recording chamber. Whole-cell patch-clamp recordings in voltage clamp mode were used to determine the electrophysiological properties and viability of the neurons, which responded with a sodium current to depolarizing voltage steps. After recording, the cytoplasm of the cell was sucked into the electrode in a stream of sterile control solution. The content of the electrode (8 μ l) was expelled into an Eppendorf tube, containing 7 μ l of a mixture prepared according to the protocol of the “first strand cDNA synthesis kit” (Pharmacia Biotech, Freiburg, Germany). After incubation for 1 h at 37°C for reverse transcription (RT) this reaction was stopped by freezing at –20°C.

Cell identification was performed by histidine decarboxylase (HDC)-cDNA amplification. For the first amplification round primers HDCup: 5'-GAT GAT GGA GCC C(A/T)G TGA ATA-3' was used with HDC lo: 5'-CTG GTC AGA GGC ATA GGC AAC A-3' in rats and with mHDC lo: 5'-TCA GAG GTG TAG GCA ACG A-3' in mice. For the second round of amplification in rats HDC up2 primer: 5'-AGT CCT CTG CAA GAC GCC TC-3' was taken in combination with HDC lo primer, generating PCR products of 457 b.p. size. Mouse HDC was amplified with the HDC up primer in combination with HDC lo2 primer: 5'-GAT GCT GTC CCA GCT GTC G-3' (expected size of amplicon 193 b.p.). In mice and rats cDNAs encoding for the TRH receptors were amplified in the first amplification round with degenerate primers Dgup: 5'-TGGCTGC(AG)GG(AG)CT(GC)CCCAA-3' and Dglo: 5'-TGGTG(AG)CCTGCTTCCTGGA-3'. For the TRHR1-specific amplification primer R 1 lo: 5'-TGGCTCTGGAAA(CT)GTGCA(GC)AG-3' was used in combination with Dgup (amplicon size 201 b.p.) and for the TRHR2-specific amplification R2up: 5'-TGAGAGCACAGACCGTGTGCACTG- 3' and R2lo: 5'-TC(CA)CCAGCAAGGGT(GC)C(AG)ATGAA-3' primers were used (amplicon size 219 b.p.). Randomly selected PCR products obtained after two amplification rounds were purified in water and sequenced. The obtained sequences corresponded to the known one for the rat or mouse (GENBANK, accession number): mouse TRHR2 receptor (BC117988), mouse TRHR1 (BC128269), rat TRHR1 (M90308) and rat TRHR2 (AB015645).

Thin-walled PCR tubes contained a mixture of first strand cDNA template (1–1.5 μ l), 10x PCR buffer, 10 pM each of sense and antisense primer, 200 μ M of each dNTP and 2.5 units Taq polymerase. The final reaction volume was adjusted to 10 μ l with nuclease-free water (Promega, Mannheim, Germany). The magnesium concentration was 3 mM in all PCR reactions. The Taq enzyme, PCR buffer, Mg^{2+} solution, and four dNTPs were all purchased from Qiagen (Erkrath, Germany). All oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Amplification was performed on a thermal cycler (Mastecycler, Eppendorf, Germany). A two round amplification strategy was used in each protocol. In each round 35 cycles of the following thermal programs were used: denaturation at 94°C for 48 s, annealing at 53°C for 48 s, and extension at 72°C for 1 min. For the second amplification round 1 μ l of the product of the first PCR was used as a template. Products were visualized by staining with ethidium bromide and analyzed by electrophoresis in 2% agarose gels.

Real-time RT-PCR analysis of TRH receptor-expression in HDC KO and WT mice

Total cellular mRNA was isolated from posterior hypothalamic slices (500–600 μ m thick) using an mRNA isolation kit (Pharmacia Biotech) from 6 – 11 week-old KO ($n=4$) and WT ($n=4$) mice according to the manufacturer's protocol. Total mRNA was eluted from the matrix with 200 μ l of RNase-free water. For the reverse-transcription 8 μ l of eluted mRNA was added to 7 μ l of reagents mixture prepared according to the protocol of the “first strand cDNA synthesis kit” (Pharmacia Biotech). After incubation for 1 hour at 37°C the reverse transcription reaction was stopped by freezing at –20°C. The reverse-transcription reactions were not normalized to contain the equivalent amounts of total mRNA. The PCR was performed in a PE Biosystems GeneAmp 5700 sequence detection system using the SYBR green master mix kit. Each reaction contained 2.5 μ l of the 10xSYBR green buffer, 200 nM dATP, dGTP, and dCTP and 400 nM dUTP, 2 mM $MgCl_2$, 0.25 units of uracil N-glycosylase, 0.625 units of Amplitaq Gold DNA polymerase, 10 pM forward and reverse primers, 5 μ l of 1:4 diluted cDNA, and water to 25 μ l. All reactions were normalized on β -actin expression, which was amplified with the primers β -actin up: 5'-CGT GAA AAG ATG ACC CAG ATC ATG TT-3'; β -actin lo: 5'-GCT CAT TGC CGA TAG TGA TGA CCT G-3',

The reactions were performed in optical tubes capped with MicroAmp optical caps. The reactions were incubated at 50 °C for 2 min to activate uracil N'-glycosylase and then for 10 min at 95 °C to inactivate the uracil N'-glycosylase and activate the Amplitaq Gold polymerase followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C. The PCR reactions were subjected to a heat dissociation protocol (PE Biosystems 5700 software). Following the final cycle of the PCR, the reactions were heat denatured over a 35 °C temperature gradient at 0.03 °C/s from 60 to 95 °C. Each PCR product showed a single peak in the denaturation curves. Standard curves for real-time PCR protocols with all primer-pairs obtained with sequential dilutions of one cDNA sample (till 1:128) were found optimal (linear regression coefficients were >0.95). Semiquantitative analysis of TRH receptor expression relative to the β -actin endogenous control was performed according to the " $2^{-\Delta\Delta Ct}$ " (Δ Fold) method as described previously {Sergeeva, Chepkova, et al. 2003 }. The non-parametrical Mann-Whitney U test was used for the comparison between averages (6–9 data points for each animal).

Surgery, polygraphic recording in the mouse and analysis of sleep-wake parameters

All experiments followed EEC (86/609/EEC) directives. Histidine decarboxylase knock-out mice were offspring of the mouse strain generated by {Ohtsu, Tanaka, et al. 2001 } and kept on 129Sv genomic background and genotyped by PCR (see Parmentier et al {Parmentier, Ohtsu, et al. 2002 }). At the age of 12 weeks and with a body weight of 30 ± 2 g, mice used for EEG and sleep-wake studies were chronically implanted, under deep gas anesthesia using isoflurane (2%, 200 ml/min) and a TEM anesthesia system (Bordeaux, France), with six cortical electrodes (gold-plated tinned copper wire, $\varnothing = 0.4$ mm, Filotex, Draveil, France) and three neck muscle electrodes (fluorocarbon-coated gold-plated stainless steel wire, $\varnothing = 0.03$ mm, Cooner Wire Chatworth, CA, U.S.A.) to record the electroencephalogram (EEG) and electromyogram (EMG) and to monitor the sleep-wake cycle. Finally, the electrode assembly was anchored and fixed to the skull with Super-Bond (Sun Medical Co., Shiga, Japan) and dental cement. This implantation allows stable and long-lasting polygraphic recordings {Parmentier, Ohtsu, et al. 2002 }.

After surgery, the animals were housed individually in barrels placed in an insulated sound-proof recording room maintained at an ambient temperature of 22 ± 1 °C and on a 12 h light/dark cycle (lights-on at 7 a.m.), standard food and water being available ad libitum. After a 7-day recovery period, mice were habituated to the recording cable for 7 days before polygraphic recordings were started. Cortical EEG (contralateral frontoparietal leads) and EMG signals were amplified, digitized with a resolution of 256 and 128 Hz, respectively, and computed on a CED 1401 Plus (Cambridge, UK). Using a Spike2 script and with the assistance of spectral analysis using the fast Fourier transform, polygraphic records were visually scored by 30-sec epochs for wakefulness (W), slow wave sleep (SWS), and paradoxical sleep (PS) according to previously described criteria validated for mice {Valatx & Bugat 1974 }; {Parmentier, Ohtsu, et al. 2002 }.

Animals were subjected to sleep-wake recordings following administration of either a vehicle (NaCl 0.9%) or montirelin (TRH analog, known as NS3, CG3703) at 1 or 3 mg/kg. Drugs were dissolved in the vehicle, fresh before each administration, and were administered ip. All administrations were performed at 10 a.m., i.e. during the sleepy period. The order of administration was randomized. Polygraphic recordings were made immediately after administration and maintained during 24 h. Two administrations were separated by a period of 7 days (washout). Each animal served as its own control and each administration was repeated two times. Statistical analysis was performed with Dunnett's t test and ANOVA for repeated measures. Significance level was set at $p < 0.05$ or 0.01. Data are presented as mean \pm standard error of the mean (SEM).

Drugs and statistical analysis

Drugs used in the present study were: R- α -methyl-histamine, gabazine (SR-95531), KB-R7943, D-AP5 and DNQX from Biotrend (Koeln, Germany); (S)- α -fluoromethylhistidine from (Merck Sharp & Dohme, France); Chlordiazepoxide, benzamil hydrochloride hydrate, tetrodotoxin and TRH were obtained from Sigma/RBI (Deisenhofen, Germany). Montirelin (CG3703) was a gift from Gruenenthal (Aachen). Drugs were diluted and stored as recommended. Neurons were recorded for at least 15 min to obtain a stable baseline before perfusion of drugs for 5–10 min in the recording chamber. Statistical analysis was performed with the non-parametrical Mann-Whitney U-test. Significance level was set at $p < 0.05$. Data are presented as mean \pm standard error of the mean (SEM).

Results

Extracellular firing rate of TMN neurons is increased by TRH

Several criteria were used to identify TMN neurons in the present study. The recordings were performed in the ventral part of the TMN, a region where histaminergic neurons are encountered almost exclusively when recording intracellularly with sharp electrodes {Eriksson, Sergeeva, et al. 2001 }. For extracellular recording ventral TMN neurons were selected on the basis of their location, regular firing in the range of 1–8 Hz (most typically 2–4 Hz and a broad triphasic action potential (2–4 ms) {Sergeeva, Klyuch, et al. 2006 }. In addition, pharmacological identification was used as the most reliable criterion: TMN neurons displayed an inhibition by the H3-receptor agonist R- α -methyl-histamine. A total of 33 neurons fulfilled these criteria and were taken for analysis.

In mouse slices 6 out of 8 TMN neurons (75%) responded to TRH (1.5 μ M) with an enhancement of firing rate to 190 ± 61 % of the control level. In rat slices 10 out of 14 cells (71%) were excited by TRH (2–10 μ M, there was no difference in the response amplitude between these two concentrations) to 262 ± 66 % of control, Fig. 1A,B,C. The effect was followed by a fast desensitization in the presence of TRH. A second TRH response was not observed in the same neuron within 1 hr (n=4, not shown). Montirelin (CG3703, 10 μ M), a biologically stable analogue of TRH, enhanced firing frequency in rat TMN neurons to 168 ± 14 % (n=4).

The TRH effect is antagonized by chlordiazepoxide

Chlordiazepoxide (CDZ, 10 μ M), an antagonist at TRH receptors attenuated the effect of TRH in rat TMN neurons; the enhancement of firing reached 215 ± 51 % (n=14) in control, and 144 ± 35 % in the presence of CDZ (p=0.19, Mann-Whitney U test, n=4). At 50 μ M CDZ, a concentration used also in previous studies for the blockade of TRH receptors {Deng, Porter, et al. 2006}, the TRH effect was abolished (n=6, p=0.0005, Mann-Whitney U test, Fig. 1D). Interestingly, CDZ (50 μ M) significantly reduced the firing rate of TMN neurons by 20 ± 5 % (n=6, p=0.065) on its own. In acutely isolated mouse TMN neurons, CDZ potentiated GABA-evoked whole-cell currents with an EC₅₀ of 0.7–1 μ M and maximally at 10 μ M (no further potentiation at 50 μ M). Therefore the observed inhibition of firing cannot be attributed to the modulatory action of CDZ at GABA_A receptors. Moreover, GABA_A receptors do not exhibit a tonic influence on TMN firing in slices, as bicuculline (50 μ M, n=4) and gabazine (up to 100 μ M, n=6) did not change the firing rate of TMN neurons. This indicates either an endogenous tone of TRH or the presence of constitutively active TRH receptors {Heinflink, Nussenzveig, et al. 1995} {Jinsi-Parimoo & Gershengorn 1997}.

Inhibition of Na⁺/Ca²⁺ exchange by benzamil blocks the TRH effect

In the presence of benzamil (20 μ M) TRH increased firing rate in 2 TMN neurons recorded in rat slices and did not affect firing frequency in 8 neurons (Fig. 1D). The difference to the occurrence of the excitatory TRH effect under control conditions (10 neurons out of 14 tested) was significant (p=0.04, Fisher's exact probability test). On the average in the presence of benzamil TRH increased firing frequency to 134.1 ± 29.4 % of control (n=10), to a significantly lower level (p=0.036) compared to the control condition (215 ± 51 % of control, n=14).

Transcriptional analysis of TRH receptor expression

Mouse and rat slices prepared in the same way as for electrophysiological recordings were used for the acute isolation of histaminergic TMN neurons. After whole-cell voltage clamp recordings of voltage dependent sodium currents TMN neurons were subjected to single-cell RT-PCR (see Sergeeva et al {Sergeeva, Amberger, et al. 2003}). Among 26 mouse TMN neurons (positive for the histamine-producing enzyme histidine decarboxylase, HDC), 9 cells expressed TRH R2 (35%), 6 cells TRH R1 (23%), 5 cells (19%) contained mRNAs for both receptor types and 6 cells (23%) were TRHR-negative (Fig. 1F; 2A). Among 19 rat TMN neurons, 4 cells were negative for TRH receptors (21%), 8 cells (42%) expressed TRH R1, 1 cell (5%) only TRH R2 and 6 cells both receptors (32%). The TRH R2 was less frequently detected in rats than in mice: 37% versus 54%, respectively, while the occurrence of TRH R1 transcripts was opposite: 74% of neurons in rats versus 42% in mice, where found to be TRH R1-positive.

Thus, the transcriptional analysis of TRH receptor expression revealed that TRH R-negative cells represent the same small population among histaminergic neurons (ca 20%), as the TMN neurons not responding to TRH (21–29%) in electrophysiological experiments (see above).

Intracellular recordings in rat slices

Stable recordings were obtained from 32 neurons with electrophysiological characteristics of TMN neurons {Haas & Reiner 1988}. Recorded in current clamp mode, they exhibited spontaneous firing at 3.8 ± 0.2 Hz (n = 29) and a typical I_h sag of 75 ± 5 %, measured as the percentage reduction from the peak at the end of a 0.5-sec-long step elicited by a -400 pA current injection. Under tetrodotoxin, their resting membrane potential was -52.5 ± 0.8 mV (n = 25). Bath application of TRH (1.5 μ M) rapidly depolarized and increased the spontaneous firing rate of the TM neurons; this effect reversed completely 20–40 min after TRH withdrawal (Fig. 3A). During a washout period of 1 hr, repeated application of TRH caused neither depolarization nor an increase in firing rate. In the presence of tetrodotoxin, which prevents firing and causes synaptic isolation, a depolarization by 15.3 ± 2.4 mV is clearly detected in 4 cells (out of 6 tested) (Fig. 3B). No change in the input resistance was observed. The following experiments were designed to elucidate the mechanism of the depolarization by TRH (summarized in Figure 3C). Only the cells which responded to TRH were used (21 of 28). First, the effect of cations that can block potassium conductances were tested. The effect of TRH was not inhibited by 500 μ M BaCl₂ (n=5; Fig. 3C) or by 3 mM CsCl (n=3). The selective blocker of the Na⁺/Ca²⁺ exchanger (NCX), KB-R7943 {Iwamoto, Watano, et al. 1996} at 80 μ M, strongly suppressed the depolarization (p<0.01), but a residual 2–6 mV depolarization remained in 5 responding cells. Voltage-current curves obtained before and during 1.5 μ M TRH intersected at -4.3 ± 6.3 mV (n=4, Fig. 3D), a potential close to the predicted one for a mixed cationic conductance. Electrophysiological identification of TMN neurons was confirmed post hoc by the co-localization of biocytin (delivered to the cell through the recording electrode) with HDC-immunoreactivity (Fig. 3E).

Frequency of sIPSCs is differently modulated by TRH in different cells

In our initial experiments sIPSCs were recorded from TMN neurons, identified by the presence of I_h and I_A currents (Fig. 4A) in rat coronal slices at room temperature. Surprisingly, they were either significantly increased ($n=2$), or decreased ($n=5$) in frequency after bath application of TRH (2.5 μ M) for 5 min. The amplitude was not changed significantly under TRH indicating a presynaptic effect. After TRH washout (5–10 min) the GABA_A receptor antagonist gabazine (10 μ M) abolished sIPSCs in all cells ($n=7$, Fig. 4B) in accordance with our previous studies {Eriksson, Sergeeva, et al. 2004 };{Sergeeva, Eriksson, et al. 2002 } showing that spontaneous synaptic currents recorded from somata of TMN neurons are exclusively carried through the postsynaptic GABA_A receptors. No postsynaptic inward currents in response to TRH were seen in these experiments indicating a high probability that Na^+ /Ca^{2+} exchange is involved in TRH-induced excitation as this transport is functionally inactive at room temperature in TMN neurons {Eriksson, Stevens, et al. 2001 }.

As several factors could contribute to the heterogeneous responses to TRH of GABAergic cells and their axons we performed the following experiments in the presence of AMPA/NMDA receptor blockers. A neurochemical study revealed recently that TMN neurons are under tonic NMDA-receptor mediated inhibition {Faucard, Armand, et al. 2006 } as NMDA receptor antagonists increase TMN histamine production and its release in different brain regions. As inhibitory NMDA receptors do not exist, these findings are explained by the tonic activation of inhibitory GABAergic cells projecting to the TMN through these receptors. As GABAergic cells may also diverge with respect to their demand for Na^+ /Ca^{2+} exchange in order to respond to TRH we performed the following experiments at 32°C. We expected to get a homogeneous modulation of sIPSCs under such conditions.

In the presence of AMPA/NMDA receptor antagonists the sIPSC frequency was significantly reduced in all investigated neurons ($n=13$), while amplitudes and decay kinetics were not significantly altered (see Fig. 4C,D), indicating a presynaptic site of action. The extent of frequency and amplitude modulation of sIPSCs by AMPA/NMDA receptor antagonists did not correlate with the again diverse effects of TRH: in 6 neurons TRH caused increases (group 1) in sIPSC frequency from 0.85 ± 0.27 Hz to 1.37 ± 0.49 Hz, in 4 cells (group 2) decreases from 0.61 ± 0.27 Hz to 0.43 ± 0.18 Hz and in 3 cells (group 3) the frequency was not significantly changed (0.87 ± 0.29 Hz)(Fig. 5). There was no difference in the control sIPSC frequencies between the 3 groups. The reduction of sIPSC frequencies obtained prior to TRH application with DNQX/D-AP5 were to $62 \pm 6.2\%$ ($n=6$); $55.3 \pm 18.7\%$ ($n=4$) and $72.9 \pm 11.6\%$ ($n=3$) of control in the first, second and third cellular group, respectively (difference between groups was not significant). Postsynaptic inward currents (24.9 ± 3.38 pA) upon TRH-perfusion were seen in 11 out of 17 cells (65%) recorded at physiological temperature; however these responses were found in neurons belonging to all three groups (see Fig. 5A), indicating that postsynaptic and presynaptic effects of TRH are not coherent at least in a slice preparation in vitro, creating large scale neuronal heterogeneity on the output of the histaminergic system.

TTX (1 μ M) abolished in TMN neurons I_{Na^+} (recorded by applying a depolarization step +40 mV of 200 ms duration from holding potential -50 mV) and significantly reduced frequency of spontaneous IPSCs in all recorded cells from 1.5 Hz to 0.5 Hz ($n=12$). In the presence of TTX a reduction of frequency of mIPSCs under TRH was never observed and in the majority of recorded neurons ($n=8$) the frequency did not change. However in four cells TRH significantly increased the frequency of mIPSCs (Kolmogorov-Smirnov test) from 0.55 ± 0.13 Hz to 0.86 ± 0.23 Hz, indicating that TRH enhanced action-potential independent release of GABA at some synapses either through presynaptic TRH receptors or indirectly, if an interaction with neuronal postsynaptic or glial receptors would trigger the release of some yet unknown transmitter. This retrograde or glial transmitter hypothesis was not elucidated further due to the low occurrence of this presynaptic facilitation phenomenon in TMN neurons in our preparation.

Can the reduction of sIPSC frequency under TRH be attributed to the release of endogenous dynorphin, which suppresses GABA release in TMN at the presynaptic site Sergeeva?. We addressed this question in experiments where TRH application was performed in the presence of the kappa-opioid receptor antagonist nor-binaltorphimine. In two out of seven investigated neurons TRH decreased sIPSC frequency from xxx to xxxx Hz, in five cells the frequency of sIPSCs was not affected by TRH. Facilitation of sIPSC frequency by TRH was not seen in these experiments. Nor-binaltorphimine significantly increased frequency of sIPSCs on its own in 3 (from xxx to xxxx Hz) and did not affect it in 5 investigated neurons indicating that endogenous dynorphin controls some GABAergic inputs to TMN neurons in slices.

Montirelin (CG3703) induces waking in mice

During the lights-on period, montirelin (TRH analogue) markedly enhanced wakefulness (Fig. 6) at the expense of slow wave sleep and paradoxical sleep in wild type mice ($HDC^{+/+}$) in a dose dependent manner. In fact, $HDC^{+/+}$ mice present a suppression of cortical slow activity ($\delta + \theta$ ranges) and spindles (8–14 Hz), resulting in a state of total cortical activation, i.e., low voltage electrical activity with dominant waves in the β and γ bands (20–60 Hz). These effects on the cortical EEG were manifested on polygraphic scoring as an almost total waking state, characterized by a significantly delayed sleep latency (Fig. 6) during more than 2 hours with 3 mg/kg. In $HDC^{-/-}$ mice, the same injections of montirelin had no significant effect on the wake duration compared to saline injection of the same animals. However, $HDC^{-/-}$ mice present low but significantly delayed sleep latencies after the administration of 3 mg/kg of montirelin (slow wave

sleep latency + 31.7 min compared to placebo vs +137.3 min for HDC^{+/+} mice, see Fig. 6). As HDC^{-/-} mice have perturbed wakefulness and a straightforward conclusion about histamines role in TRH-mediated arousal may suffer from compensatory changes in other aminergic systems of the brain or other transmitters in TMN neurons in these mice, we performed experiments with the acute depletion of TMN neurons from histamine by the ip injection of (S)- α -fluoromethylhistidine {Parmentier, Ohtsu, et al. 2002 } 3 hours prior to the montirelin injection in wild type mice. This depletion blocked the montirelin-mediated increase in waking (Fig. 6).

TRH receptor-expression in HDC knockout mice

We investigated the relative abundance of mRNAs encoding for the TRH receptors with the help of semi quantitative real-time RT-PCR. All data points in each amplification were normalized on the probe WT#1 (showing lowest receptor expression). No significant difference between HDC KO and WT mice was obtained: the levels of mRNA represented, for TRH R1 2.8 ± 0.44 vs 2.39 ± 0.5 ($p=0.47$) and for TRH R2 1.38 ± 0.4 vs 0.53 ± 0.26 ($p=0.11$), respectively.

Discussion

The present study elucidates mechanisms of TRH receptor-mediated responses and expression of TRH in individual posterior hypothalamic histaminergic (TMN) neurons. We show a direct depolarization of most histaminergic cells (ca 70%) by TRH through the activation of electrogenic Na⁺/Ca²⁺ exchange. This depolarization was not affected by cesium or barium indicating that block of a K⁺ conductance is not involved in the TRH-mediated excitation of TMN neurons. The reversal potential and the residual depolarization after NCX blockade indicate the implication of nonselective cation channels.

In several previous studies on mechanisms of the TRH action in the nervous system, inhibition of a resting K⁺ conductance was suggested as a major mechanism {Deng, Porter, et al. 2006 };{Bayliss, Viana, et al. 1992 }. However, immature motoneurons displayed activation of a non-selective cationic conductance by TRH and lack of interaction with potassium channels {Bayliss, Viana, et al. 1994 }, moreover, their depolarisation by TRH was not accompanied by a change in their input resistance. This "immature" type of the response, partially explored by Bayliss et al. {Bayliss, Viana, et al. 1994 };{Bayliss, Viana, et al. 1992 } bears similarity with the TRH responses in TMN neurons, which were, however, recorded in young adult rodents.

Single cell RT-PCR revealed expression of one or both TRH receptors in most TMN neurons, whereas a quarter of the neurons lacks TRH receptor-expression. The expression of these receptors corresponded to the ratio (70–75%) of responding to TRH TMN neurons in electrophysiological recordings. Previous in situ hybridization studies demonstrated that TRH R1 is predominantly distributed in hypothalamic areas {Heuer, Schafer, et al. 2000 } while TRHR2 seems to be widely distributed throughout the brain {Heuer, Schafer, et al. 2000 };{O'Dowd, Lee, et al. 2000 }. An immunohistochemical analysis by Gotoh and colleagues revealed that TRHR2 and to a lower extent TRHR1 are expressed in the histaminergic TMN neurons of rats {Gotoh, Fukagawa, et al. 2007 }. What is the functional meaning of the TMN neuron heterogeneity with respect to the expression of TRH receptors? TRHR1 and TRHR2 expressing cells may belong to different functional systems (feeding/neuroendocrine vs arousal and cognition) as these receptors are expressed in distinct pathways {Heuer, Schafer, et al. 2000 }, however due to the lack of specific pharmacological tools targeting one or the other receptor, the functional meaning of the heterogeneous expression of TRHRs in TMN neurons remains at present unclear.

The benzodiazepine chlordiazepoxide (CDZ) is a competitive TRHR antagonist, which dose dependently attenuated and blocked the TRH effect in TMN neurons. Interestingly, higher doses of CDZ produce a decrease of TMN neuron's spontaneous firing. This effect can occur due to a decrease of spontaneous TRH-tone on histaminergic cells or due to lowering constitutive activity of TRHRs {Heinflink, Nussenzweig, et al. 1995 };{Jinsi-Parimoo & Gershengorn 1997 }. High rates of turnover of TRHRs have been described in various cell types {Ashworth, Yu, et al. 1995 };{Drmot, Gould, et al. 1998 };{O'Dowd, Lee, et al. 2000 }. Therefore rapid agonist induced internalization of TRHRs may be responsible for the fast desensitization kinetics observed in the presence of TRH in our experiments and our failure to get a second response to TRH in a same slice. We do not know whether threshold or submaximal concentrations of TRH cause rapid receptor internalization in TMN. Considering the large cell to cell heterogeneity in TRH response amplitude and in the expression of TRHRs in TMN we performed all our experiments with nearly maximal concentrations of TRH (see dose-response curve for TRH constructed for hippocampal interneurons by Deng et al. {Deng, Porter, et al. 2006 }). At maximal concentrations (1.5–10 μ M) TRH caused depolarization or enhanced firing rate in about 70% of all recorded TMN neurons. Responses to 1.5, 2.5 and 10 μ M of TRH were statistically indistinguishable. With TRH concentrations lower than 1 μ M the fraction of neurons responding to TRH cells dropped below 50%, making the analysis of mechanisms difficult.

Experiments with two NCX inhibitors: benzamil and KB-R7943 indicated that TMN neurons are excited via NCX activation that causes depolarization. Although each of those blockers possesses some additional activities, such as antagonistic action towards non-selective cation channels of the TRPC3-type (KB-R7943, xxxxx) or epithelial Na⁺ channels (ENaC) and Na⁺/H⁺ exchanger (benzamil, xxx) we assume, that their blocking effects towards TRH responses are attributed to the NCX inhibition. First, neither ENaC (xxxx) nor TRPC3 channels Sergeeva are present in TMN neurons. Second, in accordance with the temperature-dependent activity of

NCX (xxxxxx), TRH-evoked inward currents could not be observed in the present study at room temperature. The NCX is electrogenic, with an exchange ratio of 3 Na⁺ in for every Ca²⁺ that is pushed out, and is expressed throughout the brain {Quednau, Nicoll, et al. 1997 }. Recently, we have shown that serotonin and orexin peptides induce depolarization of TMN neurons by activation of the NCX {Eriksson, Stevens, et al. 2001 };{Eriksson, Sergeeva, et al. 2001 }. In the same way, H1-receptor mediated depolarization of rat vasopressin neurons in the supraoptic nucleus occurs through the activation of NCX {Smith & Armstrong 1996 }. These receptors are all coupled to phospholipase C, and this is also true for TRHRs {Gershengorn & Osman 1996 }. The activation of NCX is likely secondary to a surge in the intracellular Ca²⁺ concentration, as the receptors are coupled to inositol 1,4,5-trisphosphate production. In the present study, no change in membrane conductance was seen in association with the NCX activation indicating that Ca²⁺ is most likely released from intracellular stores. Also in previous studies no obvious Ca²⁺-channel component was seen after NCX activation {Smith & Armstrong 1996 };{Eriksson, Stevens, et al. 2001 };{Eriksson, Sergeeva, et al. 2001 }.

In contrast to the hippocampus, where TRH enhances the frequency of sIPSCs in CA1 pyramidal neurons {Atzori & Nistri 1996 };{Deng, Porter, et al. 2006 }, TRH bi-directionally modulates frequency of sIPSCs in TMN neurons. This bi-directional nature of TRH-modulation is preserved under room temperature and AMPA/NMDA receptor antagonists. Interestingly, experiments with TTX revealed, that TTX-insensitive GABA release is facilitated by TRH whereas TTX-sensitive (action potential-dependent release) is inhibited. One of the possible explanations for the TRH-mediated suppression of GABA-release would be depolarization block of firing (inactivation of Na_v channels) due to the strong and long-lasting neuronal depolarization. This idea is supported by the rebound facilitation of sIPSC frequency after TRH withdrawal (see Fig. 5C), when the membrane potential of the presynaptic neuron returns to the resting value. Depolarization block of firing was seen in some of our sharp electrode recordings from TMN neurons, when concentration of TRH exceeded 1.5 μM. Another possibility would be the action-potential dependent release of some inhibitory neurotransmitter (modulator) acting presynaptically. One possible candidate for such action is a dynorphin. VLPO neurons (the major GABAergic input to the TMN) express both μ- and κ-opioid receptors {Mitchell, Prevot, et al. 1997 } and agonists of both receptors can suppress sIPSCs in TMN neurons {Eriksson, Sergeeva, et al. 2004 }. Thus opioid peptides may suppress GABAergic inputs to the TMN neurons supporting TRH-mediated excitation. Dynorphin (κ-opioid receptor agonist)-positive fibres are found at high density in the TMN region {Lantos, Gorcs, et al. 1995 } and in orexin/ataxin-3 mice, which show selective loss of the orexin neurons as well as prodynorphin mRNA in the lateral hypothalamic area, dynorphin expression is restricted to the orexin neurons in this area {Chou, Lee, et al. 2001 }. Orexinergic neurons are excited by TRH {Hara, Xie, et al. 2007 } and may co-release dynorphin and orexin. Whereas orexin enhances the frequency of sIPSCs recorded from TMN neurons, dynorphin inhibits it, when both peptides are co-applied, the effect of dynorphin dominates {Eriksson, Sergeeva, et al. 2001 }. Presynaptic κ-opioid receptors inhibit transmitter release from a variety of neurons activating voltage-dependent K⁺-channels {Schlicker & Kathmann 2008 }. Our experiments with the κ-opioid receptor antagonist nor-binaltorphimine indicated that endogenous dynorphin is unlikely responsible for the TRH-mediated inhibition of presynaptic GABA-release as such inhibition was observed only in 2 out of 7 neurons exposed to nor-binaltorphimine. Interestingly, facilitation of GABA-release by TRH was not observed in the presence of nor-binaltorphimine, indicating, that such facilitation may demand pre-conditioning of presynaptic terminals by dynorphin.

The TRH analogue montirelin induces robust waking in wild type mice but not in mice lacking histamine synthesis or under acute disruption of histamine synthesis. TRH receptor expression in posterior hypothalamus (Real-time PCR analysis) does not differ significantly between WT and KO mice. Therefore the difference in the behavioural response can be attributed here to the lack of histamine. A larger dose (3 mg/kg) of montirelin induces somewhat delayed sleep latencies in HDC^{-/-} mice (1/3 of those in HDC^{+/+} mice). Thus waking induced by low (physiological) TRH levels can be entirely attributed to the histaminergic system, while higher TRH levels may recruit other central or peripheral (metabolic) mechanisms. In conclusion, TRH may represent a key factor in controlling attention and arousal and the excitation of TMN neurons by TRH ascribes an important role in this action to brain histamine.

Acknowledgements:

We are grateful to Annette Scherer and Claudia Wittrock for excellent technical assistance. Supported by the DFG SE 1767, SFB 575/3 and 8, INSERM U628 and INSERM grant of JSL to RP. We wish to thank Dr. Hiroshi Ohtsu for the generous donation of HDC-knockout mice.

References:

- Airaksinen MS , Alanen S , Szabat E , Visser TJ , Panula P . 1992 ; Multiple neurotransmitters in the tuberomammillary nucleus: comparison of rat, mouse, and guinea pig . *J Comp Neurol* . 323 : 103 - 116
- Ashworth R , Yu R , Nelson EJ , Dermer S , Gershengorn MC , Hinkle PM . 1995 ; Visualization of the thyrotropin-releasing hormone receptor and its ligand during endocytosis and recycling . *Proc Natl Acad Sci U S A* . 92 : 512 - 516
- Atzori M , Nistri A . 1996 ; Effects of thyrotropin-releasing hormone on GABAergic synaptic transmission of the rat hippocampus . *Eur J Neurosci* . 8 : 1299 - 1305
- Bayliss DA , Viana F , Berger AJ . 1992 ; Mechanisms underlying excitatory effects of thyrotropin-releasing hormone on rat hypoglossal motoneurons in vitro . *J Neurophysiol* . 68 : 1733 - 1745
- Bayliss DA , Viana F , Kanter RK , Szymeczek-Seay CL , Berger AJ , Millhorn DE . 1994 ; Early postnatal development of thyrotropin-releasing hormone (TRH) expression, TRH receptor binding, and TRH responses in neurons of rat brainstem . *J Neurosci* . 14 : 821 - 833
- Broberger C , McCormick DA . 2005 ; Excitatory effects of thyrotropin-releasing hormone in the thalamus . *J Neurosci* . 25 : 1664 - 1673
- Cenni G , Blandina P , Mackie K , Nosi D , Formigli L , Giannoni P , Ballini C , Della CL , Francesco MP , Beatrice PM . 2006 ; Differential effect of cannabinoid agonists and endocannabinoids on histamine release from distinct regions of the rat brain . *Eur J Neurosci* . 24 : 1633 - 1644

- Charli JL , Joseph-Bravo P , Palacios JM , Kordon C . 1978 ; Histamine-induced release of thyrotropin releasing hormone from hypothalamic slices . *Eur J Pharmacol* . 52 : 401 - 403
- Chou TC , Lee CE , Lu J , Elmquist JK , Hara J , Willie JT , Beuckmann CT , Chemelli RM , Sakurai T , Yanagisawa M , Saper CB , Scammell TE . 2001 ; Orexin (hypocretin) neurons contain dynorphin . *J Neurosci* . 21 : RC168 -
- Deng PY , Porter JE , Shin HS , Lei S . 2006 ; Thyrotropin-releasing hormone increases GABA release in rat hippocampus . *J Physiol* . 577 : 497 - 511
- Drmota T , Gould GW , Milligan G . 1998 ; Real time visualization of agonist-mediated redistribution and internalization of a green fluorescent protein-tagged form of the thyrotropin-releasing hormone receptor . *J Biol Chem* . 273 : 24000 - 24008
- Ericson H , Watanabe T , Kohler C . 1987 ; Morphological analysis of the tuberomammillary nucleus in the rat brain: delineation of subgroups with antibody against L-histidine decarboxylase as a marker . *J Comp Neurol* . 263 : 1 - 24
- Eriksson KS , Sergeeva O , Brown RE , Haas HL . 2001a ; Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus . *J Neurosci* . 21 : 9273 - 9279
- Eriksson KS , Sergeeva OA , Selbach O , Haas HL . 2004 ; Orexin (hypocretin)/dynorphin neurons control GABAergic inputs to tuberomammillary neurons . *Eur J Neurosci* . 19 : 1278 - 1284
- Eriksson KS , Stevens DR , Haas HL . 2001b ; Serotonin excites tuberomammillary neurons by activation of Na(+)/Ca(2+)-exchange . *Neuropharmacology* . 40 : 345 - 351
- Faucard R , Armand V , Heron A , Cochois V , Schwartz JC , Arrang JM . 2006 ; N-methyl-D-aspartate receptor antagonists enhance histamine neuron activity in rodent brain . *J Neurochem* . 98 : 1487 - 1496
- Fone KC , Austin RH , Topham IA , Kennett GA , Punhani T . 1998 ; Effect of chronic m-CPP on locomotion, hypophagia, plasma corticosterone and 5-HT2C receptor levels in the rat . *Br J Pharmacol* . 123 : 1707 - 1715
- Gershengorn MC , Osman R . 1996 ; Molecular and cellular biology of thyrotropin-releasing hormone receptors . *Physiol Rev* . 76 : 175 - 191
- Gotoh K , Fukagawa K , Fukagawa T , Noguchi H , Kakuma T , Sakata T , Yoshimatsu H . 2007 ; Hypothalamic neuronal histamine mediates the thyrotropin-releasing hormone-induced suppression of food intake . *J Neurochem* . 103 : 1102 - 1110
- Greene RW , Haas HL , Reiner PB . 1990 ; Two transient outward currents in histamine neurones of the rat hypothalamus in vitro . *J Physiol* . 420 : 149 - 63
- Grimberg H , Zaltsman I , Lupu-Meiri M , Gershengorn MC , Oron Y . 1999 ; Inverse agonist abolishes desensitization of a constitutively active mutant of thyrotropin-releasing hormone receptor: role of cellular calcium and protein kinase C . *Br J Pharmacol* . 126 : 1097 - 1106
- Haas HL , Reiner PB . 1988 ; Membrane properties of histaminergic tuberomammillary neurones of the rat hypothalamus in vitro . *J Physiol* . 399 : 633 - 46
- Haas H , Panula P . 2003 ; The role of histamine and the tuberomammillary nucleus in the nervous system . *Nat Rev Neurosci* . 4 : 121 - 130
- Haas HL , Sergeeva OA , Selbach O . 2008 ; Histamine in the Nervous System . *Physiol Rev* . 88 : 1183 - 1242
- Hara J , Xie XM , Sakurai T , Kilduff TS . 2007 ; Thyrotropin releasing hormone excites hypocretin/orexin neurons via pre and postsynaptic mechanisms . *Society for Neuroscience* . 471.16 -
- Heinfliink M , Nussenzveig DR , Grimberg H , Lupu-Meiri M , Oron Y , Gershengorn MC . 1995 ; A constitutively active mutant thyrotropin-releasing hormone receptor is chronically down-regulated in pituitary cells: evidence using chlordiazepoxide as a negative antagonist . *Mol Endocrinol* . 9 : 1455 - 1460
- Heuer H , Schafer MK , O'Donnell D , Walker P , Bauer K . 2000 ; Expression of thyrotropin-releasing hormone receptor 2 (TRH-R2) in the central nervous system of rats . *J Comp Neurol* . 428 : 319 - 336
- Ishizuka T , Nomura S , Hosoda H , Kangawa K , Watanabe T , Yamatodani A . 2006 ; A role of the histaminergic system for the control of feeding by orexigenic peptides . *Physiol Behav* . 89 : 295 - 300
- Iwamoto T , Watano T , Shigekawa M . 1996 ; A novel isothiourea derivative selectively inhibits the reverse mode of Na⁺/Ca²⁺ exchange in cells expressing NCX1 . *J Biol Chem* . 271 : 22391 - 22397
- Jinsi-Parimoo A , Gershengorn MC . 1997 ; Constitutive activity of native thyrotropin-releasing hormone receptors revealed using a protein kinase C-responsive reporter gene . *Endocrinology* . 138 : 1471 - 1475
- Kamondi A , Reiner PB . 1991 ; Hyperpolarization-activated inward current in histaminergic tuberomammillary neurons of the rat hypothalamus . *J Neurophysiol* . 66 : 1902 - 1911
- Kohler C , Swanson LW , Haglund L , Wu JY . 1985 ; The cytoarchitecture, histochemistry and projections of the tuberomammillary nucleus in the rat . *Neuroscience* . 16 : 85 - 110
- Korotkova TM , Ponomarenko AA , Haas HL , Sergeeva OA . 2005 ; Differential expression of the homeobox gene Pitx3 in midbrain dopaminergic neurons . *Eur J Neurosci* . 22 : 1287 - 1293
- Kubek MJ , Garg BP . 2002 ; Thyrotropin-releasing hormone in the treatment of intractable epilepsy . *Pediatr Neurol* . 26 : 9 - 17
- Lam DD , Przydzial MJ , Ridley SH , Yeo GS , Rochford JJ , O'Rahilly S , Heisler LK . 2007 ; Serotonin 5-HT2C Receptor Agonist Promotes Hypophagia via Downstream Activation of Melanocortin 4 Receptors . *Endocrinology* .
- Lantos TA , Gorcs TJ , Palkovits M . 1995 ; Immunohistochemical mapping of neuropeptides in the premammillary region of the hypothalamus in rats . *Brain Res Brain Res Rev* . 20 : 209 - 249
- Lin JS , Sakai K , Jouvett M . 1988 ; Evidence for histaminergic arousal mechanisms in the hypothalamus of cat . *Neuropharmacology* . 27 : 111 - 122
- Mitchell V , Prevot V , Jennes L , Aubert JP , Croix D , Beauvillain JC . 1997 ; Presence of mu and kappa opioid receptor mRNAs in galanin but not in GnRH neurons in the female rat . *Neuroreport* . 8 : 3167 - 3172
- Monti JM , Jantos H , Boussard M , Altier H , Orellana C , Olivera S . 1991 ; Effects of selective activation or blockade of the histamine H3 receptor on sleep and wakefulness . *Eur J Pharmacol* . 205 : 283 - 287
- Nillni EA , Sevarino KA . 1999 ; The biology of pro-thyrotropin-releasing hormone-derived peptides . *Endocr Rev* . 20 : 599 - 648
- Nishino S , Arrigoni J , Shelton J , Kanbayashi T , Dement WC , Mignot E . 1997 ; Effects of thyrotropin-releasing hormone and its analogs on daytime sleepiness and cataplexy in canine narcolepsy . *J Neurosci* . 17 : 6401 - 6408
- O'Dowd BF , Lee DK , Huang W , Nguyen T , Cheng R , Liu Y , Wang B , Gershengorn MC , George SR . 2000 ; TRH-R2 exhibits similar binding and acute signaling but distinct regulation and anatomic distribution compared with TRH-R1 . *Mol Endocrinol* . 14 : 183 - 193
- Ohtsu H , Tanaka S , Terui T , Hori Y , Makabe-Kobayashi Y , Pejler G , Tchougounova E , Hellman L , Gertsenstein M , Hirasawa N , Sakurai E , Buzas E , Kovacs P , Csaba G , Kittel A , Okada M , Hara M , Mar L , Numayama-Tsuruta K , Ishigaki-Suzuki S , Ohuchi K , Ichikawa A , Falus A , Watanabe T , Nagy A . 2001 ; Mice lacking histidine decarboxylase exhibit abnormal mast cells . *FEBS Lett* . 502 : 53 - 56
- Parmentier R , Ohtsu H , Djebbara-Hannas Z , Valatx JL , Watanabe T , Lin JS . 2002 ; Anatomical, physiological, and pharmacological characteristics of histidine decarboxylase knock-out mice: evidence for the role of brain histamine in behavioral and sleep-wake control . *J Neurosci* . 22 : 7695 - 7711
- Quednau BD , Nicoll DA , Philipson KD . 1997 ; Tissue specificity and alternative splicing of the Na⁺/Ca²⁺ exchanger isoforms NCX1, NCX2, and NCX3 in rat . *Am J Physiol* . 272 : C1250 - C1261
- Riehl J , Honda K , Kwan M , Hong J , Mignot E , Nishino S . 2000 ; Chronic oral administration of CG-3703, a thyrotropin releasing hormone analog, increases wake and decreases cataplexy in canine narcolepsy . *Neuropsychopharmacology* . 23 : 34 - 45
- Sergeeva OA , Chepkova AN , Doreulee N , Eriksson KS , Poelchen W , Monnighoff I , Heller-Stilb B , Warskulat U , Haussinger D , Haas HL . 2003a ; Taurine-induced long-lasting enhancement of synaptic transmission in mice: role of transporters . *J Physiol* . 550 : 911 - 919
- Sergeeva OA , Amberger BT , Eriksson KS , Scherer A , Haas HL . 2003b ; Co-ordinated expression of 5-HT2C receptors with the NCX1 Na⁺/Ca²⁺ exchanger in histaminergic neurones . *J Neurochem* . 87 : 657 - 664
- Sergeeva OA , Eriksson KS , Sharonova IN , Vorobjev VS , Haas HL . 2002 ; GABA(A) receptor heterogeneity in histaminergic neurons . *Eur J Neurosci* . 16 : 1472 - 1482
- Sergeeva OA , Klyuch BP , Fleischer W , Eriksson KS , Korotkova TM , Siebler M , Haas HL . 2006 ; P2Y receptor-mediated excitation in the posterior hypothalamus . *Eur J Neurosci* . 24 : 1413 - 1426

- Sergeeva OA , Parmentier R , Vandael D , Klyuch BP , Haas HL . Excitation of histaminergic neurons by thyrotropin-releasing hormone . Soc Neurosci Abstr . 37 : (199.2) 2007 ;
- Simansky KJ , Dave KD , Inemer BR , Nicklous DM , Padron JM , Aloyo VJ , Romano AG . 2004 ; A 5-HT_{2C} agonist elicits hyperactivity and oral dyskinesia with hypophagia in rabbits . *Physiol Behav* . 82 : 97 - 107
- Smith BN , Armstrong WE . 1996 ; The ionic dependence of the histamine-induced depolarization of vasopressin neurones in the rat supraoptic nucleus . *J Physiol* . 495 : 465 - 478
- Steininger TL , Alam MN , Gong H , Szymusiak R , McGinty D . 1999 ; Sleep-waking discharge of neurons in the posterior lateral hypothalamus of the albino rat . *Brain Res* . 840 : 138 - 147
- Takahashi K , Lin JS , Sakai K . 2006 ; Neuronal activity of histaminergic tuberomammillary neurons during wake-sleep states in the mouse . *J Neurosci* . 26 : 10292 - 10298
- Valatz JL , Bugat R . 1974 ; [Genetic factors as determinants of the waking-sleep cycle in the mouse] . *Brain Res* . 69 : 315 - 330
- Vanni-Mercier G , Gigout S , Debilly G , Lin JS . 2003 ; Waking selective neurons in the posterior hypothalamus and their response to histamine H₃-receptor ligands: an electrophysiological study in freely moving cats . *Behav Brain Res* . 144 : 227 - 241
- Vogel RA , Cooper BR , Barlow TS , Prange AJ Jr , Mueller RA , Breese GR . 1979 ; Effects of thyrotropin-releasing hormone on locomotor activity, operant performance and ingestive behavior . *J Pharmacol Exp Ther* . 208 : 161 - 168

Fig. 1

TRH enhances firing rate of TMN neurons

Averaged time course diagrams of firing rates illustrating responses to bath applied TRH in mouse (A, 1.5 μ M TRH, n=6) and rat (B, 2–10 μ M TRH, n=10) TMN neurons. Non-responding cells are not included. C. Example of recordings in rat TMN neuron: note regular firing, excitation upon TRH 2 μ M application and recovery of spontaneous activity in this cell 15 min after TRH withdrawal. Averaged extracellular triphasic action potentials (80–150 APs for each of three periods) demonstrate reduction of amplitude during TRH application, indicating cellular depolarization. D. Chlordiazepoxide (CDZ, open bar indicates period of bath application), TRH receptor antagonist, reduces the TRH response in rat TMN neurons at 10 μ M (n=4) and abolishes it at 50 μ M (n=6). E. TRH applied in the presence of benzamil enhanced firing rate in a significantly smaller fraction of TMN neurons and on average (n=10) to a significantly smaller extent compared to the control group (n=14, given in grey).

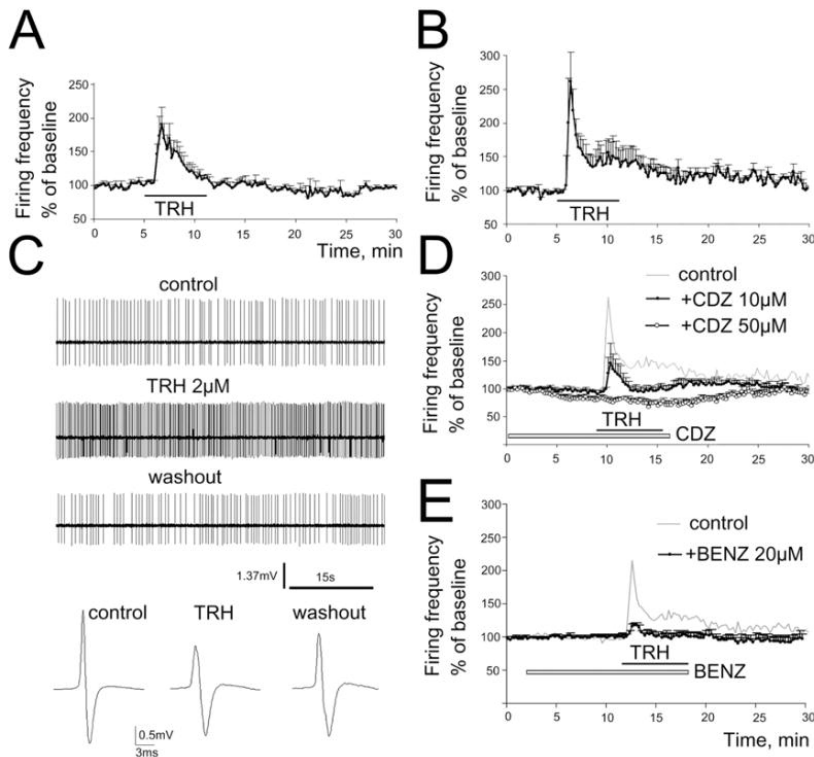


Fig. 2

Expression of TRH receptors in rat and mouse TMN neurons. A,B. Examples of single-cell RT-PCR analysis of TRH receptor expression in 5 mouse (A) and 5 rat (B) TMN neurons (photographs of corresponding cells are given over the agarose gels). Pc-positive control: posterior hypothalamus; nc-negative control; M-DNA size marker: 100 b.p. ladder (500 b.p.-most intense line); HDC-histidine decarboxylase. C. Summary of single-cell RT-PCR analysis done in 26 mouse and 19 rat TMN neurons positive for the histamine synthesizing enzyme histidine decarboxylase (HDC).

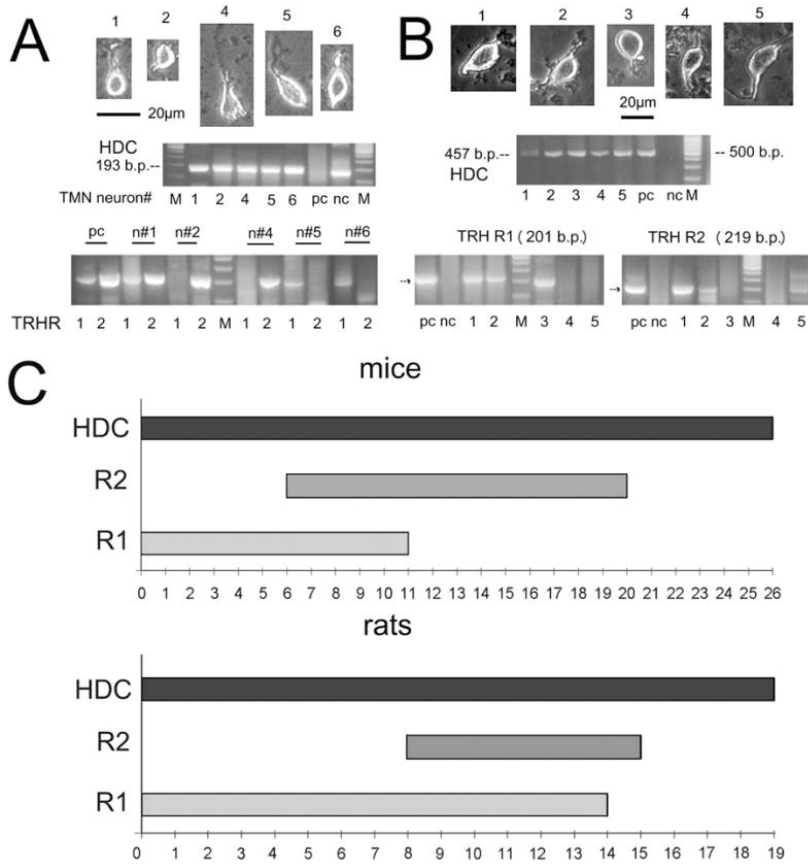


Fig. 3

Intracellular sharp-electrode recordings from rat TMN neurons. A. Example of a TMN neuron showing increased frequency in firing rate and depolarization after TRH application. Following washout and return to baseline, application of a histamine H3-receptor agonist (R- α -methylhistamine), induces markedly reduced firing rate. B. During treatment with 1 μ M TTX, TRH depolarizes the neuron, indicating a postsynaptic site of action. During the maximal depolarization the membrane potential is manually returned to the resting level. No change in input resistance is noted. C. Depolarization induced by TRH and effects on it by 500 μ M BaCl₂, 3 mM CsCl or 80 μ M KB-R7943 (Right, representative depolarization under each condition after preincubation with 1 μ M TTX, mean \pm SE for responding cells, numbers beside bars indicate number of cells. While BaCl₂ and CsCl induce no difference in the TRH-depolarization of TMN neurons, the selective blocker of the NCX, KB-R7943 strongly depresses the depolarization. **p<0.01, Student's two-tailed t test. D. The voltage-current plots recorded in the same neuron before and during TRH treatment. The curves intersect at -4.3 ± 6.3 mV, which is close to the reversal potential predicted for a mixed cationic conductance E. TMN neuron stained with both biocytin (red) and HDC (green). Scale bar, 25 μ m

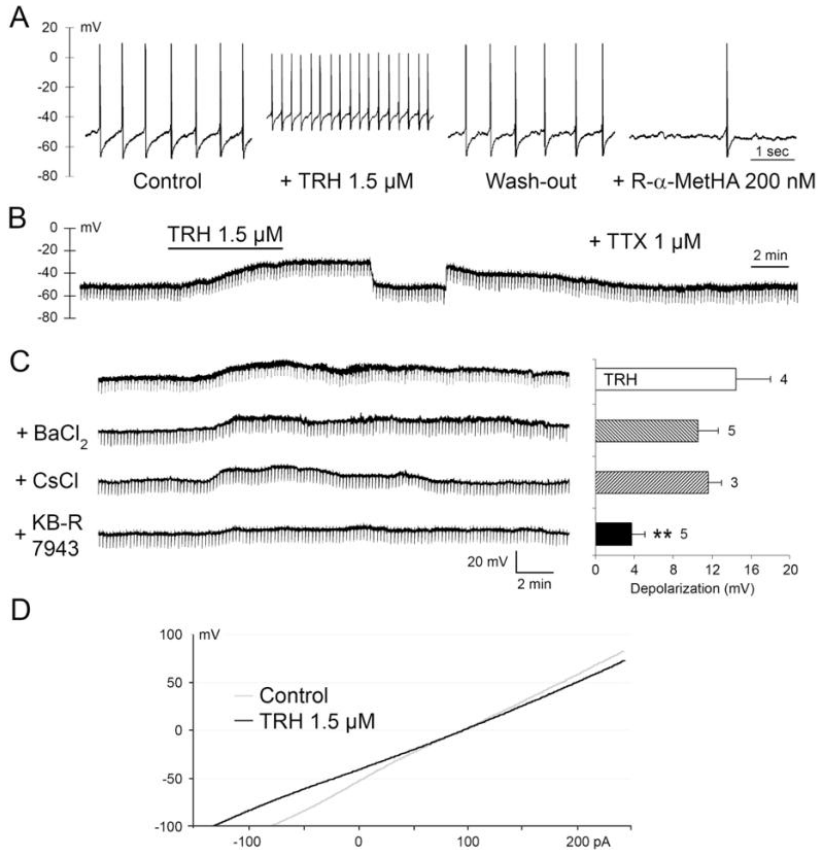


Fig. 4

Whole-cell voltage clamp sIPSCs recordings from TMN neurons in rat thin slices. A. Photograph of TMN neuron approached with a patch pipette (scale bar 20 μm) and identified with the stimulation protocol shown at the right side. Hyperpolarization-activated inward current (I_h) becomes obvious after voltage jumps from -50 to -90 mV and to more negative values. Maximal outward I_A current is seen after return to the holding membrane potential from -120 mV. B. Example of TRH ($2.5 \mu\text{M}$)-induced depression of sIPSCs frequency (experiment done at room temperature). The GABA_A receptor antagonist gabazine ($10 \mu\text{M}$) blocks spontaneous synaptic activity. C. AMPA and NMDA receptor antagonists (DNQX and D-AP5, respectively) reduce frequency of sIPSCs without affecting their kinetics or amplitude. D. Summary of experimental values for the frequency, amplitude and decay time of sIPSCs during control period and under DNQX/D-AP5 obtained in 13 TMN neurons.

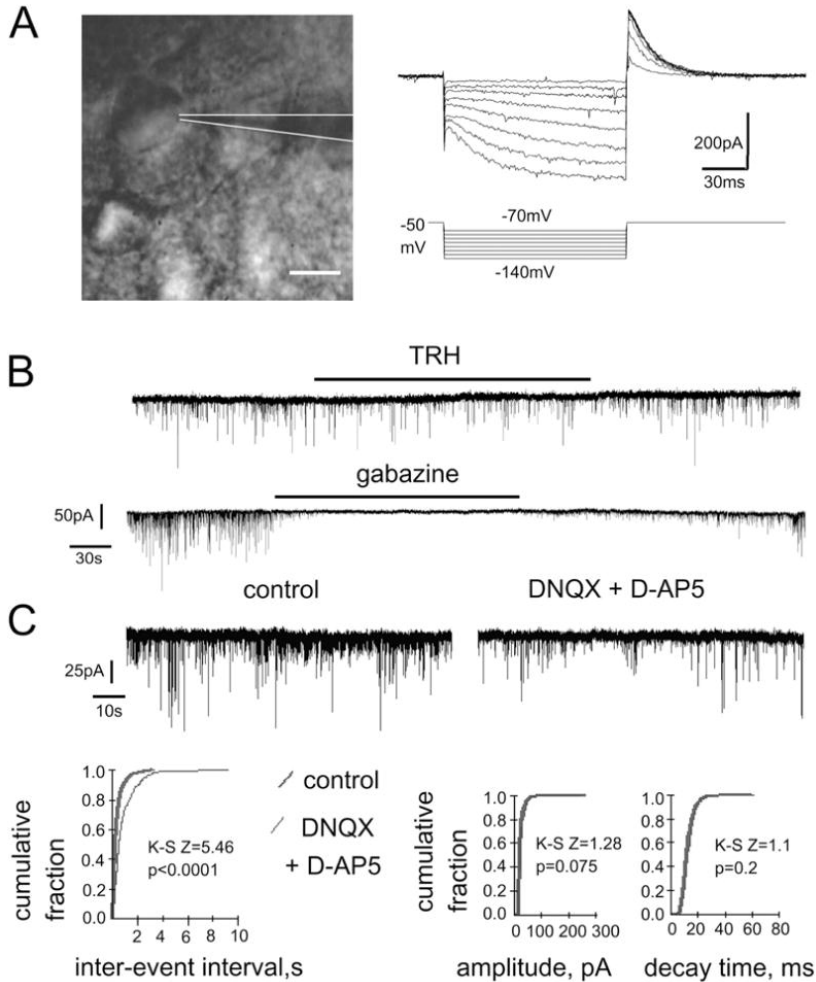


Fig. 5

TRH induces inward currents and modulates frequency of sIPSCs in TMN neurons recorded in whole-cell voltage-clamp at 32°C. A. Example of TRH-mediated inward current in TMN neuron where frequency of sIPSCs was up-regulated. Summary diagram at the right side shows three neuronal groups: with no change, decrease and increase in sIPSC frequency under TRH (control 100%). Dark grey bars show percent changes in neurons, where no direct postsynaptic currents in response to TRH were recorded. Light grey bars are superimposed to the amplitudes of TRH-evoked currents measured in the same cells. B. Example of neuron with an increased sIPSC frequency in response to TRH and summary of frequency changes in all cells belonging to the same group (at the right side). C. The same for a cell with decreased sIPSC frequency upon TRH application. As no difference in sIPSC occurrence (suppression or enhancement) by TRH was obtained with Fisher's exact probability test ($p=0.36$) between neurons recorded at room- or more physiological temperature, all recordings were pooled. Cells with control sIPSC frequency below 0.1 Hz were not included in summary diagrams.

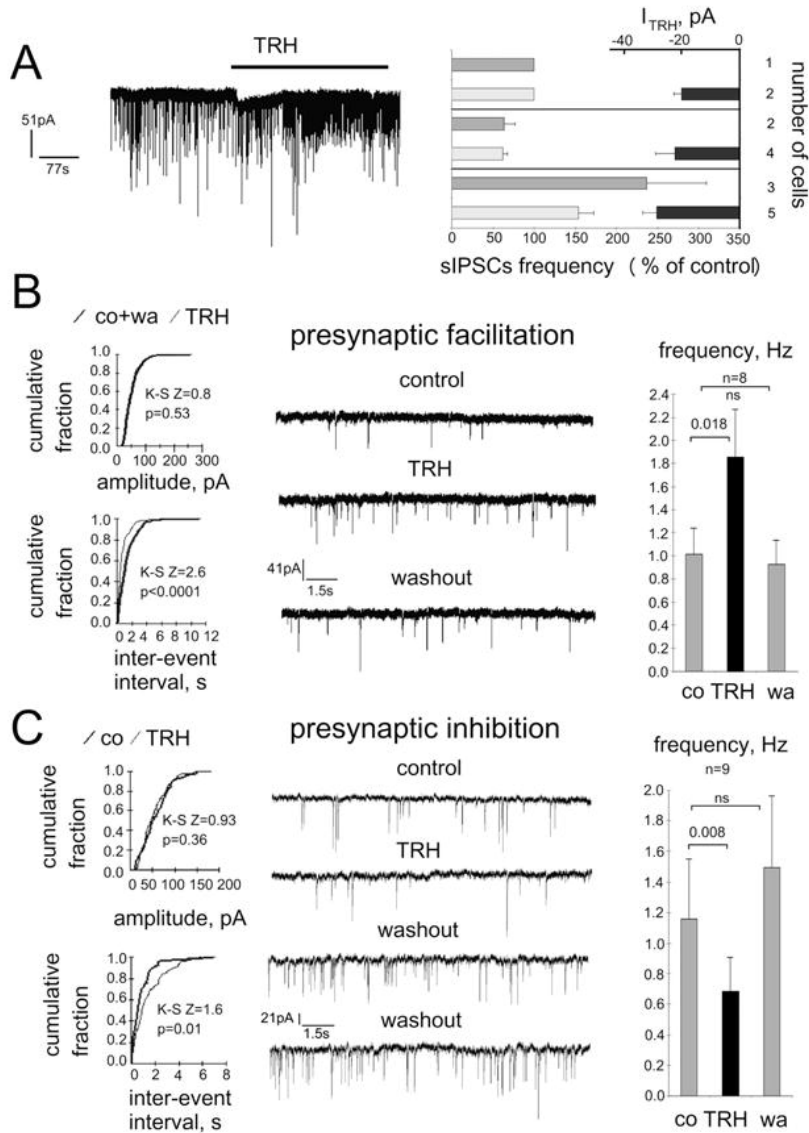


Fig. 6

The TRH analogue Montirelin lacks waking effect in histamine deficient mice ($HDC^{-/-}$). Bottom, typical example illustrating the waking effect induced by montirelin (1 mg/kg ip) in wild type mice ($HDC^{+/+}$) and its absence in $HDC^{-/-}$ mice. Top, Mean (\pm SEM) hourly cumulative values of waking during one hour before and 4 hours after the injection of placebo (NaCl no symbols), montirelin at 1 mg/kg (open symbols) or 3 mg/kg (filled symbols) in $HDC^{+/+}$ and $HDC^{-/-}$ mice. Middle, mean latencies to slow wave sleep (SWS) and paradoxical sleep (PS) after compound injection ($n = 8$ and 6 in 4 and 3 animals for $HDC^{+/+}$ and $HDC^{-/-}$ mice respectively). Note the clear and significant effect induced in $HDC^{+/+}$ mice after montirelin injections and its absence in $HDC^{-/-}$ mice (a, b, Montirelin vs NaCl in the same group, $p < 0.01$, 0.05 , Dunnett's t test, c, d, $p < 0.05$ $HDC^{+/+}$ vs $HDC^{-/-}$, Montirelin 1 mg/kg + α -FMH ((S)- α -fluoromethylhistidine) 60 mg/kg vs Montirelin 1 mg/kg, two tailed t test after ANOVA for repeated measures).

