Electrophysiological and molecular analysis of aminergic neurons controlling arousal

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Summary

One of the most important roles of histaminergic and dopaminergic systems is regulation of sleep-waking cycle (Brown *et al.,* 2001). In my thesis I and colleagues showed the effect of several well-known wake (or sleep) - promoting neurotransmitters and substance on the activity of HAergic and DAergic neurons.

Adenosine does not change the firing rate or membrane potential of TMN neurons in hypothalamic brain slices (Sergeeva *et al.,* 2006). These results support the idea about indirect modulation of HAergic systems with adenosine. In contrast, ATP, ADP and UTP excite TMN neurons in hypothalamic slices (Sergeeva *et al.,* 2006). With a help of expression analysis we found that $P2Y_1$ and $P2Y_4$ receptors are the major target for purines in TMN neurons. The other point of this work is found interactions between TMN neurons and glial cells (Sergeeva *et al.,* 2006). Our immunostaining revealed that metabotropic $P2Y_1$ receptors are widely expressed both in neurons and nuclear of the glial cells. Moreover, the effect of ATP and ADP was reduced but was not eliminated completely by prior application of $P2Y_1$ antagonist. According to this data we proposed that glial $P2Y_1$ receptors are activated after uptake of nucleotides with followed increase of glutamate release (Sergeeva *et al.,* 2006).

In other work we showed that TRH increased the firing rate and induced membrane depolarization directly through TRH1 and TRH2 receptors (Parmentier *et al.,* in preparation). On other hand, TRH mediates frequency but not amplitude of spontaneous inhibitory postsynaptic currents indicating indirect effect (Parmentier *et al.,* in preparation).

Modafinil is a novel wake-promoting drug but the mechanism of it action remains unclear. In our work we showed that modafinil does not change the activity of HAergic neurons and inhibit DAergic neurons from substantia nigra and ventral tegmental area. This action does not involve the adrenergic system, but is related to D2-like receptor activation. Considering our data, we suggest that D2-like receptors are the major if not unique target of modafinil in the DAergic neurons.

All our findings are relevant for better understanding of the role of adenosine, ATP, TRH and modafinil as well HAergic and DAergic systems in the sleep-waking regulation.

Table of contents

1. Introduction

1.1 Sleep and waking

1.1.1 Role of sleep and waking

About one third of our life we spend sleeping. The sleep-waking cycle is one of the most important biological rhythms controlled by the brain. While the role of waking is more or less obvious, the role of the sleep is under discussion. Only one thing we can say for sure, we do need sleep. Sleep deprivation investigations showed that men totally deprived of sleep for several days show irritability, blurred vision, slurred speech and memory lapses. In experiments with rats sleep deprivation caused death. Autopsies failed to reveal the reason of the death (Boonstra *et al.,* 2007). Such experiments did not tell us much why we need sleep, only that we have a very powerful mechanism to drive us to sleep.

1.1.2 Mechanism of sleep-waking regulation

In the 20th years of the past century von Economo predicted that the region of the hypothalamus near the optic chiasm contains sleep-promoting neurons, whereas the posterior hypothalamus contains neurons that promote wakefulness. Investigations on animals proved Economo's theory, however the definite pathways remained unknown. A dominant role of the hypothalamus in sleep-waking regulation is now generally accepted after the discoveries of two systems located in the hypothalamus that are critically involved in sleep-waking regulation (Saper et al., 2001). GABAergic neurons from ventrolateral preoptic area (VLPO) promote sleep (Sherin *et al.,* 1996, 1998, Szymusiak *et al.,* 1998), whereas histaminergic neurons from the tuberomamillary nucleus (Haas and Panula 2003) and orexinergic neurons from the perifornical area promote wakefulness (Sakurai *et al.,* 1998, de Lecea *et al.,* 1998).

1.1.3 Arousal control system

In vivo extracellular electrophysiological recordings revealed that firing of neurons in all monoaminergic systems correlates with behavioral state. The first group includes: noradrenergic (NAergic) neurons from the locus ceruleus (LC), serotonergic (5-HT) neurons from the dorsal raphe (DR) and histaminergic (HAergic) neurons from the tuberomamillary nucleus (TMN). The activity of these nuclei is highest during wakefulness and slowing or stopping completely during sleep (Lin *et al.,* 1988, Takahashi *et al.,* 2006, Saper *et al.,* 2001). The second group includes the cholinergic neurons in the pedunculopontine and laterodorsal

tegmental nuclei (PPT-LDT). These neurons fire rapidly during wakefulness and rapid eye movement (REM) sleep and are silent during SWS (Saper *et al.,* 2001). Retrograde and anterograde tracings revealed that monoaminergic systems are interconnected. For example, TMN neurons project to the LC and the DR nucleus as well as to dopaminergic (DAergic) neurons in the substantia nigra (SN) and ventral tegmental area (VTA) (Haas and Panula, 2003). Pharmacological investigations also support the idea of mutual interactions between monoaminergic systems. For example, 5-HT neurons are excited by histamine and HAergic neurons are excited by serotonin (Eriksson *et al.,* 2001, Brown *et al.,* 2002). NA does not activate TMN neurons directly, but inhibits GABAergic inhibitory postsynaptic potential (IPSPs) (Stevens *et al.,* 2004).

For a long time, the involvement of the dopaminergic system in sleep-waking cycle regulation was neglected. Previous investigations showed that the firing rate of VTA/SN DAergic neurons does not correlate with behavioral state in rats (Miller *et al.,* 1983). Furthermore, neurotoxic lesions of the VTA did not decrease wakefulness (Lai *et al.,* 1999). However, sleep disturbances in Parkinson's disease and schizophrenia and their alleviation with dopaminergic medication suggest the involvement of the DAergic system in sleep-waking regulation (Dzirasa *et al*., 2006). Besides, it was found that the dopamine level increases during waking and REM sleep (Lena *et al.,* 2005). All these data indicate the importance of the DAergic system in sleep-waking regulation.

Recently, it has been shown that DAergic neurons of the ventral periaqueductal gray matter (vPAG) are wake-active. This was shown by measuring the level of c-Fos protein expression as an indicator of neuronal activity. The authors revealed that about half of these cells expressed c-Fos during wakefulness but not during sleep (Lu *et al.,* 2006). Originally, these neurons have been considered an extension of the VTA, as they share many efferent projections with the VTA, such as to the striatum and the cerebral cortex. However, small differences in the projection pattern exist between these structures. For example, the ventrolateral preoptic area is one of the main targets of vPAG neurons, while the SN and the VTA neurons do not project to the VLPO (Lu *et al.,* 2006).

Retrograde and anterograde tracings revealed that vPAG DAergic neurons form contacts with orexinergic (OXergic) and monoaminergic neurons except for the HAergic ones. Proceeding from these data, vPAG neurons have been introduced as a new member of the sleep–waking control system (Saper *et al.*, 2005a). At the moment, there are no data about recording of DAergic neurons from vPAG. Possible pharmacological and

electrophysiological properties of these neurons are based on similarity with DAergic neurons from the VTA (Lu *et al.,* 2006).

Although the VTA/SN DAergic neurons are not included in the sleep-waking control system, their contribution to the sleep-waking regulation cannot be ignored. Activation of these neurons initiates locomotor behavior, and this activation is associated with a high level of arousal and attention (Lu *et al.,* 2006). The release of acetylcholine, known to activate DA neurons, is maximal during waking and REM sleep (Lena *et al.,* 2005). Burst firing of DAergic neurons typical of positive reward could also occur during dreams.

Monoaminergic neuron activity is strongly regulated by the orexinergic system. Retrograde labeling and immunohistochemistry investigations showed that the orexinergic neurons project to the LC, the DR, the VTA/SN, the pVAG and the TMN (Saper *et al.,* 2001, Lu *et al.,* 2006). Sakurai and co-workers identified two types of orexin receptors - orexin-1 (Ox1R) and orexin-2 (Ox2R) receptors (Sakurai *et al.,* 1998). They belong to the G protein-coupled receptor group and exhibit differences in affinity for agonist, distribution and signal transduction pathways. Ox1R is coupled exclusively to the Gq subclass, whereas Ox2R may also couple to Gi/o (Sakurai *et al.,* 1998). Ox1R are expressed in the LC and the DR neurons, both Ox1R and Ox2R are presented in the TMN and SN/VTA neurons. In all cases orexin increases the firing rate of the target monoaminergic neurons (Hagan *et al.,* 1999, Eriksson *et al.,* 2001, Brown *et al.,* 2002, Korotkova *et al.,* 2003). All these observations gave the basis to suggest that the oxrexinergic system together with monoaminergic systems constitute arousal control system (Saper *et al.,* 2005a, b).

1.1.4 Sleep control system

c-Fos protein expression and electrophysiological recordings of GABAergic neurons from VLPO showed that their activity is low during wakefulness, increases during NREM and further increases during the REM sleep (Sherin *et al.,* 1996, Szymusiak *et al.,* 1998). Anterograde tracing showed that neurons from the VLPO cluster (VLPOc) intensely project to the TMN, while neurons which are located in the extended VLPO (VLPOex) target the DR, the LC and the VTA (Sherin *et al.,* 1998). The relationship between the VLPO and the major monoamine groups appears to be reciprocal. The VLPO neurons inhibit histaminergic, noradrenergic and serotonergic by GABA and are inhibited by noradrenaline and serotonin

(Saper *et al.,* 2005a). Although, VLPO neurons do not respond to histamine, TMN neurons contain GABA and galanin, which might inhibit VLPO neurons (Brown *et al.,* 2001).
Recently, one more possible system important for sleep-waking regulation has been reported. It is located together with orexinergic neurons in the lateral hypothalamus and contains melanin concentrating hormone (MCH). Neurons from this system have projections similar to orexinergic neurons and are most active during REM sleep. Many of these cells contain GABA and probably reduce the activity of monoaminergic neurons (Boissard *et al.,* 2003).

1.1.5 Flip-Flop model

VLPO neurons are active during sleep and suppress the activity monoaminergic neurons. Monoamine neurons on the contrary fire rapidly during wakefulness and inhibit the activity of the VLPO neurons. This mutual inhibition between the VLPO and monoaminergic systems produces a circuit system reminiscent of a "flip-flop" switch in electronic circuits. The two halves of a flip–flop circuit strongly inhibit each other and create a feedback loop (Saper *et al.,* 2001). Orexin neurons do not have a direct projection to the VLPO region, but they control monoaminergic systems and might work like a finger that holds the switch into the aroused state during wakefulness. This system is bistable, with two possible patterns avoiding intermediate states. Flip-flop is resistant to switching during the day when input signals to the VLPO and the monoaminergic cells fluctuate transiently. However, large scale influences, such as circadian rhythms might gradually shift the relative balance of mutual inhibitions. Such model fits well with behavioral studies: animals are in clear waking or sleeping state, with a relatively short time spent in transitions. Such fast switching seems to have some evolutionary advantages: the animal would be an easy target for predators if it would not be fully alert. Quick awaking is required for defense against enemies (Saper *et al.,* 2005a).
Lesion studies showed that breaking off any side of the flip–flop circuit might produce abrupt and unstable fluctuations in behavioral state and cause disorders such as narcolepsy. Individuals with narcolepsy experience frequent and unwanted transitions into sleep, particularly into REM sleep (SOREM= sleep onset REM) during wakefulness, and they tend to awake more frequently from sleep as well (Saper *et al.,* 2005b).

1.2 Detailed description of histaminergic and dopaminergic systems

1.2.1 Histaminergic system (HAergic)

1.2.1.1 Location and morphology of HAergic neurons

Histamine is one of the biogenic amines in the brain. Besides sleep-waking regulation it plays a key role in many behavioral processes such as food intake, endocrine homeostasis, learning and others (Brown *et al.,* 2001, Haas and Panula, 2003). First ideas about the neuronal histaminergic system appeared 40 years ago. However, investigation of the histaminergic system was neglected because the histamine producing neurons were not identified (Haas and Wolf, 1977, Haas and Panula, 2003).

The exact location of HAergic neurons was defined after development of antibodies against histamine producing enzyme L-histidine decarboxylase (HDC) and histamine itself (Watanabe *et al.,* 1984, Panula *et al.,* 1984). These studies showed that histaminergic neurons are located in a small region of the posterior hypothalamus in the tuberomamillary nucleus (TMN) (Fig. 1A). Ericson *et al.,* (1987) reported about 2200 HDC immunopositive cells in rat's TMN. Of these, around 1500 cells are located in the ventral part of TMN (TMV), 600 cells are in the medial part (TMM) and 100 cells are diffusely distributed between these areas (TMdiff) (Fig. 1B). Several authors use the name "lateral part of the TMN" instead of "ventral part of the TMN" (Wada *et al.,* 1991). In the text below, both names are used. After discovery each subgroup of TMN neurons was further divided into different parts. The ventral part of the TMN was subdivided into the rostral (TMVr) and the caudal (TMVc) part. The medial TMN subgroup was subdivided into the dorsal (TMMd) and the ventral (TMMv) part (Ericson *et al.,* 1987). At present time, there is no evidence to suggest that different parts within each subgroup play distinct roles (Wada *et al.,* 1991, Brown *et al.,* 2001).

The majority of medial and lateral neurons is densely packed in the nucleus. In semithin sections, cut through the center of the TMN, a number of HDC positive cells or dendrites are found in close apposition to other HDC positive cell bodies or dendrites. The distance between the individual TMN cells appears to increase with the distance from the dense core of the nucleus. In the outer parts of this zone, scattered TMN neurons are intermingled with other nonhistaminergic neurons (Watanabe *et al.,* 1984, Ericson *et al.,* 1987).

A detailed examination of the individual TMN neurons reveals large (diameter > 25 μM) medium (18-25 μM) and small (diameter < 18 μM) neurons that are intermingled within the TMN nucleus. The cells of medium and large size are the most frequent. The large neurons

have round, triangular or polygonal shape. The cells of medium size have oval or round form, and the small neurons have ovoid or fusiform shape. Although the neurons of all sizes and forms are present in both medial and lateral parts of the TMN, there is a significant difference between subgroups with regard to soma size. The medial part of TMN contains a higher percentage of small and medium-size cells compared to the ventral part (Ericson *et al.,* 1987).

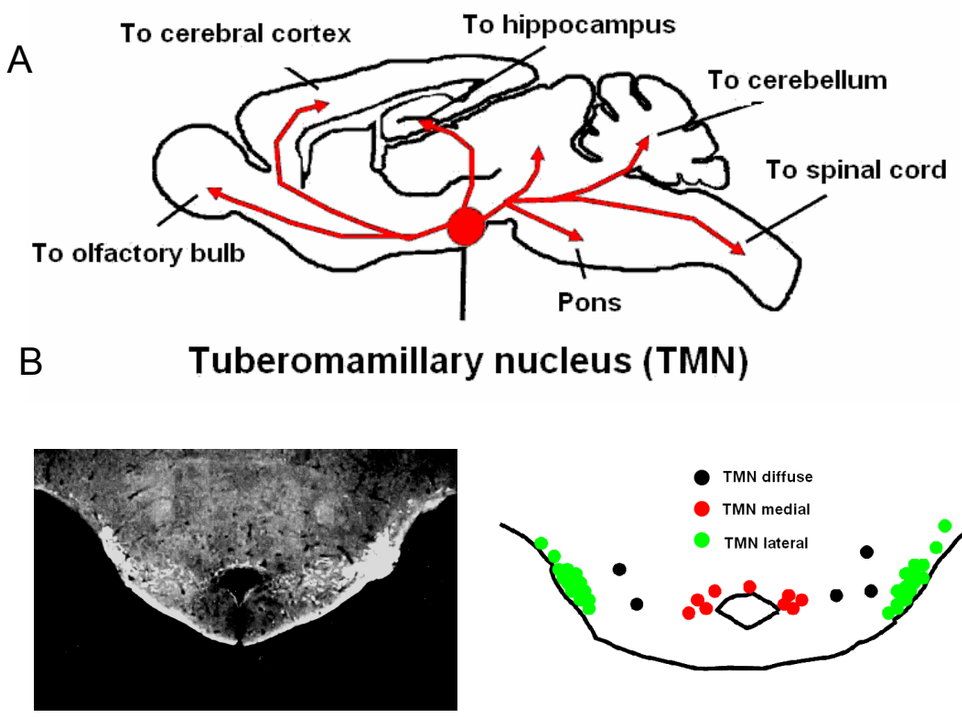

Fig. 1. Location and projections of histaminergic neurons.

A. HAergic neurons are located in the tuberomamillary nucleus of the posterior hypothalamus and project to almost all parts of the brain and the spinal cord.

B. Coronal section of the brain, containing HAergic neurons. Immunohistochemical staining for histamine (left) and scheme (right). The rat brain contains about 2200 histaminergic neurons. Of these, around 1500 cells are located in the lateral part of the TM, 600 cells are in the medial part of the TMN and 100 cells are diffusely distributed between these two areas.

Three or four major dendrites come from the soma. The dendrites of TMN neurons are simple with few branches. The length of dendrites varies from about several tens micrometer up to 300 micrometers. Many dendrites form horizontally oriented bundles, which extend in mediolateral as well as rostrocaudal directions (Watanabe *et al.,* 1984). TMN neurons are located so close to third ventricle (3V) and ventral surface of the brain that direct contact with cerebrospinal fluid (CSF) is possible. Substances presented in CSF may influence the activity of TMN neurons (Ericson *et al.,* 1991, Brown *et al.,* 2001).

There are two morphological features of TMN axons. The first is that the axon leaves typically not from the soma but from one of dendrites (Ericson *et al.,* 1987). The second is that there is usually no synaptic specialization as seen with all aminergic systems in the brain (Diewald *et al.,* 1997).

1.2.1.2 Projections of HAergic neurons

Retrograde and anterograde tracings and immunostaining showed that like other monoaminergic system, histaminergic neurons form long and highly divergent diffuse projections to many brain areas (Fig. 1A) (Panula *et al.,* 1989, Ericson *et al.,* 1991). HDC positive, mostly unmyelinated, varicose or nonvaricose fibers are detected in almost all cerebral regions, particularly limbic structures. Individual neurons project to widely divergent areas (Watanabe *et al.,* 1984).

Fibers arising from the tuberomamillary nucleus constitute two ascending pathways (ventral and dorsal) and one descending pathway (Panula *et al.,* 1989). The ventral ascending pathway remains on the ventral surface of the brain, providing innervation to the hypothalamus, diagonal band, septum and olfactory bulb. The dorsal pathway leaves the TMN dorsally, following the lateral side of the third ventricle to innervate the thalamus, hippocampus, amygdala and rostral forebrain structures. The descending pathway in rats is associated with the medial longitudinal fasciculus and provides input to the brain stem and spinal cord (Panula *et al.,* 1989, Ericson *et al.,* 1991). At the moment, there is no reason to suggest that different subgroups of histaminergic neurons display specific projections (Wada *et al.,* 1991).

Sleep-active GABAergic neurons from the ventrolateral preoptic area provide a major input to the tuberomamillary nucleus (Sherin *et al.,* 1996). Further investigations have shown that TMN neurons receive also GABAergic inputs from the diagonal brand of Broca and the

lateral hypothalamus (Brown *et al.,* 2001). Histaminergic neurons have a very dense orexin innervation originating from the lateral hypothalamus. Finally, the histaminergic system makes contacts with other monoaminergic systems (Haas and Panula, 2003).

1.2.1.3 Electrophysiological properties of HAergic neurons

The first electrophysiological investigation of HAergic neurons were done by Reiner and McGeer in 1987 (extracellular recordings) and Haas and Reiner in 1988 (intracellular recordings). It was proven with immohistochemical analysis that all recorded neurons were indeed histaminergic (Haas and Reiner, 1988). In these and following works, it was shown that TMN neurons have very stable pacemaker-like firing with frequency rate from 0 to 5 Hz in the absence of extrinsic activity (Stevens *et al.,* 2001). Resting membrane potential is low, about -50 mV. Action potentials have an amplitude of 75-80 mV with a mid-amplitude duration 1.8 ms (Haas and Reiner, 1988). During spontaneous activity, the action potential arises from a slower depolarizing potential, then rises in a more rapid depolarization, which generates an overshoot. TMN neurons are also characterized with a shoulder on the falling phase of the action potential. A perfusion with Ca^{2+}-free solution with Cd^{2+} or Co^{2+} showed that this shoulder exists due to activation of a voltage-dependent Ca^{2+} conductance (Stevens *et al.,* 2001). An afterhyperpolarization (AHP) following the action potential decays slowly with a time constant of 100–200 ms. The AHP decay is followed by a slow depolarization leading to the next action potential. No time is spent at a stable resting potential in firing cells (Haas and Reiner, 1988, Stevens *et al.,* 2001).

TMN neurons also exhibit a transient outward current (Ia current) (Haas and Reiner, 1988, Greene *et al.,* 1990, Jackson and Bean, 2007). The decay of the transient outward current was best fitted by double-exponential curve with separation onto the fast and the slow decaying components. The fast decaying current was completely blocked by 1 mM 4-aminopyridine (4-AP). The slow decaying current was insensitive to 4-AP. These data show that the transient outward current is composed of at least two currents with different pharmacological properties (Greene *et al.,* 1990). Ia current delays the return to the resting potential after hyperpolarization. Prolongation of interspike interval may serve to prevent excitation of the TMN neurons and awaking during rapid eye movement sleep (Haas *et al.,* 1991).

The other remarkable feature of TMN neurons is the inwardly rectifying current activated by hyperpolarization (Ih) which is carried by Na^{+} and K^{+} ions. This current plays an important

role in pacemaker activity in heart and many neurons (Pape, 1996, Siegelbaum and Robinson, 2003). However, the voltage dependence of Ih in TMN neurons demonstrates that the current is inactive at the membrane potential positive to -80 mV (Kamondi and Reiner, 1991). This value is more negative than the value of the threshold for action potential generation (-52 mV). Thus, the Ih current seems to not play an important role in the firing rate of TMN neurons. One of the possible roles of the Ih current is to shunt and inactivate inputs to TMN neurons during REM (Kamondi and Reiner, 1991, Haas *et al.,* 1991).

1.2.1.4 Histamine receptors

Four types of histamine receptors were identified (H_1 - H_4). The first three of them are present in the brain (H_1-H_3) and many types of neurons express at least one of them. The fourth type is detected in cells of the immune system and mast cells (Haas and Panula, 2003). Studies of neurochemistry and distribution of the H_1 receptor were made feasible with the design of reversible and irreversible radiolabeled probes such as [^{3}H] mepyramine, [^{125}I] iodobolpyramine, and [^{125}I] iodoazidophenpyramine. A high density of the first type of histaminergic receptor was found in areas involved in arousal: (thalamus, LC and DR). The H_1 receptor was initially defined in the design of so-called *antihistamines*, most of which display prominent sedative properties. H_1R mediate an excitatory action on whole brain activity and are coupled to $G_{q/11}$, which, when activated, stimulates phospholipase C (PLC). Activation of PLC leads to the formation of the two second messengers: diacylglycerol (DAG) and inositol-1,4,5-triphosphate (Ins(1,4,5)P3) (Hill *et al.,* 1997, Brown *et al.,* 2001).
Identification of the second type of histamine receptors (H2R) was based on the different pharmacological profiles responsible for stimulating gastric acid secretion (Black *et al.,* 1972). The molecular properties of the H_2 receptor remained largely unknown for a long time. However, reversible labeling of the H_2 receptor was achieved by using [^{125}I] iodoaminopotentidine. The H_2R is coupled to adenylyl cyclase via G_s protein, so its activation leads to enhanced production of the second messenger - cyclic AMP (cAMP). A high density of H_2 receptors is found in hippocampal formation and amygdala. H_1 and H_2 receptors are colocalised in several areas of the brain including pyramidal and granule cells in the hippocampal formation and monoaminergic cell groups (LC, DR, SN/VTA) (Brown *et al.,* 2001, 2002). Like the H_1 receptor, the H_2R usually mediates an excitatory action. However, in several types of neurons, for example, in serotonergic neurons of DR, H_2

receptor activation leads to inhibition of firing (Lakoski *et al.,* 1984). At the moment, little is known about the nature of this inhibition (Brown *et al.,* 2001).

The third type of histamine receptors was initially detected as an autoreceptor controlling histamine synthesis and release in the brain (Arrang *et al.,* 1983). Reversible labeling of this receptor was achieved using the highly selective agonist [^{3}H] (R) α-methylhistamine. H_3 receptors are coupled to $G_{i/o}$ and high voltage-activated Ca^{2+} channels. (Hill *et al.,* 1997).

The H_3 receptor gene shows very low homology with other biogenic amine receptors. The H_3 receptor gene contains two introns in its coding sequence. Two splice variants (H_{3L} and H_{3S}) were identified. They differ by a stretch of 30 amino acids in the third intracellular loop. The existence of these variants may partly account for the apparent H_3-receptor heterogeneity in binding or functional studies (Morisset *et al.,* 2000).

Autoradiography in the rat brain showed high densities of H_3R in the nucleus accumbens, striatum, olfactory tubercles and the substantia nigra, whereas only moderate levels were found in the hypothalamus, including TMN nucleus, where H_3 receptors were found on the cell bodies of the histamine neurons. H_3-receptor activation in TMN neurons causes inhibition of their activity and of histamine release. They also inhibit the release of other neurotransmitters including serotonin, dopamine, acetylcholine, noradrenaline, galanin glutamate and GABA in the CNS and periphery (Hill *et al.,* 1997, Brown *et al.,* 2001). Inverse agonists of H_3 receptors thioperamide and ciproxifan have the opposite effect – increasing histamine synthesis and release (Morisset *et al.,* 2000, Haas and Panula, 2003).

1.2.2 Dopaminergic system (DAergic)

The dopaminergic neurons are largely concentrated in the substantia nigra and ventral tegmental area. Smaller groups were also found in the periaqueductal gray matter and hypothalamus. Electrophysiological properties of DAergic neurons of VTA and SN have been described extensively (Grace and Onn, 1989, Lacey *et al.,* 1989). As it was mentioned before, at the moment there are no data on electrophysiological properties of DA neurons from pVAG. Different subpopulations of DA neurons diverge in their pharmacological and electrophysiological properties and projections (Korotkova *et al.,* 2004, Lu *et al.,* 2006). Within this and the next chapters I will focus on the properties of DA neurons from the VTA.

DAergic neurons project to the nucleus accumbens, amygdala, striatum, hippocampus and cortex and play an important role in many behavioral processes. They are crucial in brain

mechanisms of reward and emotional arousal. The firing rate of these neurons correlates with the availability of primary rewards. The activity of neurons is increased during aroused and rewarding waking situations, for example, when the animals find a hidden food. Drugs that stimulate release of DA such as amphetamine and cocaine also lead to an aroused and positively rewarding state (Wisor *et al.,* 2001, Szabadi, 2006).

1.2.2.1 Electrophysiological properties of DAergic neurons

The electrophysiological properties of DAergic neurons are very similar to the properties of HAergic neurons and differ from GABAergic neurons located in the VTA. DAergic cells recorded *in vitro* have membrane potential about -57mV with spike threshold -53 mV. The firing rate is pacemaker-like with an average frequency of about 2.5 Hz. The action potential is long with spike width, measured at half-peak amplitude, 0.9 ms. and an average amplitude of 77mV (Grace and Onn, 1989, Korotkova *et al.,* 2004). DAergic cells show pronounced Ih and Ia currents (Koyama and Appel, 2006). *In vivo* DAergic neurons show other electrophysiological characteristics: they fire irregularly in the single spiking mode or in a burst pattern (group of 3 to 8 action potentials) (Grace and Onn, 1989). As was mentioned above, the activity of DAergic neurons from VTA does not correlate with the sleep-waking cycle (Miller *et al.,* 1983).

1.2.2.2 Structure and classification of dopamine receptors (D)

The physiological role of dopamine is mediated by five distinct subtypes of G protein-coupled receptors. According to their pharmacological, biochemical and physiological properties, they are divided into two subfamilies. The D1-like receptor subfamily couples to G_s and activates adenylyl cyclase. The second receptor subfamily, D2-like, is coupled to G_i and inhibits adenylyl cyclase. The D_1-like subfamily consists of two dopamine receptors D_1 and D_5. The D_2-like subfamily consists of the D_2, D_3, and D_4 dopamine receptors (Dziedzicka-Wasylewska, 2004). Members of one subfamily share comparatively high homology. The D_1 and D_5 receptors share 80% identity in their transmembrane domains. The D_2 and D_3 receptors have about 75% identity in their transmembrane domains, and the D_2 and D_4 receptors share about 53% identity in the transmembrane domains (Missale *et al.,* 1998).

The genes for the D_1 and D_5 receptors are intronless. The gene for the D_2 receptor contains six introns, the D_3 gene has five introns, and the D_4 gene has four introns. There are two alternatively spliced isoforms of the D_2 receptor in the brain: a short form (D_{2S}) and a long form (D_{2L}). The D_{2L} contains additional 29 amino acids (Gyros *et al.,* 1989). Although the D_{2S} and D_{2L} variants are usually grouped together simply as D_2 receptors, they differ in terms of their transduction mechanisms, affinities for several drugs and distribution in the CNS (Gyros *et al.,* 1989, Martres *et al.,* 1992). For D_3 receptor splice variants encoding nonfunctional proteins have been identified (Schwartz *et al.,* 1993). The D_4 receptor does not have alternatively spliced isoforms in the rat's brain (Missale *et al.,* 1998). However, the D_4 receptor is polymorphic in humans. The human gene of this receptor contains a sequence with variable numbers of tandem repeats (van Tol *et al.,* 1992).

The pharmacological difference between subfamilies is considerable, but it is extremely low within one subfamily. Many agonists and antagonists allow to differentiate subfamilies, but do not allow to differentiate receptors within a subfamily. The difference within a family is represented by a variable shift in the affinity of certain agonists and antagonists (Dziedzicka-Wasylewska, 2004).

1.2.2.3 Pharmacology of D1-like receptors

The affinity of agonists for D_1 and D_5 receptors is almost identical. The most accurate difference is given by dopamine itself, which has 10-fold higher affinity for the D_5 than for the D_1 receptor. The most efficient antagonist to distinguish D_1 and D_5 receptors is butaclamol. In the presence of butaclamol, compounds generally show a slightly higher affinity for the D_1R than for the D_5R (Missale *et al.,* 1998).

The commonly used agonist to distinguish D_1-like and D_2-like receptors is SKF-38393. This substance shows a three orders higher affinity for the D_1-like receptor than for the D_2-like. The commonly used antagonist to distinguish D_1-like and D_2-like receptors is SCH-23390. This substance shows a 4 orders higher affinity for the D_1-like receptors than for the D_2-like (Missale *et al.,* 1998). In most of the cases D_1-like receptors are coupled to a G_s protein which activates adenylyl cyclase.

1.2.2.4 Pharmacology of the D2-like receptors

As in the case of D_1-like receptors, D_2-like receptors have an almost identical pharmacological profile within the subfamily. Dopamine itself presents the most accurate difference between receptors in the D_2-like subfamily. Dopamine has a 20-fold higher affinity for the D_3 than for the D_2 receptor. The selective D_2-like receptor agonist quinpirole possesses an even higher difference in affinity. It is about 100 times more potent at D_3 than at D_2 receptor (Sokoloff *et al.,* 1990). Quinpirole plays an important role in pharmacological investigations because it is the best discriminating compound between D_2-like and D_1-like receptors subfamilies. It has a moderate affinity to D_2-like receptors and does not activate D_1-like receptors. The commonly used D_2-like receptor antagonist is sulpiride. This substance shows moderate affinity for the D_2-like receptors and does not block D_1-like receptors (Missale *et al.,* 1998). In contrast to D_1-like receptors, D_2-like receptors usually are coupled to G_i which inhibits adenylyl cyclase.

1.3 Regulation of neuronal activity by neurotransmitters

1.3.1 Purines

1.3.1.1 Role of ATP and adenosine in sleep-waking regulation

Adenosine 5'-triphosphate (ATP) is a multifunctional molecule which is necessary for all living systems. The most important role of ATP is energy storage and transfer. ATP is also incorporated in the processes of DNA replication and transcription as energy supplier and "building blocks". In addition, nucleotides and adenosine act as extracellular signaling molecules, the effect of which is mediated by purinergic receptors (Illes *et al*., 2000).

One of the theories for homeostatic sleep regulation suggests that adenosine is an endogenous sleep pressure factor that is accumulated during waking and promotes sleep, while high ATP levels promote waking (Benington and Heller, 1995, Huston *et al.,* 1996). Intracerebroventricular (icv) injection of adenosine increases sleep time and EEG slow wave activity. An increase in adenosine level during waking seems not to be necessary in the whole brain but may be restricted to certain areas (Haas and Selbach, 2000). Systemic administration of adenosine receptor antagonists, such as caffeine and theophylline, suppresses sleep. Sleep deprivation studies showed an increased level of extracellular adenosine and up-regulation of the A_1 adenosine receptor mRNA (Basheer *et al.,* 2004). Energy restoration is proposed as one of the functions of sleep. However, while the

importance of adenosine in sleep-waking regulation is widely accepted, the precise mechanism of action is far from clear. Investigations on KO mice lacking adenosine A_1 receptor did not reveal any difference in sleep patterns or in responses to sleep deprivation compared to WT mice (Stenberg *et. al.,* 2003).

Effects of adenosine in several brain structures were tested with electrophysiological recordings. Arrigoni and co-authors showed that adenosine has a postsynaptic inhibitory effect on cholinergic and noncholinergic neurons from laterodorsal tegmental nuclei (LDT) (Arrigoni et al., 2006). The firing rate of both types of neurons was inhibited by adenosine. In cholinergic neurons an effect of adenosine was mediated by an inwardly rectifying K^+ conductance. In noncholinergic neurons adenosine reduced the Ih current. Application of the Ih current inhibitor ZD7288 abolished the effects of adenosine (Arrigoni et al., 2006). This observation supports the idea that adenosine might also promote sleepiness by direct inhibition of wakefulness-promoting neurons. The other possibility is that wake-active neurons could be inhibited indirectly. Arrigoni and co-authors also showed that cholinergic neurons are inhibited via presynaptic adenosine receptors (Arrigoni *et al.,* 2001).

In vivo investigations revealed that TMN neurons are indirectly inhibited by adenosine. c-Fos expression studies, microdialysis and EEG showed that CGS21680 adenosine A_2 receptor agonist increases activity of GABAergic neurons of VLPO and inhibits activity of histaminergic neurons (Scammell *et al.,* 2001, Hong *et al.,* 2005). Application of picrotoxin blocks CGS21680-induced inhibition of histamine release, proposing that inhibition of TMN neurons is mediated by excitation of GABAergic cells from VLPO (Hong *et al.,* 2005). To probe this suggestion, the direct effect of adenosine in HAergic neurons was tested during my PhD student project.

1.3.1.2 Purinergic receptors

One of the roles of ATP, adenosine and other nucleotides in the CNS is extracellular signaling, which occurs via purinergic receptors. Purinergic receptors are divided into: P1, P2X and P2Y receptors. P1 receptors are activated most potently by adenosine. They belong to G protein-coupled receptors (Fredholm *et al.,* 2000, Haas and Selbach, 2000). P2 receptors are activated by di- and triphosphates divided into P2X and P2Y receptor, according to the structure and the signal transduction mechanism. P2X are ligand-gated ion channels (Gever *et al.,* 2006) and P2Y are G protein-coupled receptors (Hussl and Boehm, 2006). P2X

receptors mediate fast signaling while P2Y mediate slow signaling in neuronal and glial systems (Illes *et al*., 2000).

1.3.1.3 Adenosine receptors

Currently, adenosine receptors are classified as A_1, A_{2A}, A_{2B} and A_3. A1 receptors couple to Gi and through the alpha beta subunit directly to potassium channels (activation) and to Ca^{2+}-channels (inhibition) (Greene and Haas, 1991). Furthermore, A_1 and A_3 receptors inhibit adenylyl cyclase, while A_{2A} and A_{2B} receptors stimulate it (Fredholm *et al.,* 2000). All genes encoding for adenosine receptors consist of two exons and one intron. The identity of amino acid sequence is less than 50% between adenosine receptors (Fredholm *et al.,* 2000).

Adenosine is an agonist for all P1 receptor, but the use of adenosine itself in pharmacological investigations is restricted due to its extensive metabolism by enzymes. Adenosine is, however, the structural core of all known agonists. Modified agonists have higher affinity to specific receptor subfamilies. Antagonists of adenosine receptors are divided into xanthines and non-xanthines (Klotz, 2000). The most well known unspecific antagonist of adenosine receptor is caffeine: "most widely consumed behaviorally active substance in the world" (Fredholm *et al.,* 2000).

1.3.1.4 Ligand-gated P2X receptors

P2X receptors are ligand-gated ion channels. Each functional P2X receptor consists of three subunits. Seven separate genes ($P2X_{1-7}$) coding P2X subunits have been identified. Subunits share less than 50% identity and range in length from 379 to 595 amino acids. The subunits share a common topology, possessing two plasma membrane spanning domains, a large ligand binding extracellular loop and intracellular N and C termini (Roberts *et al*., 2006, Khakh *et al.,* 2001). With the exception of $P2X_6$, each subunit can form a functional homomeric receptor. A P2X receptor made up of only $P2X_1$ subunits is simply termed as a $P2X_1$ receptor. $P2X_6$ cannot form a functional homomeric receptor when expressed alone, but can co-assemble with other subunits to form functional heteromeric receptors. With the exception of $P2X_7$, all of the P2X subunits are able to form heteromeric P2X receptors with at least one other subunit type. For example, a P2X receptor made up of $P2X_2$ and $P2X_3$ subunits is named $P2X_{2/3}$ receptor (Khakh *et al.,* 2001).

The properties of P2X receptors depend on the subunit composition of the channel. For example, $P2X_1$ and $P2X_3$ receptors desensitize rapidly in the presence of ATP, whereas the $P2X_7$ receptor channel mostly remains open for as long as ATP is bound. Different subunits also exhibit different sensitivity to purinergic receptors agonists such as ATP, 2MeSATP or antagonist such as suramine and pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) (Roberts *et al*., 2006, Khakh *et al.,* 2001).

1.3.1.4 P2X receptors in TMN neurons

Kanjhan and co-authors reported high levels of $P2X_2$ receptor protein and its mRNA expression in the TMN nucleus (Kanjhan *et al.,* 1999). The first description of ATP pharmacology and the expression-pattern of P2X receptors in histaminergic neurons came from our institute in 2003 (Vorobjev *et al.*, 2003a,b). It has been reported that all neurons isolated from TMN respond to ATP with a fast inward current. The averaged maximum response was 0.5±0.06 nA and the EC_{50} was observed at 54±1.6 μM of ATP (Vorobjev *et al.,* 2003a).

Single cell RT-PCR analysis revealed that single TMN neurons express all P2X receptors except $P2X_7$. However, $P2X_7$ was present in the total mRNA. All neurons express $P2X_2$. Other receptors are present in less than 35% of the cells. No correlation was found between sensitivity to ATP and the expression pattern of P2X receptors in single TMN neurons (Vorobjev *et al.,* 2003a).

The response to ATP in TMN neurons is modulated by Zn^{2+} in a reversible and dose-dependent manner (Vorobjev *et al.,* 2003b). The modulation depends on both ATP and Zn^{2+} concentrations. At 10 or 30 μM of ATP, Zn^{2+} induced a potentiation at concentrations from 3 to 30 μM. 30 μM Zn^{2+} induced a maximal potentiation. Higher concentrations of Zn^{2+} decreased the potentiation (Vorobjev *et al.,* 2003b).

1.3.1.5 G protein-coupled P2Y receptors

A great number of G protein-coupled receptors sensitive to extracellular nucleotides have been cloned from various species. They were categorized as P2Y receptors. P2Y receptors mediate slow changes of synaptic transmission and neuronal excitability (Illes *et al.,* 2000). Originally, fifteen receptors (from $P2Y_1$ through $P2Y_{15}$) have been described. However, several of these receptors were subsequently identified as species homologues of other P2Y

receptors. At the moment, eight mammalian P2Y receptor subtypes ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) have been characterized (Abbracchio *et al.,* 2006). They consist of 328 to 538 amino acids. The P2Y-receptor family differs from other known families of G protein-coupled receptors by a high diversity in the amino acid sequences. For instance, the human $P2Y_1$ and $P2Y_{11}$-receptors share only 33% identical amino acid residues despite similarities in their pharmacological profiles. Homologies from different species have much higher similarity. For example, 83% of the amino acid residues of the human and rat $P2Y_1$ receptors are identical (Abbracchio *et al.,* 2006, Hussl and Boehm, 2006). Despite the broad diversity in the amino acid sequence P2Y receptors share similar properties from the pharmacological point of view. They can be grouped as receptors for purines ($P2Y_{1, 11, 12, 13}$), pyrimidines ($P2Y_{6, 14}$), or both nucleotides ($P2Y_{2, 4}$). Furthermore, they can be classified as receptors preferring triphosphates ($P2Y_{2, 4, 11}$) or diphosphates ($P2Y_{1, 6, 12, 13, 14}$) (Hussl and Boehm, 2006). The other way to distinguish P2 receptors is their sensitivity to antagonists. Unfortunately, subtype selective P2 receptor antagonists are not available. All known P2 receptor antagonists block several subtypes of P2 receptors (Ralevic and Burnstock 1998; Jacobson *et al.,* 1999). However, the combined use of subtype preferential agonists and antagonists allows to characterize each subtype of a native P2Y-receptor.

The $P2Y_1$ receptor is the most frequently occurring P2Y receptor in the mammalian brain. The RT-PCR analysis revealed a higher expression of $P2Y_1$ receptors in neurons compared to other purinergic receptors. This receptor is selectively activated by adenine nucleotides, being insensitive to uridine nucleotides. The typical rank order of agonist potency is 2-MeSADP>2-MeSATP>ADP>ATP. The most selective antagonist is 2'-deoxy-*N*6-methyladenosine-3',5'-bisphosphate (MRS 2179) (Abbracchio *et al.,* 2006).

$P2Y_2$ receptors are activated by triphosphate nucleotides ATP and UTP with equal potency. ADP, UDP or 2-MeSADP have weak or no activity. Suramin blocks $P2Y_2$-receptor, but affinity is 10 times lower in comparison with $P2Y_1$-receptor (Abbracchio *et al.,* 2006).

Like $P2Y_2$ receptors, $P2Y_4$ receptors are activated by UTP and ATP in rodents, whereas the human receptors have a strong preference for UTP over ATP. $P2Y_4$ receptors also are not activated by diphosphates. $P2Y_4$ is suramin-insensitive and is blocked by Reactive Blue. Sometimes $P2Y_2$ and $P2Y_4$ are combined in a separate functional subgroup of P2U receptors (Abbracchio *et al.,* 2006).

$P2Y_6$ receptors are activated by UDP and UTP. UDP is approximately 100-fold more potent than UTP. ADP, ATP and 2-MeSADP have almost no effect. $P2Y_6$ is blocked by suramine, PPADS and Reactive Blue (Abbracchio *et al.,* 2006).

$P2Y_{11}$ receptors display a rank order of agonist potency of ATP>2-MeSATP>ADP. The observed agonistic activity of UTP depends on the signaling cascade that is activated by the receptor. $P2Y_{11}$ is blocked by suramine and Reactive Blue (Abbracchio *et al.,* 2006).

$P2Y_{12}$ receptors are activated by 2-MeSADP, ADP and, in some cases, ATP. The efficacy of ATP is species-dependent with high intrinsic activity at rat, but not at human receptors. The most potent direct $P2Y_{12}$ antagonist is ARC69931MX, but this is also an antagonist of $P2Y_{13}$ receptors (Abbracchio *et al.,* 2006). $P2Y_{13}$ receptors are activated by 2-MeSADP, ADP, and ATP. The rank order of agonist potency is different for human and rat receptors (Abbracchio *et al.,* 2006). The $P2Y_{14}$ receptor is sensitive to various UDP sugars, but not to adenine or uridine nucleotides (Abbracchio *et al.,* 2006).

To date, there exist no investigations of P2Y receptors in TMN neurons. My thesis contains an electrophysiological and molecular characterization of P2Y receptors in TMN neurons and glial cells.

1.3.2 Thyrotropin-releasing hormone

Thyrotropin-releasing hormone (TRH) is a tripeptide hormone (*p*-Glu-His-Pro-NH_2) produced in the paraventricular nucleus (PVN) of the hypothalamus. It is enzymatically processed from a larger precursor, prepro-TRH, which contains multiple copies of the TRH sequence and several distinct non-TRH products (Gary *et al.,* 2003).

TRH has physiological effects in many behavioral processes. For example, in the regulation of the sleep-waking cycle, cognition, locomotion and mood. My work concentrated on the role of TRH in the sleep-waking cycle. Systemic and central application of TRH increases the time of wakefulness. A more dramatic effect is the ability of TRH to arouse animals from narcosis induced by alcohol and barbiturates. Interestingly, TRH levels rise in the medial septum when rats recover from ethanol-induced sedation (Morzorati *et al.,* 1993). However, the cellular mechanisms underlying effects on waking are largely unknown. It is reported, that TRH enhances cholinergic and noradrenergic system activity. Moreover, it is believed that a major part of the TRH effect is mediated by cholinergic or monoaminergic

mechanisms (Nillni and Sevarino, 1999). The effect of TRH on the histaminergic system has not been investigated so far.

It is believed that TRH initiates effects by interacting with two G protein-coupled TRH receptors (TRH-R): TRH-R1 and TRH-R2. The sequence alignment revealed identity about 50 % in many species (Gershengorn and Oman, 1996). Considerable differences are also found in the receptor distribution. Brain regions usually express only one of these receptors. For example, TRH-R1 expression is present in hypothalamus and septum, while cortex and thalamus mainly express TRH-R2. Despite the marked difference in structure and distribution, TRH-R1 and TRH-R2 exhibit similar binding affinities for TRH and it analogues (Sun *et al.*, 2003) and intracellular signal transduction pathways (Nillni and Sevarino, 1999). TRH receptors usually couple to Gq/11 proteins (Gershengorn and Oman, 1996). Another TRH receptor pathway is inhibition of potassium leak current, mediated by two-pore-domain potassium (K2P) channels (Goldstein *et al.,* 2001).

1.3.3 Modafinil

1.3.3.1 Modafinil: general overview

Modafinil (benzhydrylsulphinylacetamide, commercially known as Provigil, Alertec or Vigicer) is currently used for the treatment of excessive sleepiness such as occurs in narcolepsy. It increases wakefulness supposedly without the central and peripheral side effects associated with psychostimulants such as amphetamines and methylphenidate (Ballon and Feifel, 2006). Modafinil has also been reported to be effective in the treatment of multiple sclerosis and Parkinson's disease (Szabadi, 2006). The mechanism of modafinil action is under discussion. It has been proposed that modafinil increases wakefulness via an action on noradrenergic neurotransmission. Investigations in cats showed that the waking effect of modafinil is prevented or attenuated by pretreatment with α_1 or β adrenoreceptor antagonists (Lin *et al.,* 1992). On the other hand, pharmacological elimination of the noradrenaline transporter did not reduce the efficiency of modafinil (Wisor and Eriksson, 2005). Modafinil could induce waking by its action on dopamine transmission. Indeed, it exhibits a weak affinity for the dopamine transporter (DAT) and does not increase waking in mice with deletion of the DAT gene (Wisor *et al.,* 2001, Wisor and Eriksson, 2005). In other studies, it has been shown that modafinil can increase the release of serotonin, decrease GABA release and increase extracellular glutamate levels (Ferraro *et al.,* 1999, 2000).

Administration of amphetamine or methylphenidate evoked c-Fos immunoreactivity in the striatum and whole cortex, which are targets of dopaminergic projections. In contrast, administration of modafinil caused activation of only a few cells in these structures (Lin *et al.,* 1996). Injection of modafinil increased three-four times the activation of c-Fos expression in the TMN and PFx; a small increase in c-Fos level was found in the LC and a decreased c-Fos level in the VLPO. Interestingly, modafinil did not induce c-Fos expression within arousal regions such as DR (Scammell *et al.,* 2000). Microdialysis investigations also showed that icv modafinil injection increases histamine release. However, direct injection of modafinil into the TMN nucleus did not cause changes in histamine level (Ishizuka *et al.,* 2003). Proceeding from these data, authors proposed that modafinil exerts indirect effect on TMN neurons.

In vitro investigations could help in searching of mechanism of modafinil action. In our institute we showed that the modafinil does not affect on TMN neuron activity *in vitro* (next chapter, our unpublished observation). In other study, performed at our institute, is demonstrated that modafinil inhibits DAergic neurons in both the VTA and the SN. Coapplication with the sodium channel blocker tetrodotoxin did not eliminate the effect of modafinil, indicating a postsynaptic action. In this thesis the mechanism of action is described in DAergic neurons.

1.3.3.2 Modafinil does not affect the activity of HAergic neurons *in vitro*

Bath application of modafinil did not change firing rate of TMN neurons at the concentrations of either 200μM (Fig. 2A, n=3) or 500 μM (Fig. 2B, n=3). During application of 1 mM modafinil TMN neurons showed heterogeneity. Two out of four neurons did not respond to modafinil (Fig. $2C_1$). Two other neurons were strongly inhibited by modafinil, to 21±2% of baseline (Fig. $2C_2$). However, this inhibition of firing is likely due to the solvent DMSO. The final conclusion is thus that, in contrast to dopaminergic neurons, modafinil has no effect on histaminergic neurons.

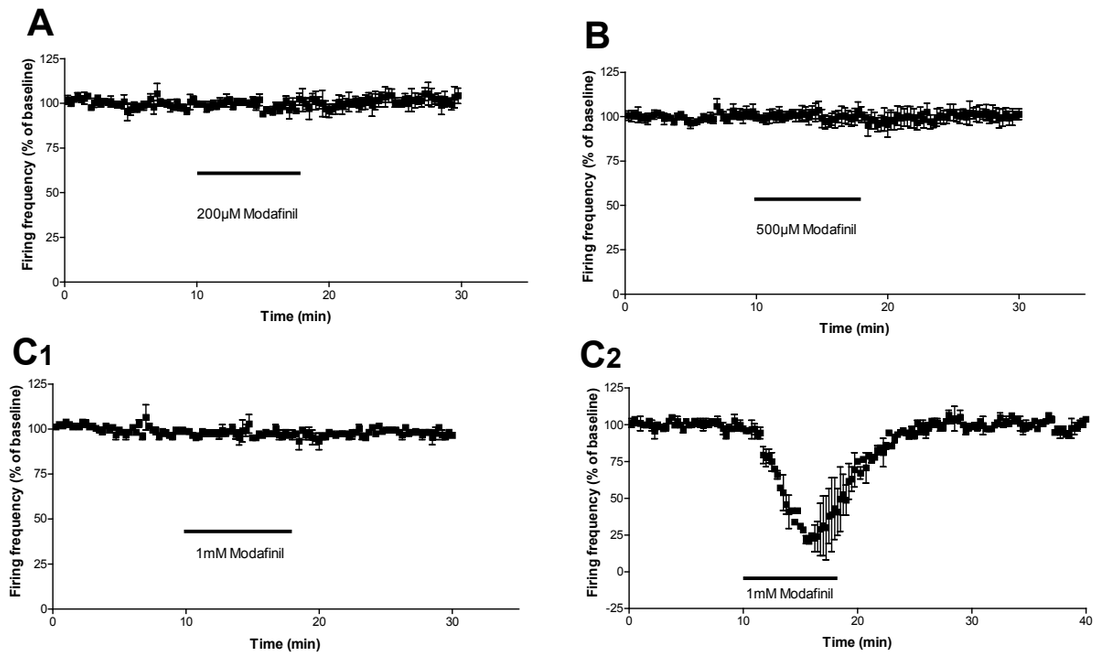

Fig. 2. Lack of effect of modafinil on TMN neurons.

A,B. Bath-applied modafinil at the concentrations 200μM (n=3) or 500 μM (n=3) did not change firing rate of TMN neurons.
C. Effect of 1mM modafinil on TMN neurons
C_1. Two out of four neurons did not respond to application of 1mM modafinil.
C_2. 1 mM modafinil caused strong inhibition in other two neurons.

This study showed that modafinil does not change the activity of TMN neurons in hypothalamic slices. In other words, TMN neurons are not the direct target of modafinil. This result is surprising because in our paper it is shown that modafinil acts via D2-like receptors in dopaminergic neurons from VTA and SN (Korotkova *et al.,* 2007). On the other hand our current data indicate the expression of D2-like receptors in TMN neurons, and their activation leads to excitation of TMN neurons (our unpublished observation).

There are several explanations of our negative results. First possibility is that modafinil acts mainly via D3 receptors. In our previous study we showed that modafinil acts via D2-like receptors, but we did not investigate the role of D2 and D3 receptors in modafinil action in DAergic neurons (Korotkova *et al.,* 2007). No pharmacological difference between D2 and D3 receptors is generally assumed; nonetheless, several works report their difference in affinity for certain drugs (Sokoloff *et al.,* 1990). Dopamine itself has 20 times higher affinity for D3 than for D2 receptors. The D2 selective receptor agonist quinpirole shows an even larger difference, about 100 times (Sokoloff *et al.,* 1990). Thus D3 receptors may play a larger role in the modafinil effect than D2 receptors.

Second, modafinil can have different affinities for different isoforms of the D2 receptor. Dopaminergic neurons express only the short form of the D2 receptor ($D2_S$) (Giros *et al.,* 1989). Our results show that TMN neurons express only the long isoform ($D2_L$). A few chemicals have different affinities for the D2 receptor isoforms; for example clozapine has a 2-3 fold higher affinity for $D2_L$ than for $D2_S$ (Malmberg *et al.,* 1993). Modafinil may be one of these drugs that have a higher affinity for short isoforms of the D2 receptor. However, this possibility does hardly explain our results.

Third, D2 receptors might have a low selectivity for modafinil compared to dopamine or quinpirole. Nowadays, there is no biochemical investigation of modafinil-dopamine receptor binding. Our pharmacological data propose that quinpirole and dopamine have inhibition constants (Ki) about 2 orders of magnitude lower than modafinil. During investigation of the modafinil action on DAergic neurons we found out that the concentrations which caused a maximal response are 5 and 200μM for quinpirole and modafinil, respectively. From our current data we conclude that the minimal concentration of quinpirole that causes excitation of TMN neurons is about 45μM. Comparing the actions of quinpirole and modafinil in DA neurons we had proposed that the minimal concentration of modafinil should be 1mM to cause an effect in TMN neurons. But applying such a high concentration, which is far from the concentration used *in vivo*,

I received puzzling results. In half cases modafinil did not change activity of TMN neurons. In the other half of the cases there was a strong inhibition of TMN neurons. This inhibition may result from the solvent medium DMSO.

Previous works showed that modafinil increases the Fos expression level three-four times in the TMN nucleus (Scammell *et al.,* 2000). Microdialysis investigations also supported this finding: i.c.v. injection of modafinil increases histamine release. However, injection of modafinil into the TMN nucleus did not change the histamine level. According to these data, authors suggested an indirect effect of modafinil on TMN neurons (Ishizuka *et al.,* 2003). Our results support this conclusion.

There are two possible ways of indirect regulation of TMN neurons by modafinil: inhibition of sleep-promoting systems or/and activation of waking active systems. Recently, it has been shown that modafinil specifically increases the inhibition of VLPO neurons by noradrenaline (Gallopin *et al.,* 2004). Investigation of Fos expression also revealed inhibition of VLPO neurons by modafinil (Scammell *et al.,* 2000). These neurons project to and inhibit TMN cells (Sherin *et al.,* 1998, Szymusiak *et al.,* 1998). So, modafinil can activate TMN neurons by inhibition of GABAergic neurons from VLPO. Another possibility of TMN neuron activation is an increase in excitatory neurotransmitter release. Indeed, an increase of glutamate and 5-HT levels after modafinil injection has been shown (Ferraro *et al.,* 1999, 2000). The enhancement of the glutamate level could results from a reduction of GABAergic tone (Ferraro *et al.,* 1999); however, an increased of serotonin level could depend on a direct D2-like receptor activation in 5-HT neurons by modafinil (Ferraro *et al.,* 2000). Indeed, it has been shown that D2-like receptors have an excitatory function in 5-HT neurons from the dorsal raphe (Haj-Dahmane, 2001). On the other hand, serotonin activates TMN neurons directly (Eriksson et al., 1999). Modafinil could activate serotonin release, and serotonin can activate TMN neurons. However, this inhibition of firing is likely due to the solvent DMSO. The final conclusion is thus that, in contrast to dopaminergic neurons, modafinil has no effect on histaminergic neurons.

Background and aims of the project

The histaminergic system plays a key role in many behavioral processes such as the sleep-waking cycle, food intake, endocrine homeostasis and others (Haas and Panula, 2003). The present study aims at the characterization of several not well explored modulators of HAergic neuron activity such as adenosine, adenosine 5'-triphosphate (ATP) and thyrotropin-releasing hormone (TRH). Furthermore, the mechanism of action of the wake-promoting drug modafinil is studied.

Within the project the following aims were formulated:

1. To examine distribution and pharmacological properties of P1 and P2Y purinergic receptors in HAergic neurons.

2. To study the direct effect of TRH on the activity of the histaminergic system *in vitro*.

3. To clarify the mechanism of modafinil action on DAergic and histaminergic neurons.

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Summary

Histaminergic neurons from the tuberomamillary nucleus innervate practically the whole brain and parts of the spinal cord. Their most prominent role is the maintenances wakefulness and attention (Brown *et al.,* 2001). They represent the major waking centre of the brain (Lin *et al.,* 2006). Histamine itself is a wake promoting substance, and experimental lesions of histaminergic neurons cause hypersomnolence (Gerashenko *et al.,* 2004). In this thesis the effects of wake promoting neurotransmitters on HAergic systems are characterized. The effect of the wake-promoting substance modafinil is described on both HAergic and DAergic systems.

1. Adenosine does not change the firing rate or membrane potential of TMN neurons in hypothalamic brain slices. Thus, this sedative sleep pressure substance acts downstrream the histaminergic waking, e.g. on cholinergic neurons.
2. ATP, ADP and UTP excite TMN neurons *in vitro*. These data explain the wake-promoting effectof ATP and related purines.
3. Single-cell RT-PCR analysis revealed the expression of $P2Y_{1,}$ and $P2Y_4$ receptors in the TMN. The mRNA encoding for other P2Y receptors was not detected in the single neurons.
4. We provide initial evidence for interactions between TMN neurons and glial cells. Immunostaining of cultured cells revealed that the $P2Y_1$ receptors are widely expressed both in neurons and the nuclei of glial cells.
5. TRH increased the action potential firing frequency and induced a membrane depolarization. TRH affects the frequency but not the amplitude of spontaneous IPSCs indicating the presence of a presynaptic effect too.
6. Single cell RT-PCR and immunohistochemistry revealed expression of one or both TRH receptors in most TMN neurons, whereas a small fraction of neurons lacked TRH receptor-expression.
7. Modafinil inhibits dopaminergic neurons directly. D2-like receptors are the major if not unique target of modafinil in DAergic cells.

Annex

1. **P2Y receptor mediated excitation in the posterior hypothalamus (2006)**
Sergeeva OA, Klyuch BP, Fleischer W, Eriksson K, Korotkova TM, Siebler M, Haas HL
Europ.J.Neurosci. 24: 1413-1426.

Contribution of Boris Klyuch:
Shared first authorship. Extracellular recording from single identified histaminergic neurons. Figs. 1,2,3,4CD, 5A-F. Significant contribution to analysis, documentation and writing of the paper.

2. Modafinil inhibits rat midbrain dopaminergic neurons through D2-like receptors (2007)
Korotkova TM, Klyuch BP, Ponomarenko AA, Lin JS, Haas HL and Sergeeva OA
Neuropharmacology 52: 626-633

Contribution of Boris Klyuch:
Shared first authorship. Extracellular recording from single identified VTA and SN neurons. Figs. 1,2, 5A-F. Significant contribution to analysis, documentation and writing of the paper.

3. Excitation of histaminergic tuberomamillary neurons by thyrotropin-releasing hormone
(to be submitted 2008)
Regis Parmentier, David Vandael, Boris P. Klyuch, Jian-Sheng Lin, Oliver Selbach, Helmut L. Haas and Olga A. Sergeeva

Contribution of Boris Klyuch:
Extracellular and intracellular whole cell recording from single identified histaminergic neurons. Figs. 1,5,6. Significant contribution to analysis, documentation and writing of the paper.

P2Y receptor mediated excitation in the posterior hypothalamus

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Abstract

Histaminergic neurons located in the posterior hypothalamus (tuberomamillary nucleus, TMN) project widely through the whole brain controlling arousal and attention. They are tonically active during wakefulness but cease firing during sleep. As a homeostatic theory of sleep involves ATP depletion and adenosine accumulation in the brain, we investigated the role of ATP and its analogues as well as adenosine on neuronal activity in the TMN. We show increased firing of rat TMN neurons by ATP, ADP, UTP and 2meSATP, indicating activation of receptors belonging to the P2Y family. Adenosine affected neither membrane potential nor firing of these cells. Single-cell reverse transcriptase-polymerase chain reaction revealed that $P2Y_1$ and $P2Y_4$ are prevailing receptors in TMN neurons. P2Y1 receptor mRNA was detected with a higher frequency in 2-week-old than in 4-week-old rats; in accordance, 2meSATP was more potent than ATP. Semi-quantitative real-time polymerase chain reaction revealed a developmental downregulation of mRNA levels for $P2Y_1$ and $P2Y_4$ receptors. Immunocytochemistry demonstrated neuronal and glial localization of the $P2Y_1$ receptor protein. Network activity measured with multielectrode arrays in primary cultures made from the posterior hypothalamus was enhanced by UTP and 2meSATP ($P2Y_4$ and $P2Y_1$ agonists, respectively). ATP caused an inhibition of firing, which was reversed in the presence of suramin or gabazine [γ-aminobutyric acid (GABA)A receptor antagonist], indicating that GABAergic neurons are preferentially activated by ATP in this network. Excitation of the wake-active TMN neurons by nucleotides and the lack of adenosine action may be important factors in sleep–wake regulation.

Introduction

ATP is the major intracellular source of energy and a signalling molecule mediating interactions between neurons as well as astrocytes and neurons. Endogenous or exogenously applied ATP is sensed directly by type 2 purinoceptors (P2 receptors): ionotropic P2X receptors (Khakh *et al.,* 2001; Khakh, 2001; North, 2002) and metabotropic G-protein-coupled P2Y receptors (von Kugelgen & Wetter, 2000), or indirectly by G-protein-coupled adenosine receptors (Dunwiddie & Masino, 2001) after a rapid conversion by ectonucleotidases (Dunwiddie *et al.,* 1997; Braun *et al.,* 1998). These different receptor

types produce various responses in central and peripheral neurons, influencing synaptic transmission. In hippocampal circuitries different receptors are involved in an inhibitory ATP action depending on the preparation: adenosine mediates the ATP action in acute slices through A1 receptors (Dunwiddie *et al.,* 1997; Masino *et al.,* 2002), while mainly $P2Y_1$ receptors are involved in cultures (Koizumi *et al.,* 2003; Zhang *et al.,* 2003). In the CA3 region of the hippocampus ATP excites interneurons through the $P2Y_1$ receptor, thus contributing to the inhibition (Kawamura *et al.,* 2004). P2Y receptors mediate a rise in intracellular calcium and are widely expressed in hippocampal astrocytes (Zhu & Kimelberg, 2001; Kawamura *et al.,* 2004).

Among other essential functions the hypothalamus is the command centre for the regulation of energy administration and behavioral state. Histaminergic neurons in the posterior hypothalamus send projections through the whole brain (Staines *et al.,* 1987; Panula *et al.,* 1989) controlling arousal and attention (Parmentier *et al.,* 2002; Haas & Panula, 2003). They are tonically active during waking and this activity is suppressed during sleep (Vanni-Mercier *et al.,* 2003; Haas & Panula, 2003). A homeostatic theory of sleep suggests that adenosine accumulation during waking increases sleep drive, while high ATP levels support waking (Benington & Heller, 1995; Huston *et al.,* 1996; Arrigoni *et al.,* 2001). We have previously shown that single tuberomamillary nucleus (TMN) neurons express variable P2X receptors, the major type (by pharmacological evidence) is the $P2X_2$ receptor (Vorobjev *et al.,* 2003). The physiological role of these receptors is at present obscure. The role of ATP and related nucleotides as well as products of ATP degradation including adenosine on neuronal firing in this wakefulness controlling nucleus is unknown. Lateral hypothalamic neurons co-release γ-aminobutyric acid (GABA) and ATP, as demonstrated by a suramin-sensitive component of inhibitory postsynaptic currents (IPSCs; Jo & Role, 2002). We were not able to detect such a component in TMN neurons as GABA-mediated currents were 10–100-fold greater than ATP ($P2X_2$)-mediated responses (Vorobjev *et al.,* 2003) and all spontaneous postsynaptic currents were blocked by gabazine, the selective GABAA receptor antagonist (Sergeeva *et al.,* 2002). Spontaneous excitatory postsynaptic currents are absent in TMN neurons recorded either in acute isolation (Sergeeva *et al.,* 2002) or in hypothalamic slices (Eriksson *et al.,* 2004). With whole-cell patch-clamp recordings we did not detect metabotropic ATP actions (P2Y

receptors) in TMN neurons (Vorobjev *et al.,* 2003). To avoid the cell-dialysis problem during whole-cell recording we studied now how ATP and its analogues influence spontaneous firing in these neurons in slices and in cultures, using non-invasive techniques, namely extracellular recording of firing rates. We describe here an excitation of TMN neurons by ATP and UTP mediated by metabotropic P2Y receptors.

Materials and methods

Slice preparation

Coronal brain slices from the posterior hypothalamus (400–450 μm thick) containing the TMN were prepared from 21–28-day-old male Wistar rats, except where mentioned otherwise. All experiments were conducted in compliance with German law and with the approval of the Bezirksregierung Duesseldorf. All efforts were made to minimize the number of animals. The animals were decapitated and the brain quickly 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 the vibroslicer (Campden Instruments, UK) 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 h 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 MΩ). According to Ericson et al. (1987) (Fig. 1A) 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 (TMv, Fig. 1A), which was visually identified under a dissecting microscope. Signals were recorded using an Axoclamp 2B amplifier (Axon

Instruments, USA), filtered between 0.5 and 10 kHz, sampled at 20 kHz and analysed with pClamp8 software (Axon Instruments). The frequency of extracellular action potentials was determined online in bins of 15 s duration.

Intracellular recordings from TMN neurons were obtained using sharp glass microelectrodes filled with 2 M KCl with resistances of 60–100 MΩ or with K-acetate (4 M, 90–120 MΩ). The spontaneous inhibitory postsynaptic potentials (sIPSPs) were recorded with KCl-electrodes and with the neuron held at −68 to −70 mV by negative current injection, which prevented the spontaneous activity, as the threshold for action potential firing in these neurons is at −52 mV (Haas & Reiner, 1988). The data acquisition was made with an Axoclamp-2A amplifier and a Digidata 1200 interface board (Axon Instruments). After further amplification, the signal was fed to a chart recorder and to a PC running Clampex 7 software.

Primary posterior hypothalamic cultures and multielectrode array (MEA) recordings

Cultures were prepared from the caudal half of the hypothalamus as described in Sergeeva et al. (2005); dissociated cells were plated in a density of $1–2\times10^5/cm^2$ onto polyethylenimine-coated MEAs in a volume of 100 µL (multielectrode arrays, Multi Channel Systems, Reutlingen, Germany) or, for immunocytochemical staining, on similarly coated cover slips and then cultured in an incubator with 5% CO_2, 95% air and 98% relative humidity, at 35.5±0.5°C.

Extracellular potentials were recorded on MEAs with a square grid of 60 planar Ti/TiN-microelectrodes (30 µm diameter, 200 µm spacing). The AC input impedance measured at a frequency of 1 kHz was < 50 kΩ according to the specifications of the manufacturer (Multi Channel Systems). Signals from all 60 electrodes were simultaneously sampled at 25 kHz, visualized and stored using the standard software MCRack provided by Multi Channel Systems. Spike detection was performed offline using the software SpAnNer (RESULT Medizinische Analyseverfahren, Tönisvorst, Germany). Individually for each channel, the threshold for spike detection was set to eight standard deviations of the average noise amplitude during a 10% 'learning phase' at the beginning of each measurement. An absolute refractory period of 4 ms and a maximum spike width of 2 ms were imposed on the spike detection algorithm. Spontaneous spike rate was averaged over

all electrodes. Recordings were performed at 37 °C in a magnesium-free HEPES-buffered solution with pH 7.4 [see single-cell reverse transcriptase-polymerase chain reaction (scRT-PCR) section]. Neurobasal medium on the MEAs was replaced by the buffer solution and measurements were started after a 20-min adaptation phase. Every measurement comprised three recordings-control, test substance and washout (second control) - each 2 min long and separated by an intermediate delay of 30 s. Firing rates are presented as mean±SEM, n refers to the number of MEAs. Recordings were made from MEAs if more than 10 channels were active. Each substance was applied to a MEA only once.

Immunocytochemistry

Hypothalamic slices or cultures containing the TMN region were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 and cryoprotected in PB with 20% sucrose, slices were cryosectioned at 25 µm thickness. Sections were mounted on gelatin-coated slides, dried and stained according to the immunofluorescence staining protocol. To reveal histamine immunoreactivity cultures were fixed in 4% EDAC (1-ethyl-3(3-dimethylaminopropyl)-carbodiimide) in 0.1 M sodium phosphate buffer (pH 7.4). The sections (or cultures) were first washed in phosphate-buffered saline (PBS) with 0.25% Triton X-100 (PBS-T) for 5 min and then preincubated with 2% normal donkey serum in PBS-T for 30 min at room temperature. This solution was also used to dilute a guinea pig polyclonal antibody to histidine decarboxylase (HDC, Acris, Bad Nauheim, Germany) to 1:1000, rabbit-anti-histamine antibody (1:1000) and affinity-purified rabbit-anti-$P2Y_1$ receptor-polyclonal antibody (1:100; both from Chemicon International, Germany), 1:500 diluted mouse anti-microtubule-associated protein-2 (MAP-2) antibody (Sigma, Germany) or 1:200 diluted rabbit-anti-metabotropic glutamate receptor (mGluR)1α antibody (Chemicon International, Germany). The antibody solution was applied to the sections for 12–16 h at 4 °C. After washing, sections were incubated with Alexa Fluor 488-labelled goat-anti-guinea pig IgG to reveal the HDC immunoreactivity or with Alexa Fluor 488-labelled goat-anti-mouse IgG to reveal MAP-2 immunoreactivity (both from Molecular Probes, Eugene, Oregon, USA) in combination with Texas red donkey anti-rabbit IgG (Dianova, Hamburg, Germany) to reveal the $P2Y_1$, or histamine, immunoreactivities for 90

min at room temperature. All secondary antibodies were diluted to 1:500. Two negative controls (single stainings) were performed in each experiment, where one of the primary antibodies was replaced with the normal serum and further incubation with both secondary antibodies was performed as usual. Stainings were analysed with conventional fluorescence microscopy. Bandpass filters were XF22, with excitation at 485±11 nm and emission at 530±15 nm, for Alexa Fluor 488 and XF43 (580±13.5 nm/630±15 nm) for Texas red (Omega Optical, Brattleboro, VT, USA). We detected no leakage of signal through the incorrect filter for either fluorochrome, even at very strong fluorescence.

ScRT-PCR

Acutely isolated hypothalamic neurons were prepared from the brains of 11–14-day-old or 22–28-day-old male Wistar rats (n=6). Transverse slices (450 μm thick) containing the TMN region were cut and incubated for 1 h in a solution containing (in 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 sodium current to depolarizing voltage steps (for the method see Sergeeva et al. (2003). 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 μL) was expelled into an Eppendorf tube, containing 7 μL 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 RT this reaction was stopped by freezing at -20 °C. Cell identification was performed by HDC-cDNA amplification. For the first amplification round primers HDCup: 5'-GAT GAT GGA GCC CAG TGA ATA-3'and HDC lo: 5'-CTG GTC AGA GGC ATA GGC AAC A-3' were used. For the second round of amplification

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 bp size.

The cDNA species belonging to the P2Y family were amplified in the first round with a degenerate primer mixture: $P2Y_{24}$up: 5'-TAC ATG TT(CT) CAC CTG GCA (GT)T(GT) TC-3', $P2Y_{16}$up: 5'-TAC A(CT)(GC) (CT)T(GC) AA(CT) TTG GC(AT) CTG GC-3', $P2Y_{246}$lo: 5'-GA(CT) GTG GAA (AG)GG CA(GC) GAA GC(AT) GA-3' and $P2Y_1$lo: 5'-CAC ATG GAA AGG GAT GTA AGA CAC-3'. Subtype-specific primers were used for the second amplification round: $P2Y_1$Se: 5'-GCA GGC TCA AGA AGA AGA ATG CCA-3'and $P2Y_1$ASe: 5'-ACA GCC CAA GAT CAG CAC CA-3' (size of amplimer 226 bp); $P2Y_2$Se: 5'-ACC AGC GTG AGA GGG ACC CG-3' and $P2Y_2$ASe: 5'-CGG TTT GAG CAG CCG TCG G-3' (size of PCR product 164 bp); $P2Y_4$Se: 5'-CTG GAT TTG CAA GCC TTC TCT GCC-3' and $P2Y_4$ASe: 5'-ACC ATG ACT GCC GAA CTG AAG TAG A-3' (amplimer size 171 bp); $P2Y_6$Se: 5'-CCT CTT CTA TGC CAA CCT ACA CGG C-3' in combination with $P2Y_6$ASe: 5'-ACA GTG CGG TTG CGC TGG AT-3' (amplimer size 215 bp). All four subtypes were amplified from the positive control (TMN whole). Only $P2Y_1$ and $P2Y_4$ receptor mRNAs were detected in individual TMN neurons. PCR products (positive control used as a template) obtained after two amplification rounds were purified in water and sequenced. The obtained sequences corresponded to the known ones for the rat (GENBANK, accession numbers): U22830, U56839, Y11433 and 48675856 for the $P2Y_{1,2,4 \text{ and } 6}$, respectively.

For the amplification of $P2Y_{12}$ and $P2Y_{13}$ cDNAs Dglo: 5'-AAG AA (GC) ACA GC (CA) ATGA (CT)GAT-3' was used as common degenerate primer in the first amplification round either with $P2Y_{12}$up1: 5'-ACC AAC AGG AGG CCA AAA G-3' or with $P2Y_{13}$up1: 5'-TCT CCA TTT CCA TCT GGC TC-3'. In the second amplification round the following subtype-specific primers were used: $P2Y_{12}$up2: 5'-TGG CAC GAG ATA GTC AAT TAC A-3', $P2Y_{12}$lo2: 5'-TGT TCA CCC TTT TCT TGG G-3' (size of PCR product 151 bp); $P2Y_{13}$up2: 5'-ATC TTG AAC AAA GAG GCA ACG G-3', $P2Y_{13}$lo2: 5'-ACC TTC TTG GCA ATC ACC G-3' (size of PCR product 164 bp). Sequenced PCR products matched the known sequences for the rat $P2Y_{12}$ and $P2Y_{13}$ receptors (GENBANK accession numbers AF313450 and AY639875, respectively)

For the first amplification round of the first group of mGluRs the following primers were used: mGluR lo1: 5'-AAA CAT GCA (AC)CC CAG GGC CAC', mGluR5 up: 5'-GGA TAT AAT GCA TGA CTA TCC A-3' and mGluR1 up: 5'-CCA TTT TGT CCT ACC CG-3' were used. For the second, subtype-specific, amplification upstream primers were used with mGluR lo2 primer: 5'-GTC ATG AT(CT) TTG TAG TTG CT-3'. Obtained PCR products had characteristic size of 247 bp (for mGluR1-) and 252 bp (for mGluR5-amplimers). Sequenced PCR products matched the known respective sequences for the rat mGluR receptors (GENBANK accession numbers M61099 and D10891 for the mGluR1 and mGluR5, respectively).

Thin-walled PCR tubes contained a mixture of first strand cDNA template (1–1.5 μL), 10×PCR buffer, 10 pm each of sense and anti-sense primer, 200 μM of each dNTP and 2.5 units Taq polymerase. The final reaction volume was adjusted to 10 μL 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 (Mastercycler, Eppendorf, Germany). A two-round amplification strategy was used in each protocol. In each round 35 cycles of the following thermal programmes 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 μL of the product of the first PCR was used as a template. Products were visualized by staining with ethidium bromide and analysed by electrophoresis in 2% agarose gels.

Real-time PCR

Total cellular mRNA was isolated from the TMN region using an mRNA isolation kit (Pharmacia Biotech) from rats of varied age. Total mRNA was eluted from the matrix with 200 μL of RNase-free water and subjected to the reverse transcription (see above). 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 μL of the 10× SYBR 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 (see subtype-specific primers used for the second round of amplification of $P2Y_1$ and $P2Y_4$ cDNAs), 5 μL of 1:4 diluted cDNA and water to 25 μL. All reactions were normalized on β-actin expression, which was amplified with 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'. PCR samples were placed in optical tubes capped with MicroAmp optical caps and 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). Semi-quantitative analysis of P2Y receptor expression relative to the β-actin endogenous control was performed according to the '$2^{-\Delta\Delta Ct}$' (ΔFold) method as described previously (Sergeeva *et al.,* 2003).

Drugs and statistical analysis

The drugs used in the present study were: adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'-diphosphate potassium salt (ADP), 2-methylthioadenosine 5' -triphosphate tetrasodium salt (2meSATP), 2-methylthioadenosine 5' -monophosphate triethylammonium salt (2meSAMP), uridine-5' -triphosphate (UTP), suramin sodium salt, PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium salt), reactive blue 2 (cibacron blue), adenosine, gabazine (SR-95531) (all obtained from Sigma/RBI, Deisenhofen, Germany). DPCPX (8-cyclopentyl-1,3-dipropylxanthine) (R)-α-methylhistamine dihydrobromide, CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) disodium salt, EHNA hydrochloride (erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride), MRS2179 (2'-deoxy-N 6-methyladenosine 3'5'-bisphosphate), MPEP (2-methyl-6-(phenylethynyl)pyridine hydrochloride) and LY 367385 ((S)-(+)-α-amino-4-carboxy-2-methylbenzeneacetic acid) were obtained from Biotrend (Koeln, Germany) and ciproxifan (cyclopropyl-[4-(3-1H-imidazol-4-yl)propyloxyphenyl]ketone) from Bioprojet (Paris, France). Drugs were diluted and stored as recommended by the producers. Drugs were perfused for 5–10 min through the recording chamber. Neurons were recorded for at least 15 min to obtain a stable baseline before application of drugs. Maximal effects of the drugs (at maximal concentrations) were achieved within 2–3 min after drug application into the perfusion tube. Statistical analysis was performed with the non-parametrical Mann–Whitney U-test. The significance level was set at $P < 0.05$. Data are presented as mean ± standard error of the mean (SEM).

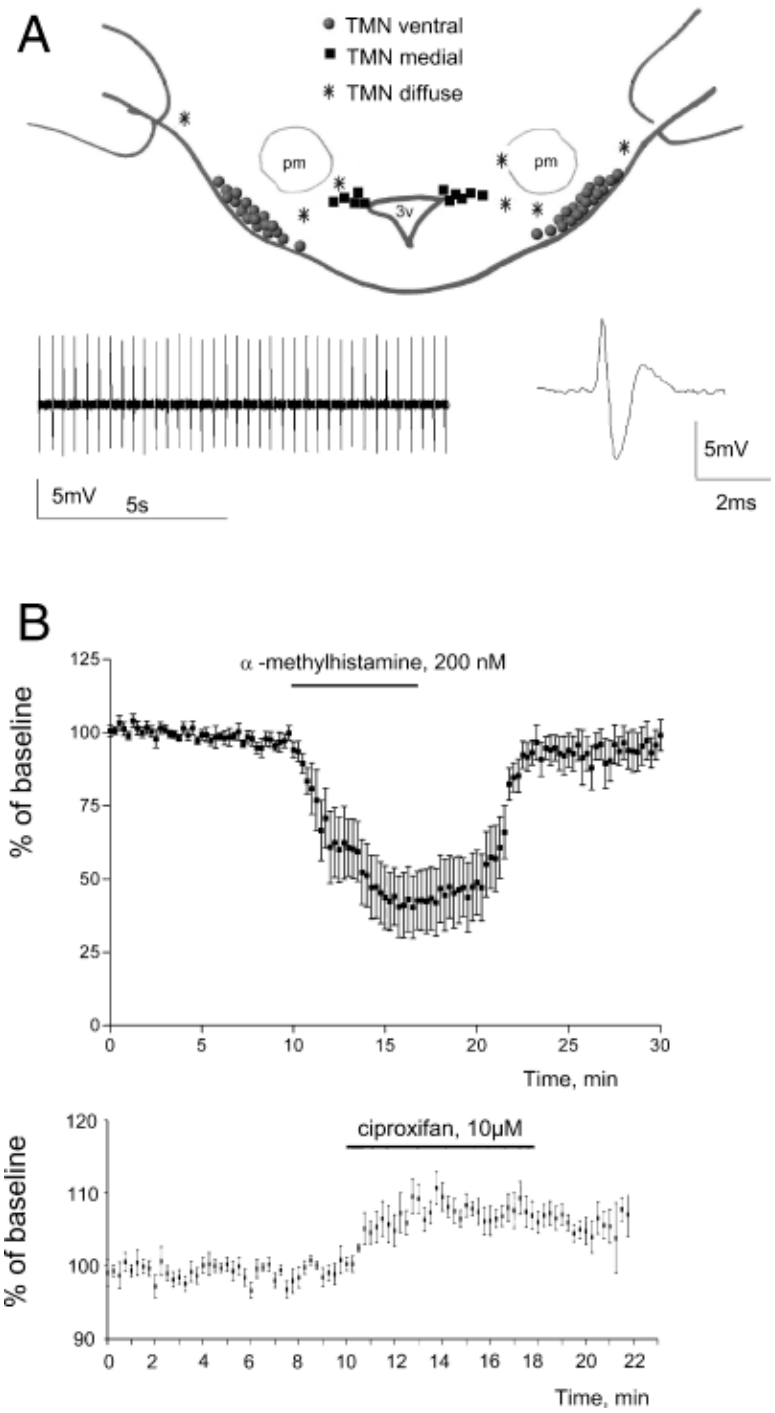

Fig. 1. Electrophysiological and pharmacological identification of tuberomamillary nucleus (TMN) neurons. (A) Schematic drawing of the coronal hypothalamic slice containing the TMN: pm, premamillary nucleus; 3V, third ventricle (top). Example of typical extracellular recording from ventral TMN neuron and typical shape of action potential recorded with an extracellular electrode (bottom). (B) Examples of averaged time course diagrams of firing rates in control and responses to α-methylhistamine (H3R agonist, n = 7) and ciproxifan (H3R antagonist, n = 9) application. α-Methylhistamine reduced the firing of TMN neurons by 59.6 ± 10.7% (n = 9), while ciproxifan enhanced the firing rate by 10 ± 3% of control level (n = 7). Firing rates are normalized to baseline (control, 100%) level.

Result

Extracellular recordings in slices

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 during intracellular recordings. For extracellular recording ventral TMN neurons (Fig. 1A) were selected on the basis of their location, regular firing in the range of 1–6 Hz (most typically 2–4 Hz) (Lin *et al.,* 1988; Haas & Panula, 2003) and a broad action potential (2–4 ms) (Stevens & Haas, 1996; Stevens *et al.,* 2001). In addition, pharmacological identification was used as the most reliable criterion: TMN neurons displayed an inhibition by the H3-receptor agonist α-methylhistamine and mostly an increased firing rate in the presence of H3-receptor (H3R) antagonists (Fig. 1B) (Lin *et al.,* 1988; Eriksson *et al.,* 2000; Haas & Panula, 2003). A total of 126 neurons (out of 134 recorded in 122 slices from the TMN) fulfilled these criteria and were taken for analysis. In the majority of the experiments α-methylhistamine was used for the pharmacological identification.

ATP (10 μM−1 mM) increased firing in all tested TMN neurons (n=26). The action of ATP was dose-dependent (Fig. 2): at 1 mM the firing of TMN neurons was increased by 123±5% (n=4), at 400 μM by 60±6% (n=4), at 50 and 100 μM by 33±2% (n=7). Responses to the latter two concentrations did not differ significantly in their amplitudes, therefore they were averaged as one group. Two $P2X_2$ receptor-type antagonists were tested. PPADS at 30 μM reduced the initial peak response to ATP (100 μM, Fig. 2C; measured at 1.5 min after applying drug to the bath) by 25% (from 133±2%, n=7 to 108±7%, n=4, P=0.01), but did not influence the later response (measured at 4.5 min in control and in the presence of antagonist). Responses were 118±3%, n=7 and 121±7%, n=4, respectively, P=0.59. Suramin (50–100 μM) did not affect the ATP (100 μM) responses (n=5, Fig. 2D). These experiments indicate that $P2X_2$ receptors (which are most sensitive to suramin and PPADS) are not the major driving force for the ATP-mediated excitation of TMN neurons. Cibacron blue (reactive blue 2, 100 μM) caused a slowly progressing inhibition of spontaneous firing, which was not reversing within a 40-min washout period. After 10 min preincubation with cibacron blue and in its continued presence, ATP (100 μM) increased the firing of TMN neurons by 40.7±6.7% in three neurons and was ineffective in four

others. The run-down of neuronal activity in the presence of the antagonist made the analysis of these experiments difficult. The several known side-effects of cibacron blue (see Discussion) may be responsible, but we cannot rule out the possibility of an excitatory tone through the P2Y receptors.

Next, we investigated the possibility that an inhibition of these neurons by adenosine (which derives from ATP through ectonucleotidases; Dunwiddie *et al.,* 1997; Braun *et al.,* 1998) can partially mask ATP responses. We applied adenosine (100 μM) and saw no effect on the neuronal firing in four experiments (Fig. 2). In nine TMN neurons recorded in slices with sharp electrodes (input resistance 202±93 MΩ, firing rate 2.6±0.8 Hz) adenosine added at 100–1000 μM for 10 min to the bath solution had no action either on the membrane potential and input resistance nor on the firing rate. In four of these experiments the blocker of adenosine deaminase EHNA (20 μM) was applied 10 min before and during adenosine (200 μM) application. Again, no effect of adenosine was observed. EHNA had no effect by itself. In two TMN neurons held at −70 mV sIPSPs were recorded. Neither adenosine (200 μM) alone nor in the presence of EHNA affected the frequency or amplitude of sIPSPs.

Several agonists of metabotropic P2Y receptors were investigated in the next experimental session in order to get indications which receptor types are involved in the ATP action. ADP (1 mM, n=4) and 2meSATP (1–100 μM, n=11) activated all investigated neurons, although with a smaller response magnitude than for ATP (1 mM; Fig. 3). UTP activated the majority of TMN neurons (13 out of 16 cells), in some cases more in some less effectively than ATP [at 1 mM it increased the firing by 109±46% over the baseline (n=6), at 100 μM by 57±46% (n=4)]. Three neurons did not respond to UTP, but were excited by ATP. In order to test whether ATP and UTP acted through the same receptor type, we used sequential and combined application of these two substances. The rapid decrease of the maximal ATP response amplitude during continuous presence of the agonist could be attributed either to desensitization or to the degradation of ATP by ectonucleotidases (Dunwiddie *et al.,* 1997; Braun *et al.,* 1998). A repeated application of ATP during the decaying response (doubling the concentration) resulted in a reduced response (84.3±6.8% of the first peak, n=3, Fig. 3D), indicating that desensitization rather then ATP degradation is responsible. When UTP at 1 mM was given at the same phase of

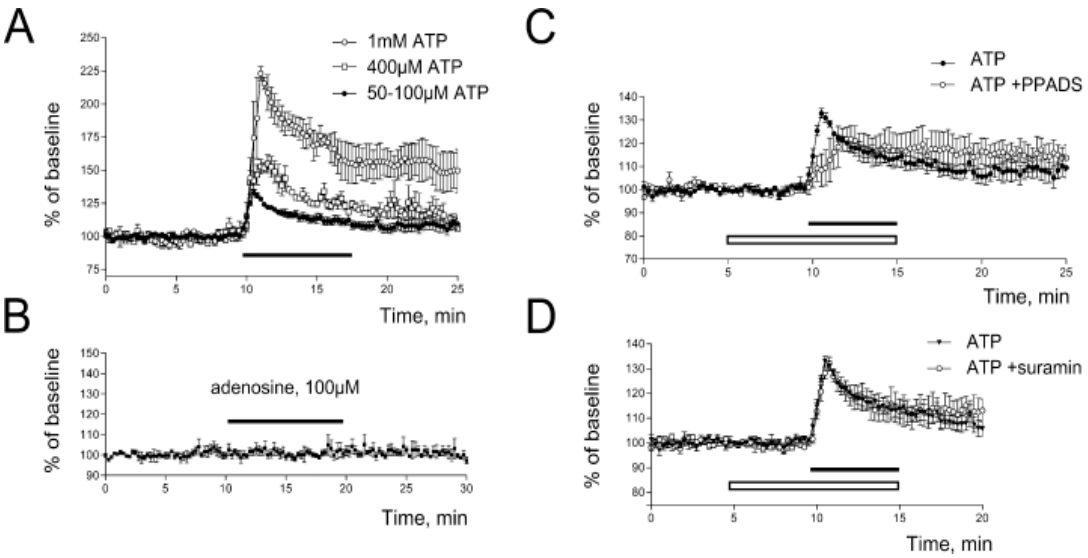

Fig. 2. ATP dose-dependently activates TMN neurons while adenosine has no effect. (A) Averaged responses to different ATP concentrations. (B) Lack of effect of adenosine on firing of TMN neurons. (C) P2X2 receptor antagonist PPADS (30 μM) significantly inhibits fast excitation by ATP, but does not affect the later phase of the response. (D) Suramin (100 μM) does not affect ATP-mediated excitation.

the initial ATP response, the second (UTP) peak was 12.9±3.8% (n=5) higher than the first (ATP-evoked) peak. The difference between second peaks, evoked either by ATP or by UTP, was significant (P=0.037).

Next, we applied first a maximal dose of UTP (1 mM) and then UTP in combination with ATP (1 mM), which, in contrast to UTP, activates multiple P2Y and P2X receptors (Fig. 3E). The second (ATP) peak represented 138.4±6.6% (n=8) of the first (UTP) peak (taken in Fig. 3D as 100%), indicating activation of more than only one ($P2Y_4$) receptor by ATP. It is known that UTP activates $P2Y_2$, $P2Y_4$ and $P2Y_6$, but not $P2Y_1$ receptors in the rat (von Kugelgen & Wetter, 2000). Thus, these experiments indicated the involvement of more than one receptor type in the excitation by ATP. Further experiments were aimed at identification of these different receptors and to determine their pharmacological properties. P2Y receptor-mediated responses in TMN neurons were not observed at room temperature (Fig. 3F). Neither ATP (n=4, 100 μM) nor ADP (n=3, 100 μM) or UTP (n=3, 100 μM) caused a change in firing rate in TMN neurons at room temperature.

Receptor expression at the single-cell level

scRT-PCR (n=23) revealed the presence of $P2Y_1$ and $P2Y_4$ mRNAs in individual TMN neurons (Fig. 4A), identified on the basis of HDC expression. Four neurons were P2Y-receptor-negative. In six neurons $P2Y_1$ and $P2Y_4$ cDNAs were detected, in five cells only $P2Y_1$, and in eight cells only $P2Y_4$ mRNAs were detected. The mRNA encoding for the $P2Y_2$ and $P2Y_6$ receptors was not detected in the neurons, but it was amplified from the positive control (total mRNA isolated from the TMN). The mRNAs encoding for the $P2Y_{12}$ and $P2Y_{13}$ receptors were detected in 21% and 14% of neurons (n=14). Thus, $P2Y_4$ and $P2Y_1$ receptors were expressed most frequently (corresponding mRNAs were detected in 74% and 58% of P2Y-positive TMN neurons, n=19). We noticed that in neurons isolated from the younger rats (10–14 days old) $P2Y_1$ mRNA occurred at a higher frequency (seven out of eight neurons) than in neurons isolated from the older rats (3–4 weeks old) (four neurons out of 15) (P=0.008, Fisher's exact probability test). The detection frequency of $P2Y_4$ receptor mRNA did not differ between neurons isolated from younger (five out of eight cells) and older rats (nine out of 15 neurons) (P=0.343, Fisher's exact probability test).

Real-time RT-PCR analysis of P2Y receptor expression

We investigated the relative abundance of mRNAs encoding for the $P2Y_1$ and $P2Y_4$ receptors at different developmental stages with real-time RT-PCR (Fig. 4B). All data points were normalized on the probe obtained from a 4-week-old rat. Downregulation of mRNA levels for the $P2Y_1$: 4.8±0.59 (P0=postnatal day 0), 2.13±0.37 (P7), 1.98±0.41 (P12) and 1.14±0.22 (P28); and for the $P2Y_4$ receptor: 5.6±1 (P0), 3.5±0.7 (P7), 2.58±0.35 (P12), 0.93±0.11 (P28) was observed (six–nine data points in each case obtained as averages in different amplifications; total mRNA isolated from the posterior hypothalamus from three rats for each age was used as a template).

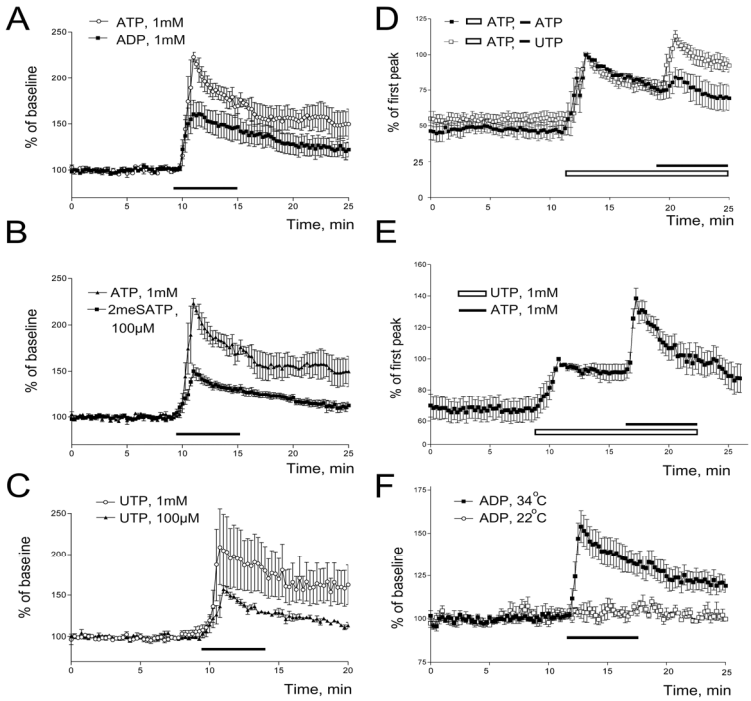

FIG.3. Effects of different nucleotides on TMN neuron firing. Responses are given in comparison with ATP (1 mM) responses (A–C). (D) Double-application experiments demonstrate that run-down of the ATP (1 mM) peak response is largely attributable to receptor desensitization, as the second ATP application elicits a much smaller response. All values are normalized on the first firing frequency peak. Application of a different agonist during response run-down (UTP, 1 mM) induces a larger enhancement than ATP itself. (E) Effects of UTP and ATP are additive, indicating involvement of different nucleotide receptors. (F) ADP increases the firing rate in TMN neurons at 34 °C, but not at room temperature (22°C).

$P2Y_1$ receptor expression coincides with higher potency of 2meSATP compared with ATP

As we found a higher expression of $P2Y_1$ with real-time PCR and a more frequent occurrence with scRT-PCR in juvenile rats we determined threshold concentrations of ATP and 2meSATP (an agonist more potent than ATP at the $P2Y_1$ receptor) also in 10–14-day-old rats. 2meSATP changed the firing frequency by 40.8±9.2% (n=4) already at concentrations of 0.25–1 μM, whereas ATP was active starting at 5–10 μM, increasing firing of TMN neurons by 19.5±1.5% (n=5) over the baseline (Fig. 4C).

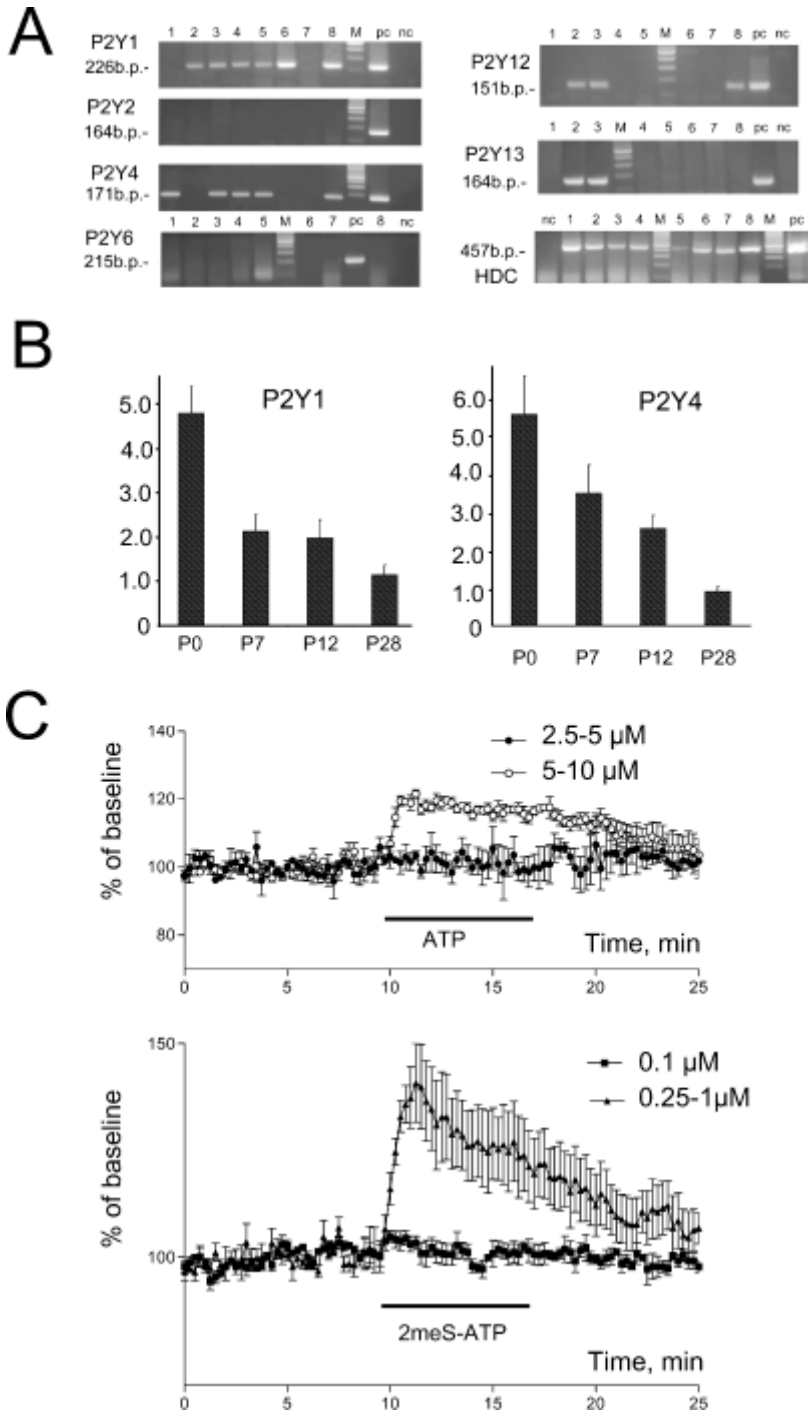

Fig. 4. Expression of P2Y receptors in TMN neurons. (A) Agarose gel-photographs illustrating the expression of six different P2Y receptors in eight TMN neurons identified by the presence of histidine decarboxylase (HDC) mRNA (M-DNA size marker, 100 bp ladder, Promega; pc, positive control: TMN whole, nc, negative control). (B) Real-time RT-PCR data illustrate developmental downregulation of P2Y1 and P2Y4 receptor mRNAs in rats of different ages (P12 j postnatal day 12). (C) Averaged time course diagrams for the effects of threshold concentrations of ATP (n j 5 neurons) and 2meSATP (n j 7 neurons), recorded in slices of 7–10-day-old rats. Action of concentrations at or below threshold is illustrated for comparison.

$P2Y_1$ receptor-mediated responses in TMN neurons are largely mediated through glutamate

Selective antagonists for the different types of P2Y receptors are needed for the understanding of their different physiological functions. The only available selective antagonist MRS2179 is directed towards $P2Y_1$ receptors, whose presence in TMN neurons we have demonstrated. We tested MRS2179 on ATP as well as ADP and 2meSATP responses. Surprisingly, even for the two latter selective agonists at P2Y1 receptors, the antagonism was weak, significant only for 2meSATP (Fig. 5A–C). The peak response to ATP (100 μM) in control and in the presence of MRS2179 represented 132.8±2.7% (n=5) and 130.2±12.2% (n=3) of the baseline firing, respectively (P=0.55). The peak firing rate with ADP (100 μM) and ADP in the presence of this antagonist represented 153.9±9.2% (n=8) and 137.9±1.6% (n=3) of the control level (P=0.745). MRS2179 (30 μM) significantly decreased (but did not abolish) the response to 2meSATP (100 μM): the firing rate was increased to 152±6.4% (n=9) in control vs. 133±3.9% (n=6) in the presence of the antagonist (P=0.02, Fig. 5).

Several recent studies demonstrated that activation of astrocytes causes release of glutamate or ATP and concomitant activation of neurons located in the close neighbourhood (Parpura & Haydon, 2000; Newman, 2003; Bowser & Khakh, 2004; Liu *et al.,* 2004). Metabotropic glutamate receptors of the type1 contribute to the neuronal responses to ADP through P2Y1 receptors in hippocampal interneurons due to a glia–neuron interaction (Bowser & Khakh, 2004). Could the ATP action seen here be mediated through glial cells, releasing glutamate? We applied the AMPA receptor antagonist CNQX (20 μM) in four experiments 10 min before and 10 min together with ATP (100 μM): responses to ATP were not different from control (40±10% increased firing, n=4). Our scRT-PCR analysis revealed that both subtypes of group 1 mGluR are co-expressed in the majority of investigated TMN neurons (18 out of 23 HDC-positive neurons, 78%). In four cells only mGluR1 and in one cell none of the mGluR mRNAs was detected (Fig. 5G). Thus, the mRNA encoding for mGluR1 was most frequently detected in TMN neurons (96% of cells).

Co-application of MRS2179 with antagonists of mGluR1 and mGluR5, LY 367385 (20 μM) and MPEP (10 μM), respectively, abolished (n=4) the enhancement of the firing rate by ADP (100 μM, n=8, Fig. 5D). UTP (100 μM) responses were not different from

control upon application of the same mixture of antagonists (Fig. 5E), representing 150.3±7.5% of baseline activity (n=6) in control and 134.2±9.2% (n=3) in the presence of antagonists (P=0.276).

P2Y12 and P2Y13 receptors cDNAs were detected in a small fraction of TMN neurons (scRT-PCR data). 2meSAMP has been reported to block the P2Y12 and P2Y13 receptor-mediated ADP action (Takasaki *et al.,* 2001; Marteau *et al.,* 2003). 2meSAMP applied to the TMN neurons enhanced the firing rate in seven TMN neurons by 23.6±3.7%. This excitatory action had different kinetics compared with the P2Y receptor-mediated effects and could not be attributed to the blockade of potassium channels (tested in patch-clamp whole-cell recordings on six acutely isolated TMN neurons), a possible action in the light of the structural similarity of the 2meSAMP triethylammonium to tetraethylammonium (Sergeeva, unpublished observation). The ADP (100 μM) response was not blunted by 2meSAMP preapplication (Fig. 5F). The ADP peak response represented 139.3±2.4% of baseline in the presence of 2meSAMP vs. 153.9±9.2% (n=8) in control experiments (P=0.92).

Immunohistochemistry

In slices from young rats (0, 7 and 14 days old, one rat per age) $P2Y_1$ staining was not very clear, due to the high neuropile or glia staining (not shown). On the other hand, cultured neurons and glia gave a clear immunoreaction towards the P2Y1 antibody (Fig. 6A and B). Surface and nuclear expression of the $P2Y_1$ protein was visualized in glial cells grown in the marginal zone of hypothalamic cultures. Immunohistochemistry with the anti-mGluR1α antibody revealed a neuronal localization of these receptors in posterior hypothalamic cultures (Fig. 6D).

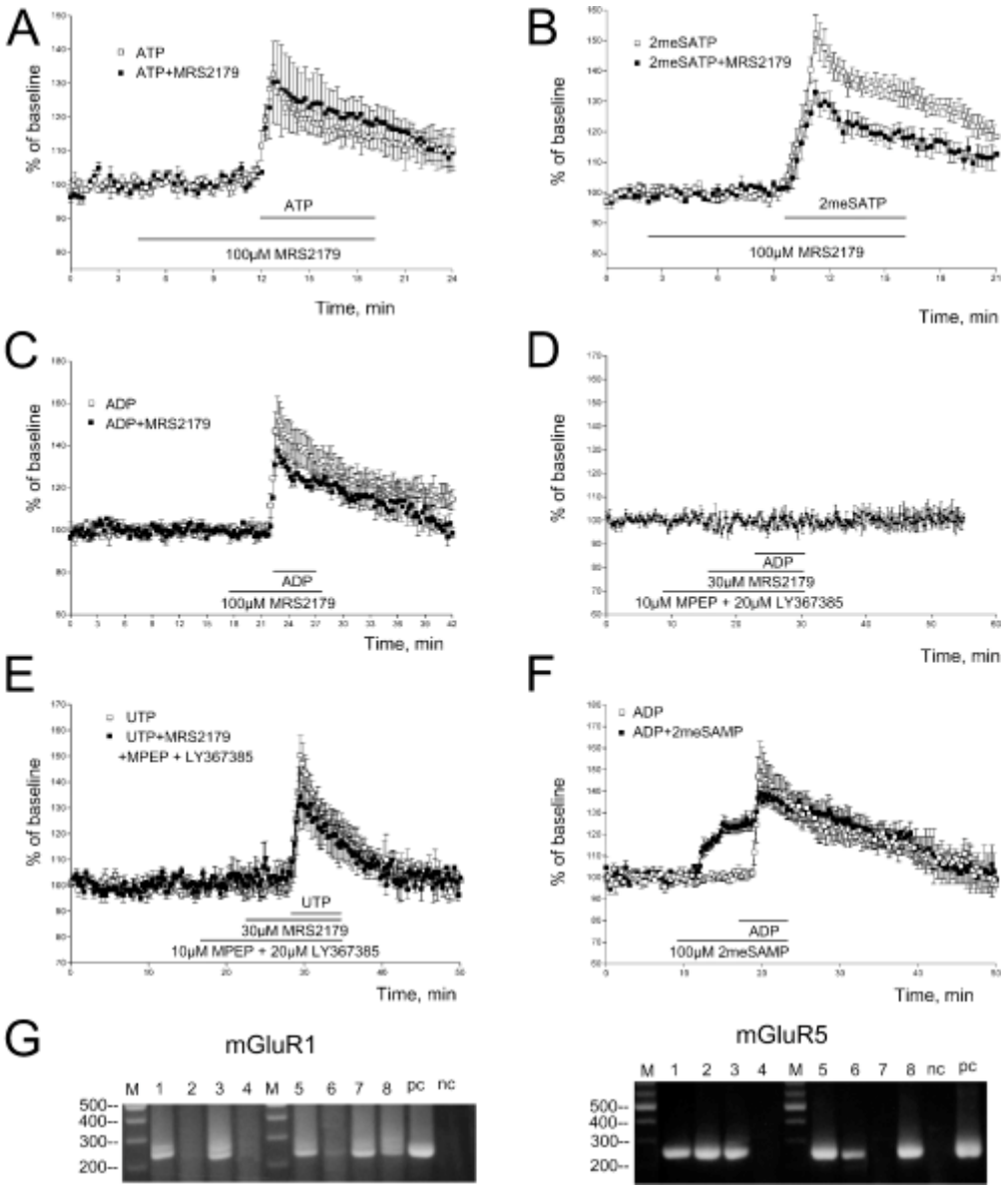

Fig. 5. P2Y1 receptor-mediated responses are abolished by co-application of MRS2179 and mGluR1 antagonists, whereas the P2Y12／13 receptor antagonist 2meSAMP is ineffective. (A–C) Averaged superimposed time course diagrams of firing rates in control experiments (application of ATP, 100 lm, n j 7; 2meSATP, 10–100 lm, n j 9; and ADP, 0.1–1 mm, n j 8) and in the presence of P2Y1 receptor antagonist (three, six and three experiments in A–C, respectively). (D) Mixture of P2Y1 receptor and mGluR1 and mGluR5 antagonists abolishes ADP responses in TMN neurons. (E) UTP (100 lm) responses in control (n j 6) and in the presence of P2Y1 receptor and mGluR antagonists are not different (n j 3). (F) Preapplication of the P2Y12 and P2Y13 receptor antagonist 2meSAMP does not blunt the ADP response (n j 4). (G) Agarose gel-photographs illustrating the expression of mGluR1 and mGluR5 receptors in eight identified TMN neurons. DNA size marker (M) lines in base pairs (bp) are given at the left side of the gels.

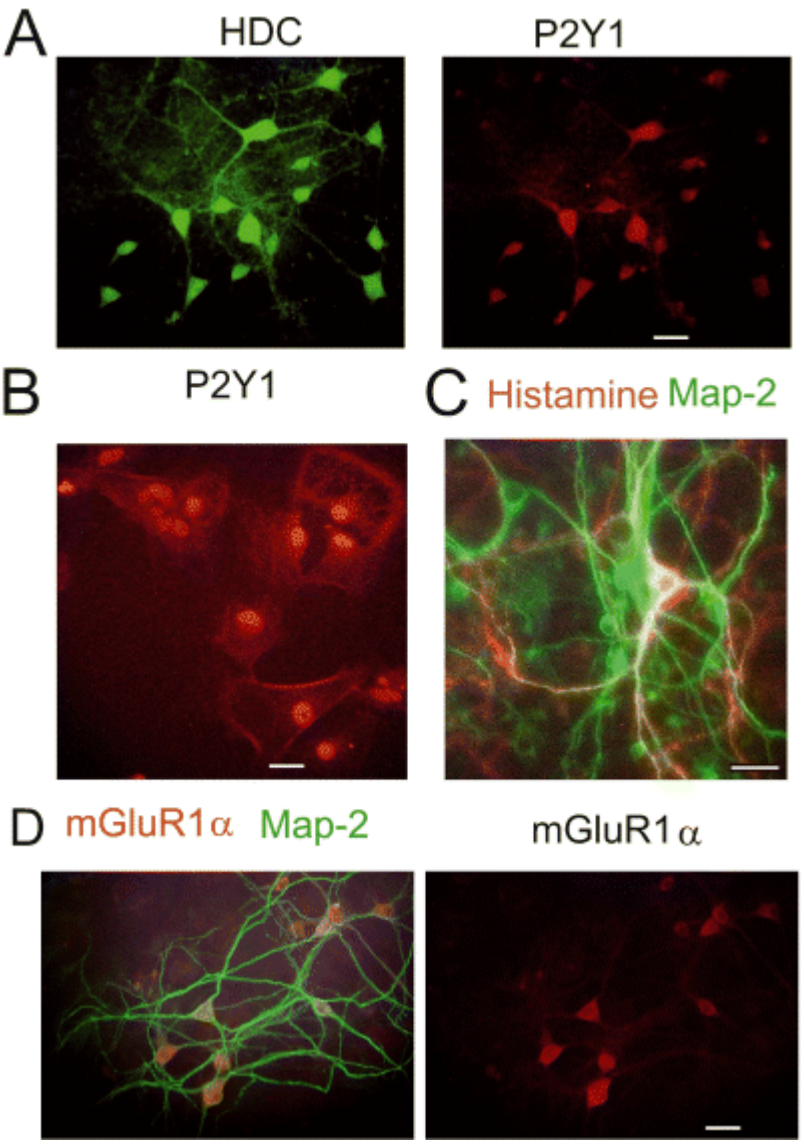

Fig. 6. Co-localization of P2Y1 and histidine decarboxylase (HDC) immunoreactivity in cultured neurons. (A) Double staining for HDC (green) and P2Y1 (red) demonstrates expression of the P2Y1 protein in cultured histaminergic neurons. (B) Surface and nuclear expression of the P2Y1 receptor protein in glial cells in the marginal zone of the same primary hypothalamic cultures. (C) Double staining for the mouse microtubule-associated protein-2 (MAP-2, green, neuronal marker) and rabbit-anti-histamine (red) antibodies in hypothalamic cultures. Scale bar: 20 lm in all images. (D) Double staining for the mouse MAP-2 (green) and rabbit anti-mGluR1a antibodies in hypothalamic cultures demonstrate neuronal localization (mainly somatic) of the mGluR1 protein.

MEA recordings

The proportion of histamine to non-histamine cells in MEA recordings was determined by the application of α-methylhistamine or ciproxifan to the cultural dish.

About 14% of the active array-electrodes from seven MEAs (30 out of 239) recorded either increased neuronal firing with the H3 receptor antagonist or decreased firing with the H3 agonist α-methylhistamine. Stainings of parallel-cultures to MEAs with the MAP-2 and rabbit-anti-histamine antibodies revealed that 7.2% of the total number of neurons, 21 out of 311 neurons, counted in 100-μm^2 fields, one per coverslip in 20 selected, were histaminergic (Fig. 6C). These data are generally in keeping with the numbers obtained from pharmacological identification of cultured TMN neurons on MEAs.

ATP (100μM−1mM) decreased posterior hypothalamus network activity recorded from MEAs to 68.7±8.5% from control (n=9; whole MEA activity 1680±424 spikes/min; Fig. 7). The adenosine receptor A1 antagonist DPCPX (1 μM) had no influence on the ATP-mediated inhibition (n=3), nor had it an action on its own (n=3). On the contrary, in striatal cultures grown on MEAs the inhibitory action of ATP was reversed partially or completely by the same dose of DPCPX (Sergeeva, unpublished observation). Furthermore, adenosine (100 μM) had an inhibitory action on the striatal network activity (unpublished observation), but not on posterior hypothalamic networks. In the presence of adenosine (100 μM) the firing rate represented 102±7% of control (n=4, control whole MEA activity 1740±690 spikes/min) in posterior hypothalamic networks. Suramin ($P2X_2$ antagonist at lower doses) at 10–20 μM enhanced network activity by 19±4.6% (n=8, control whole MEA activity 1260±188 spikes/min). When applied to the cultured neurons together with ATP (100 μM) in the same concentration, suramin reversed the inhibitory action of ATP to an excitatory one: in six such experiments the activity reached 154.5±15% of control (control whole MEA activity 1874±485 spikes/min). At higher doses suramin (100 μM) inhibited network activity to 66±10.2% (n=5, whole MEA activity 1534±317 spikes/min). Experiments with suramin revealed a dual action of endogenous ATP in hypothalamic neuronal circuitries.

The inhibitory action of ATP was reversed to an excitatory one in the presence of a $GABA_A$ receptor antagonist: in the presence of gabazine (10 μM), 100 μM ATP increased the firing rate by 76.3±11.4% (n=6, control whole MEA activity 1432±253 spikes/min) over the baseline level. Despite the reduction of whole MEA activity by ATP, some channels, presumably recording GABAergic neurons, showed increased activity.

UTP (100 μM) enhanced network activity by 45.2±14.4% (n=5, whole MEA activity 1720±397 spikes/min). In the presence of 100 μM 2meSATP the firing rate was increased by 25±9% (n=5, control firing rate 1792±315 spikes/min), and in the presence of 1 μM 2meSATP the firing rate was increased by 20±15% (n=4, control whole MEA activity 1509±529 spikes/min). Thus, the posterior hypothalamus network activity is increased by agonists at $P2Y_1$ and $P2Y_4$ receptors, while ATP has an apparent inhibitory action, most likely due to the preferential action on GABAergic neurons, presumably through $P2X_2$ receptors that are most sensitive to suramin.

Discussion

The TMN plays a central role in the maintenance of wakefulness and attention. Our findings elucidate the influence of ATP and adenosine, opposite markers of energy

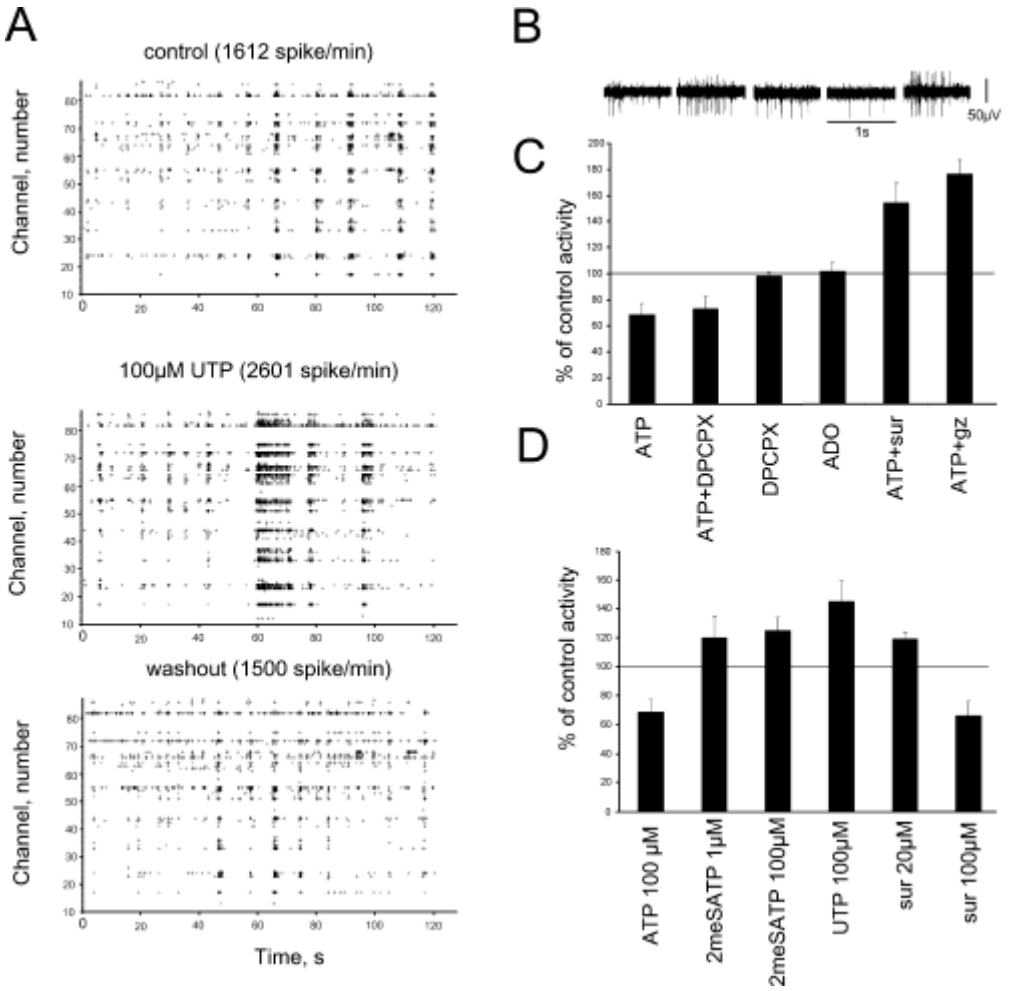

Fig. 7 MEA recordings demonstrate excitatory action of UTP and 2meSATP as well as a dual action of ATP on posterior hypothalamus network activity. (A) Example of spike raster plots illustrating control activity and activity in the presence of UTP. (B) Examples of simultaneous recordings from five channels, illustrating typical signal / noise ratios and activity patterns recorded at single electrodes. (C and D) Averaged data are represented as histograms, showing effects of different drugs on total firing rates (whole MEA spikes/min). ADO, adenosine; gz, gabazine; sur, suramin.

neuronal network activity was reported for the hippocampal stratum radiatum (Bowser & Khakh, 2004). We report now for the posterior hypothalamic network an excitatory effect of $P2Y_1$ and $P2Y_4$ receptor agonists. Surprisingly, ATP inhibited the hypothalamic neuronal network. In contrast to forebrain structures this action was not dependent on ATP degradation yielding adenosine formation, as adenosine and the A1 receptor antagonist DPCPX had no action here. This inhibition most likely depends on $P2X_2$ receptor-mediated activation of GABAergic neurons, as it was reversed by low concentrations of suramin (which is rather selective for the $P2X_2$ receptor) and by the $GABA_A$ receptor antagonist gabazine. ATP and GABA can be co-released at inhibitory synapses in the lateral posterior hypothalamus (Jo & Role, 2002): spontaneous and evoked IPSCs could be partially blocked by suramin in this study.

In TMN neurons recorded in slices, effects of ATP were always excitatory, presumably due to the lack of inhibitory inputs to these cells in slices: application of bicuculline ($GABA_A$ receptor antagonist) does not change the firing of TMN neurons (our unpublished observation). The presence of endogenous ATP and different types of ATP-sensing receptors in hypothalamic cultures was evident from experiments with suramin, which excited neurons at lower and inhibited them at higher concentrations. The lack of suramin action on either basal activity or ATP responses in slices can be explained by the inactive inhibitory inputs to the TMN neurons and by the insensitivity to this antagonist of $P2Y_4$ receptors (von Kugelgen & Wetter, 2000), which prevail in young adult rats used in these experiments. Yet another antagonist, PPADS, is also inactive towards $P2Y_4$ receptors (von Kugelgen & Wetter, 2000), while it blocks $P2Y_1$ receptors. Peak ATP responses were significantly different in control and in the presence of PPADS, demonstrating the presence of PPADS-sensitive and PPADS-resistant receptor populations. Cibacron blue (Reactive blue 2) is an effective antagonist at $P2Y_4$ and $P2Y_1$ receptors, while at other P2Y receptor types it shows low activity (von Kugelgen & Wetter, 2000). In four experiments we saw no action of ATP in the presence of this antagonist. This compound displayed additional (side-) effects on neuronal activity in our slice and MEA recordings: a slow and irreversible inhibition of firing. It is not clear whether this was related to P2Y receptors or other actions, such as inhibition of ATPase activity (Barret *et al.,* 1993), stimulation of

mitochondrial Ca^{2+} uptake (Schoff, 1995), inhibition of adenine nucleotide translocase and mitochondrial inner membrane anion channels (Beavis, 1992).

ATP, UTP and ADP did not activate TMN neurons at room temperature, indicating the involvement of a temperature-dependent effector. One possibility is a $Na+/Ca^{2+}$ exchanger downstream to the P2Y receptor activation. We have previously shown that this electrogenic exchange provides the major excitatory drive to TMN neurons following serotonin 2C and orexin-receptor activation (Eriksson *et al.,* 2001, 2002; Sergeeva *et al.,* 2003).

The $P2Y_1$ antagonist MRS2179 did not change significantly responses to ATP and ADP, the response to 2meSATP was incompletely blocked. Co-application of this antagonist with two specific mGluR1 and mGluR5 antagonists demonstrated that the response to $P2Y_1$ agonists is due to a surge in extracellular glutamate, most likely secondary to glial activation. A similar experimental paradigm was applied in a previous study by Bowser & Khakh (2004) in order to block $P2Y_1$ receptor-mediated responses in hippocampal interneurons. It remains to be elucidated why some $P2Y_1$ receptors are not sensitive to MRS2179. One possibility is a major role of intracellular glial $P2Y_1$ receptors in the ADP action, which are activated after uptake of nucleotides into the cell. Our immunostainings of glial cells (Fig. 6) demonstrate indeed a strong nuclear signal for the $P2Y_1$ receptor protein. Our hypothesis of intracellular localization of (glial) ADP receptors is further supported by the lack of prominent actions of suramin and PPADS (two highly potent $P2Y_1$ antagonists, which cannot permeate the cellular membrane due to their polar nature; von Kugelgen & Wetter, 2000). $P2Y_{12}$ and $P2Y_{13}$ receptors, which are known to be resistant to MRS2179, are less probable candidates for the ADP-mediated responses in TMN neurons: first, they were only occasionally detected in TMN neurons with scRT-PCR; second, the antagonist of these receptors, 2meSAMP, did not blunt the ADP response. At the present state we cannot exclude the involvement of a yet unknown type of glial ADP receptor in glia–neuron communication.

A developmental downregulation of $P2Y_1$ receptors was reported previously for hippocampal astrocytes (Zhu & Kimelberg, 2001). The early postnatal peak in $P2Y_1$ and $P2Y_4$ receptor expression in hypothalamic neurons reported in the present study may indicate an involvement of these receptors in neuronal migration or differentiation. In

conclusion, we demonstrate a $P2Y_1$ and $P2Y_4$ receptor-mediated excitation of TMN neurons in hypothalamic slices and cultures by nucleotides, while adenosine does not affect these neurons. Our findings bear relevance for the understanding of sleep–wake regulation, the control of arousal and attention, and their link to energy homeostasis.

Acknowledgments

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Abbreviations

ACSF, artificial cerebrospinal fluid; GABA, γ-aminobutyric acid; HDC, histidine decarboxylase; IPSC, inhibitory postsynaptic current; IPSP, inhibitory postsynaptic potential; MAP-2, microtubule-associated protein-2; MEA, multielectrode array; mGluR, metabotropic glutamate receptor; PB, phosphate buffer; PBS, phosphate-buffered saline; PBS-T, PBS with 0.25% Triton X-100; scRT-PCR, single-cell reverse transcriptase-polymerase chain reaction; TMN, tuberomamillary nucleus.

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Modafinil inhibits rat midbrain dopaminergic neurons through D2-like receptors

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Abstract

Modafinil is a well-tolerated medication for excessive sleepiness, attention-deficit disorder, cocaine dependence and as an adjunct to antidepressants with low propensity for abuse. We investigated the modafinil action on identified dopaminergic and GABAergic neurons in the ventral tegmental area (VTA) and substantia nigra (SN) of rat brain slices. Modafinil (20 μM) inhibited the firing of dopaminergic, but not GABAergic neurons. This inhibition was maintained in the presence of tetrodotoxin and was accompanied by hyperpolarization. Sulpiride (10μM), a D2-receptor antagonist, but not prazosine (20μM, an ά1-adrenoreceptor blocker) abolished the modafinil action. Inhibition of dopamine reuptake with a low dose of nomifensine (1μM) reduced the firing of DA neurons in a sulpiride-dependent manner and blunted the effect of modafinil. On acutely isolated neurons, modafinil evoked D2-receptor-mediated outward currents in tyrosine-hydroxylase positive cells, identified by single-cell RT-PCR, which reversed polarity near the K^+ equilibrium potential and were unchanged in the presence of nomifensine. Thus modafinil directly inhibits DA neurons through D2 receptors.

1. Introduction

Modafinil [(diphenyl-methyl)-sulfinyl-2-acetamide; Modiodal, Provigil] is a wake-promoting substance used for the treatment of excessive sleepiness associated with narcolepsy (Mignot and Nishino, 2005), multiple sclerosis (Kraft and Bowen, 2005) and Parkinson's disease (Nieves and Lang, 2002, Ferraro *et al*., 1998). Further therapeutic potential of modafinil includes attention-deficit disorder (Swanson *et al*., 2006), cocaine dependence and withdrawal (Vocci and Elkashef, 2005), postanesthetic sedation and depression (for review see Ballon and Feifel, 2006). The mechanism of action of modafinil is controversial. Early studies have indicated that modafinil acts different from established psychostimulants (Akaoka *et al*., 1991, Lin *et al*., 1992) and possesses only minimal potential for abuse (Scammell and Matheson, 1998, Jasinski, 2000, Deroche-Gamonet *et al*., 2002). Several hypotheses were suggested for the mechanism of modafinil action by in vivo and in vitro studies. Modafinil can increase serotonin release (Ferraro *et al*., 2000, Ferraro *et al*., 2002), decrease GABA release and enhance glutamate release in various brain regions (Ferraro *et al*., 1997, Ferraro *et al*., 1998, Ferraro *et al*., 1999), and activate orexin-containing hypothalamic neurons (Chemelli *et*

al., 1999). Studies employing pharmacological tools or genetic ablation of ά-1B-adrenoreceptors suggested that modafinil increases wakefulness by activating central noradrenergic transmission (Duteil *et al*., 1990, Lin *et al*., 1992, Stone *et al*., 2002). This hypothesis left several questions unresolved, mainly why modafinil does not affect the peripheral sympathetic system (Duteil *et al*., 1990) and why in narcoleptic patients modafinil effectively treats excessive daytime sleepiness but fails to prevent the loss of muscle tone that occurs during cataplexy. On the other hand, a number of studies indicate that modafinil interacts with the dopamine system. In an elegant study by Wisor and Eriksson (2005) pharmacological elimination of the noradrenaline transporter-bearing forebrain projections in mice did not influence the efficacy of modafinil action; at the same time evidence was obtained that dopamine-dependent adrenergic signaling is important for the wake-promoting action of modafinil. In another study Wisor *et al*. (2001) reported that deletion of the DA transporter (DAT) gene in mice causes a 20% increase in wakefulness; moreover wake -promoting effects of classical stimulants (such as amphetamine) and modafinil are abolished. Dopamine-specific reuptake blockers can promote wakefulness in normal and narcoleptic animals better than noradrenaline-selective blockers (Nishino and Mignot, 1997). The action of modafinil seems to be distinct from these drugs of abuse with respect to addiction potential (Nishino *et al*., 1998, Jasinski, 2000). In contrast to amphetamine, modafinil did not influence the firing pattern of DA neurons (VTA) of anesthetized rat (Akaoka *et al*., 1991). The action of amphetamine *in vivo* was attributed to enhanced catecholaminergic function and inhibition of DA neurons, while modafinil demanded $ά_1$- and β-adrenoreceptor activity (Lin *et al*., 1992). On the other hand, blockade of catecholamine synthesis with the tyrosine hydroxylase inhibitor ά-methyl-DL-p-tyrosine methyl ester (άMPT) abolished the amphetamine, but not the modafinil, action (Lin *et al*., 1992). This finding is difficult to interpret if the action of modafinil is restricted to the blockade of dopamine uptake (Wisor *et al*., 2001, Wisor and Eriksson, 2005). We investigated now the modafinil action on DA neurons recorded in brain slices in situ and in acute isolation. We demonstrate D2-receptor-mediated inhibition on VTA and SN dopaminergic, but not GABAergic neurons.

2. Materials and methods

2.1. Slice preparation

Coronal brain slices were prepared from 3–4-week-old male Wistar rats. All experiments were conducted in compliance with German law and with the approval of the Bezirksregierung Duesseldorf. The animals were quickly decapitated and the brain transferred to a modified artificial cerebrospinal fluid (ACSF), in which all NaCl had been replaced by 207 mM sucrose (Aghajanian and Rasmussen, 1989). 400-μm-thick slices were cut at the level of the rostral VTA (−5.4 to −6.0 mm from Bregma using a vibroslicer (Campden Instruments, UK)). After slicing they were placed into ACSF containing (in mM) 124 NaCl, 3.7 KCl, 25.6 $NaHCO_3$, 1.24 NaH_2PO_4, 2 $CaCl_2$, 1,3 $MgSO_4$, and 10 glucose, saturated with 95% O_2/5% CO_2 for ≥1 h at room temperature before being transferred to the recording chamber at 32°C, where they were constantly perfused with the same ACSF at a flow rate of 1 ml/min.

2.2. Slice electrophysiology

Extracellular recordings were obtained using glass microelectrodes filled with 2M NaCl (resistance 5 – 10 MΩ). Positioning of the electrodes was controlled through a dissecting microscope using the accessory optic tract as a marker, which is the border between the substantia nigra (SN) and VTA. Signals were recorded with an Axoclamp 2B amplifier (Axon Instruments, USA), filtered between 0.5 and 10 kHz, sampled at 20 kHz and analyzed with pClamp8 software (Axon Instruments). The frequency of extracellular action potentials was determined online in bins of 15 s duration. Drugs were bath applied. Neurons were recorded for at least 10 min at a stable baseline firing rate before application of drugs. Modafinil was applied in all cases for 7 min, time of application of all substances is indicated by bars in corresponding pictures.

Patch pipettes (3–6 MΩ) were pulled from borosilicate glass (GB 150F-8P, Science Products, Hofheim, Germany) and filled with an intracellular solution containing in mM: potassium gluconate 135, NaCl 5, $MgCl_2$ 2, HEPES 10, EGTA 0.1, Na_2ATP 2, NaGTP 0.5 (pH 7.25 with KOH ca. 5 mM, 275 mOsm). Whole-cell recordings were made by advancing the electrode “blindly” into the brain slice (i.e. without visual control) with positive pressure applied to the interior of the pipette via a side port in the electrode holder to keep it clear of

tissue. Intracellular signals were recorded using an Axoclamp 2B amplifier (Axon Instruments). Membrane potential measurements were adjusted for a −15 mV liquid junction potential between pipette solution and bath solution (calculated using pClamp8 Software, Axon Instruments). Series resistance varied from 6 to 40 MΩ—the bridge balance was periodically maintained and monitored during current-clamp experiments. Continuous recordings of membrane voltage were made using a Gould TA 550 chart recorder (Gould Electronics, Cleveland, OH, USA). Dopaminergic and GABAergic neurons differ in a number of characteristics which can be used to identify them, as previously described (Grace and Onn, 1989, Korotkova *et al*., 2005). Briefly, dopamine cells fired at lower frequencies in comparison with GABAergic neurons; spike width was broader in dopaminergic neurons than in GABAergic neurons; dopaminergic neurons recorded in whole-cell mode possessed a prominent Ih current and their spike thresholds were more positive in dopaminergic than in GABAergic cells. The study was made on drug-naive neurons. Pharmacological identification of neurons was performed at the end of the experiment: dopaminergic neurons were completely inhibited by the D2 receptor agonists quinpirole (10 μM) or dopamine (30 μM) whereas presumed GABAergic VTA neurons were unaffected by quinpirole or dopamine and inhibited by the μ-receptor agonist Tyr-d-Ala-Gly-NMe-Phe-Gly-ol (DAMGO, 1 μM).

2.3. Acute isolation of VTA neurons, whole-cell patch-clamp recordings and single-cell-RT–PCR

For preparation of isolated cells the VTA was dissected and incubated with papain in crude form (0.3–0.5 mg/ml) for 10 min at 37° C (for details see Korotkova *et al*., 2003). 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. The selected cell was digitally photographed on an inverted microscope and then approached with the micropipette (resistances between 2 and 5 MΩ after filling with the intracellular solution, see above). In a few experiments CsCl-based intracellular solution was used (concentrations are given in mM): 140 CsCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES/CsOH, adjusted to pH 7.2. After establishment of the whole-cell configuration, the cell was voltage-clamped using an EPC-9 amplifier at −60 mV (all membrane potentials given through the paper represent the corrected

values for the liquid junction potential) and viability of the neuron was tested by applying a depolarizing current step of +60 mV for 15 ms. If a prominent sodium-current was observed, the cell was lifted into the application system, where it was continuously perfused with a sterile control bath solution. All solutions flowed continuously, gravity-driven, at the same speed and lateral movements of the capillaries exposed a cell either to control or test solutions (for more details see Sergeeva *et al*., 2002). Data were collected with commercially available software (TIDA for Windows, HEKA, Lambrecht, Germany). At the end of whole-cell recording, the cell was sucked into the patch pipette and subjected to reverse transcription, followed by the PCR amplification of TH-, GAD65- and GAD67-cDNAs, according to the previously published protocols (Sergeeva *et al*., 2002, Korotkova *et al*., 2003).

2.4. Drugs and statistical analysis

Dopamine hydrochloride and nomifensine maleate salt were obtained from Sigma/RBI (Deisenhofen, Germany). Prazosine hydrochloride, (−)-quinpirole hydrochloride, (RS)-(±)-sulpiride and DAMGO came from Biotrend (Koeln, Germany) and modafinil from Laboratoire L. Lafon (France). Statistical analysis was performed with the non-parametric Mann–Whitney U-test. Averaged values of maximal inhibition by modafinil measured within a 5 min period were taken for the group comparison. Significance level was set at $p < 0.05$. Data are presented as mean±standard error of the mean (S.E.M.).

3. Results

Bath application of modafinil (20 μM) inhibited all dopaminergic neurons to 68.6±5.1% of control in VTA (n=7) and to 61.0±6.0% in SN (n=3, Fig. 1A,C). Effects of modafinil on DA neurons from both neuronal groups were similar. Bath application of modafinil (20 and 50 μM) did not affect the membrane potential or firing rate of GABAergic VTA neurons (n=6). The inhibitory action of modafinil on midbrain DA neurons resembled that of amphetamine-like psychostimulants, in keeping with recent results on reinforcing properties of modafinil depending on behavioral demands (Stoops *et al*., 2005, Kruszewski, 2006).

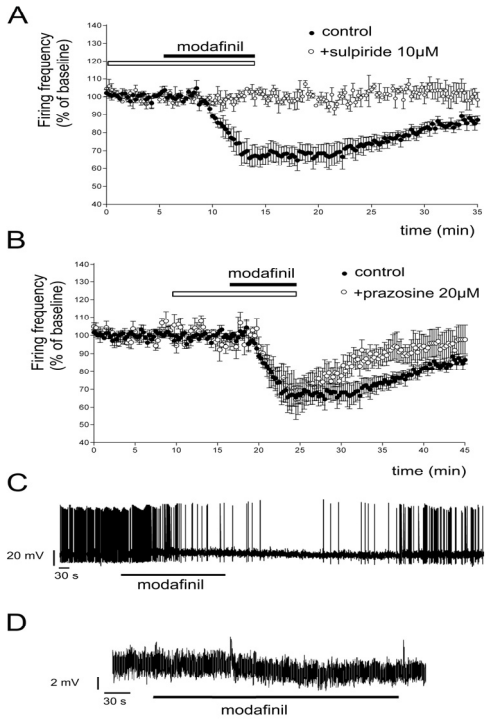

Fig. 1. Bath application of modafinil (20 μM, black bars) inhibits dopaminergic neurons in VTA and SN. (A) Action of modafinil is abolished by sulpiride (time of application of the antagonist is indicated by the open bar). (B) Prazosine does not block the modafinil response. (C) Example of extracellular recording of DA neuron firing illustrating action of modafinil. (D) Modafinil evokes hyperpolarization of DA neuron recorded in TTX (0.5 μM) in current clamp mode. Membrane potential of recorded cell was −50 mV.

The reduction of firing rate was abolished in the presence of 10 μM of sulpiride (antagonist of D2-like receptors, n=5) (Fig. 1A). As dopamine and serotonin re-uptake systems

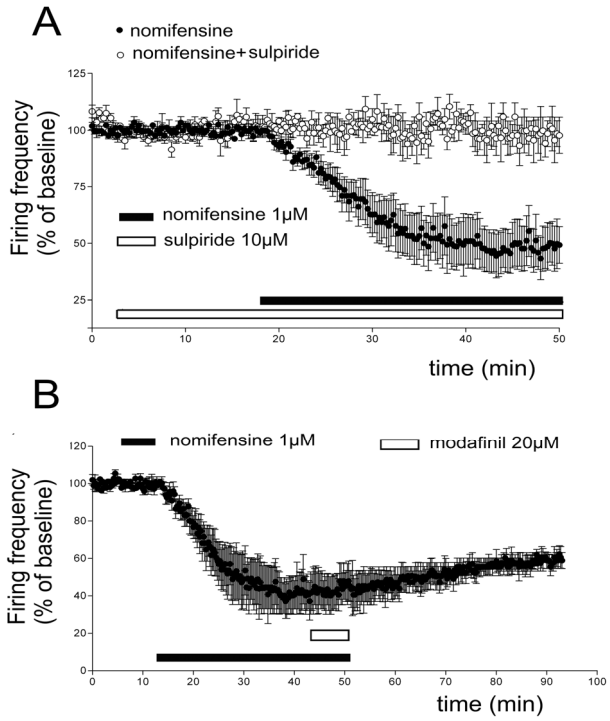

Fig. 2. Dopamine reuptake blockade interfere with modafinil action. (A) Dopamine reuptake blocker nomifensine inhibits firing of DA neurons in sulpiride-dependent manner. (B) Action of modafinil is blunted by nomifensine preapplication.

The reduction in firing rate in DA neurons was accompanied by hyperpolarization measured with whole-cell patch-clamp recordings in slices (2.8±0.3 mV, n=5); the prior application of the voltage-gated sodium channel blocker tetrodotoxin (0.5 μM) did not prevent the hyperpolarization caused by modafinil, indicating a direct postsynaptic effect (n=3, Fig. 1D). As modafinil exhibits a weak affinity for the dopamine transporter (DAT) (Mignot *et al*., 1994, Wisor *et al*., 2001), we tested whether the selective dopamine reuptake inhibitor nomifensine (1 μM) can blunt the effect of modafinil on DA neuron firing. Nomifensine reduced the firing rate of DA neurons by 30±6% (n=3, Fig. 2A) alone but had no action in the presence of sulpiride (n=4). Modafinil, when applied after 20 min of preincubation with nomifensine (steady-state inhibition was achieved at this time point), did not cause further (additional) inhibition of firing of DA neurons (n=4), indicating that blockade of dopamine reuptake or a direct action of modafinil at D2-like receptors are possible sites of modafinil action. D2 receptors undergo desensitization in the presence of nomifensine due to the accumulation of dopamine in extracellular space.

Acutely isolated neurons from the VTA were identified after recording by single-cell RT–PCR analysis of TH and GAD65/67 expression as previously described (Korotkova *et al*., 2003). Successful amplification of TH yielded a PCR product of the expected size 220 bp in 17 out of 28 neurons (Fig. 3B). Products of GAD65-amplification (306 bp) were detected in 8 TH-negative neurons, in 4 neurons GAD65 was co-expressed with the GAD67 (PCR product of 502 bp size, Fig. 3B). DA neurons were bigger in size (major axes 20–26 μm, average 23±0.7 μm) compared to the GAD-positive cells (major axes below 20 μm, average 15±1, n=8). The half-width (HW) of the inward current in response to a depolarizing voltage step (60 mV) from the −60 mV holding potential was found to be significantly different (p=0.0008) between GAD− and GAD+ neurons: GAD-TH+ cells demonstrated broader inward current (HW: 2.76 ± 0.28 ms, n=8) in comparison to GAD-positive neurons (HW: 1.48±0.12 ms, n=8).

DA neurons, recorded at −60 to −70 mV holding potentials, responded to modafinil (20–50 μM) with an outward current of 11.4±2.4 pA amplitude (n=10) and to modafinil at 200 μM with an amplitude 29±8.9 pA (n=9). Due to the run-down of responses we did not apply modafinil more than three times in one neuron; in some cases these responses were measured at different holding potentials (see below). TH-negative neurons did not respond to modafinil (n=8) (Fig. 3). Responses to modafinil were blocked by sulpiride (10μM, n=3). The antagonist

was pre-applied during 20 s and then co-applied during 10 s with modafinil. The current–voltage relation of the modafinil-evoked currents showed an inward rectification with a reversal potential that approximately equaled the K^+ equilibrium potential (calculated value is −96 mV). Fitting data points located near the reversal potential with a linear regression function (Fig. 3D, dotted line on the current–voltage plot) gave −97.6 mV for the reversal potential. The characteristics of modafinil-evoked currents were similar to those in a previous study with D2-like receptor ligands (quinpirole and dopamine) on cultured substantia nigra neurons (Kim *et al*., 1995), where D2-receptors couple to inwardly rectify K^+ channels.

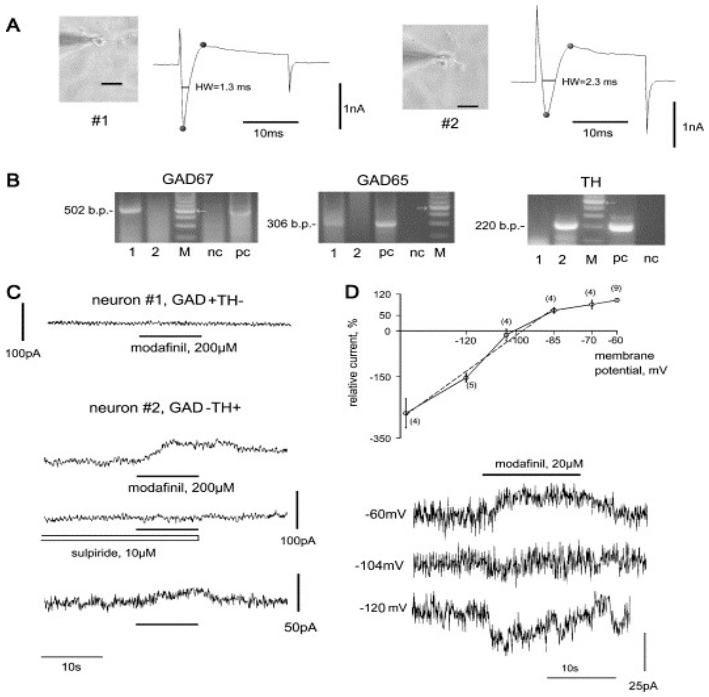

Fig. 3. Modafinil-evoked currents in acutely isolated VTA neurons. (A) Photographs of two neurons (scale bars 20 μm) with corresponding recordings of currents induced by a depolarizing voltage step (+60 mV) from the holding membrane potential −60 mV. Half-width (HW) of fast inward currents was measured at the middle of the distance between peak value and maximal value of the following outward current (two filled circles on the current traces). (B) Examples of PCR products obtained after tyrosine hydroxylase

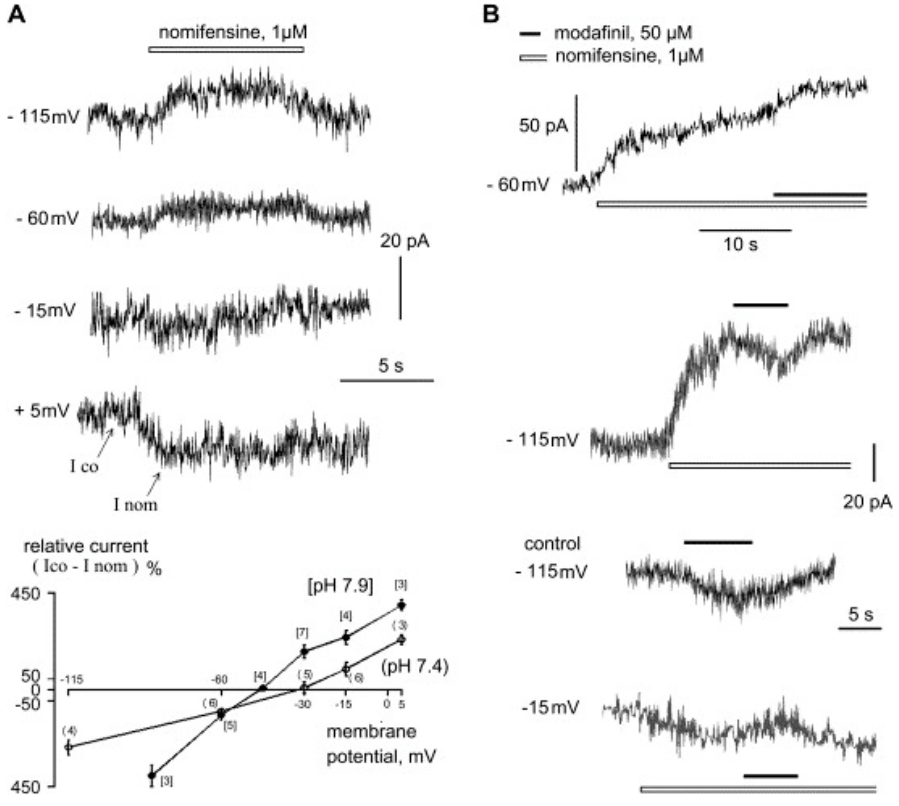

Fig. 4. The DAT antagonist nomifensine does not interfere with modafinil action in acutely isolated midbrain DA neurons. (A) Nomifensine blocks leak conductance associated with DAT. Representative responses to nomifensine at different holding potentials and averaged data in current–voltage plots. Reversal potential of leak conductance was determined in extracellular solutions buffered at two different pH values. Number of cells is given in parentheses near the data points in current–voltage plots for the leak conductance. All responses were normalized to the amplitude of the response measured in the same neuron at −60 mV. (B) Action of modafinil is not affected by nomifensine. Representative responses to modafinil in the presence of nomifensine at different holding potentials (amplitude was measured from the steady-state level of nomifensine response immediately preceding modafinil application). Control modafinil response measured at the same holding potential, −115 mV, is given for comparison.

prevent binding of amphetamine-like substrates to the DAT. Application of the DAT antagonist nomifensine (1 μM) to DA neurons at a holding potential of −60 mV caused an outward current of 17.3±4.1 pA (n=18), which reversed its polarity near −30 mV (Fig. 4A), becoming inwardly directed at more positive membrane potentials. This effect resembled the block of the previously described tonic leak conductance associated with DAT by cocaine (Sonders *et al*., 1997). This study demonstrated that the DAT-associated constitutive leak conductance is carried by alkali cations and protons: changing the extracellular pH from 7.5 to 8.5 produced a −38.5 mV shift in the reversal potential of this leak conductance (Sonders *et al*., 1997). In accordance, when we applied nomifensine at different holding potentials in an extracellular solution buffered at pH 7.9 the leak conductance reversed at about −45 mV (Fig. 4A). Co-application of modafinil (50 μM) with nomifensine (1 μM) after pre-incubation with nomifensine (10–20 s) performed at holding potential −60 mV yielded no difference (p=0.75, Mann–Whitney U-test) in the amplitudes of modafinil-evoked outward currents compared to control, when modafinil was applied alone: averaged responses represented 15.1±2.4 pA (n=10) and 13.6±2.2 (n=8) in nomifensine and control, respectively. As some modafinil responses were not easy to dissect from a not always reversible progressing nomifensine-evoked shift of the baseline current (n=4, see also Fig. 4B) we took advantage of the different reversal potentials of modafinil and nomifensine responses. Thus, at a holding potential of −115 mV both currents could be observed at different polarity in all cases (n=6, Fig. 4B). The inwardly directed modafinil-evoked current represented 25.5±13 pA (n=6) and 24.6±5.3 pA (n=5) in nomifensine and control, respectively. Thus, modafinil responses were not blunted by nomifensine in acutely isolated cells placed into a medium lacking dopamine, providing evidence that under these experimental conditions modafinil acts only through the D2-like receptors.

4. Discussion

We demonstrate a direct inhibition of midbrain dopaminergic neurons by modafinil. This action does not involve the adrenergic system, but is related to D2- receptor-activation. Considering all available publications on modafinil actions in the brain we suggest that D2-like receptors are the major if not unique target of modafinil. These findings are relevant for understanding the dopaminergic control of sleep–wake regulation.

A number of studies have pointed out the importance of dopamine reuptake in conjunction with adrenergic signaling in the modafinil action (Mignot *et al*., 1994, Nishino *et al*., 1998, Ponomarenko *et al*., 2004, Wisor and Eriksson, 2005). Earlier studies did not detect an involvement of the dopaminergic system but emphasized the role of catecholamines in the cat (Lin *et al*., 1992) and rat (Akaoka *et al*., 1991). Blockade of catecholamine synthesis with the tyrosine hydroxylase inhibitor ά-methyl-DL-p-tyrosine methyl ester (άMPT) abolished the amphetamine, but not modafinil action indicating the existence of a different modafinil action site, besides dopamine or catecholamine reuptake systems (Lin *et al*., 1992). Our present data provide evidence that modafinil is an agonist at D2-like receptors, which explains the aforementioned findings in cats. The role of dopamine in sleep regulation is still poorly understood (Jones, 2003, 2005). Recently the anatomical localization of wake-active DA neurons was revealed: 50% of DA neurons in the ventral periaqueductal gray matter (vPAG) expressed the Fos protein during natural wakefulness or wakefulness induced by environmental stimulation, but none expressed Fos during sleep (Lu *et al*., 2006). Neurotoxic lesions of the VTA do not decrease behavioral wakefulness (Lai *et al*., 1999). In addition, firing of dopaminergic neurons in the ventral tegmental area (VTA) and substantia nigra (SN) does not correlate with levels of behavioral arousal in rats (Miller *et al*., 1983). Dopaminergic neurons of the VTA play a key role in the rewarding properties of drugs of abuse, including ethanol and cocaine (Bunney *et al*., 2001). Therefore our data are important for the future development of medications lacking addiction properties. Amphetamine, methylphenidate, cocaine and its analogues (Einhorn *et al*., 1988, Bunney *et al*., 2001, Prieto-Gomez *et al*., 2004) inhibit DA neurons through inhibition of dopamine-reuptake, concomitant DA accumulation and activation of D2 receptors. On the other hand, these compounds have additional activities not only on reuptake of catecholamines, but at some distinct sites too, like the inhibitory action of amphetamine at metabotropic glutamate receptors (Paladini *et al*., 2001). In contrast to these established compounds, modafinil is not a drug of abuse; it can be used for the treatment of cocaine addiction and withdrawal symptoms (Sofuoglu and Kosten, 2006). Amphetamine-like psychostimulants increase wakefulness (Nishino *et al*., 1998, Boutrel and Koob, 2004), but their action seems to be different from modafinil on the systemic level and with respect to addiction. Thus, amphetamine alters glucose utilization in a wide variety of brain regions, while the modafinil action is restricted to the hippocampus, centrolateral nucleus of the

thalamus and central nucleus of the amygdala (Engber *et al*., 1998). As prazosine did not interact with modafinil, noradrenergic and dopaminergic systems did not seem to interact in our recordings from VTA neurons.

Nevertheless, such interaction is evident from in vivo experiments (Lin *et al*., 1992, Wisor and Eriksson, 2005). VLPO neurons are inhibited by noradrenaline in rat brain slices in vitro (Gallopin *et al*., 2000). Modafinil was not active on its own there, but enhanced the inhibitory action of noradrenaline (Gallopin *et al*., 2004), an effect attributed to the block of noradrenaline reuptake in noradrenergic terminals on sleep-promoting neurons in the VLPO. Such a mechanism could be at least partially responsible for the wake-promoting effect of modafinil. As noradrenaline reuptake does not take place in VTA (John *et al*., 2006) our prazosine data were highly expected.

In acutely isolated DA neurons modafinil acted exclusively through D2-like receptors (present study). We excluded the possibility that modafinil acts as a DAT antagonist: reversal potentials of nomifensine- and modafinil-evoked responses were different and there was no interference between them. The possibility remains that modafinil acts as a weak substrate of DAT, causing responses of very small amplitude which we were not able to measure. The substrate translocation current is always inwardly directed and u-shaped with a minimal amplitude at about −20 mV and maximal values at maximal hyperpolarization (Sonders *et al*., 1997). Responses to modafinil recorded in the present study at −115 mV were inwardly directed and they were not reduced in the presence of nomifensine, making such an interaction with DAT rather unlikely. Nevertheless, in order to entirely exclude DAT as a site of modafinil action one would need to conduct experiments on heterologously expressed DAT, like in the study by Sonders *et al*. (1997). DAT could be involved indirectly in modafinil actions through D2 receptors as they modulate the function of DAT (Mayfield and Zahniser, 2001).

The major currently accepted hypothesis explaining the modafinil action implies a weak affinity at DAT (Mignot *et al*., 1994). This is supported by the lack of the wake-promoting modafinil action on DAT deficient mice (Wisor *et al*., 2001). This hypothesis is not consistent with the fact that functional D2 receptors are missing in DAT deficient mice (Jones *et al*., 1999), therefore D2-like receptors may be the major if not sole site of modafinil action. The D2-receptor hypothesis can explain a number of findings at the systemic level, which are not easily explained by the DAT hypothesis. First, maintenance of the modafinil wake-

promoting action in cats treated with a tyrosine hydroxylase inhibitor occurs (Lin *et al*., 1992). Second, increased serotonin release would be expected due to the excitatory function of D2-like receptors on dorsal raphe serotonin neurons (Haj-Dahmane, 2001). Third, inhibition of GABA-release in the striatum (Ferraro *et al*., 1998) can be easily attributed to D2 receptor activation. Fourth, the importance of ά-1B-adrenoreceptors in the modafinil action could be related to the permissive role of these receptors in locomotor and rewarding effects of psychostimulants and opiates (Drouin *et al*., 2002). These receptors are strongly expressed in the mid layers of the cerebral cortex, in the lateral and central amygdaloid nuclei and in the thalamus (Pieribone *et al*., 1994). Last, DAT substrates, including amphetamine and methylphenidate, have, in contrast to modafinil, a more generalized action throughout the brain, and carry addictive properties.

Modafinil taken together with other D2 agonists is beneficial in the treatment of Parkinsonian patients with excessive daytime sleepiness by improving the fatigue (Nieves and Lang, 2002). Modafinil-like drugs are therefore promising medications for Parkinson's disease.

In conclusion, we describe a novel agonistic action of modafinil at D2-like receptors, which can explain the differences between classical psychostimulants and modafinil and contributes to understanding the involvement of different neurotransmitter systems in wake-promoting action. We suggest that D2-like receptors are the major if not unique site of modafinil action. This may be the key for the beneficial action in Parkinson's disease.

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Excitation of histaminergic tuberomamillary neurons by thyrotropin-releasing hormone

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Abstract

The histaminergic tuberomamillary nucleus (TMN) controls arousal and attention. Firing of TMN neurons is state-dependent: they are regularly active during waking and silent during sleep. Thyrotropin-releasing hormone (TRH) is known to promote arousal and combat sleepiness associated with narcolepsy. Single-cell RT-PCR (scRT-PCR) and immunohistochemistry 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 (50μM). 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 but an antagonist of the Na+/Ca2+ exchanger, KB-R7943, blocked it. The frequency of spontaneous inhibitory GABAergic postsynaptic potentials was either increased or decreased by TRH, indicating diverging GABAergic inputs with respect to TRH modulation. We conclude, that direct excitation of rodent TMN neurons is receptor-mediated and demands activation of nonselective cationchannels as well as electrogenic Na+/Ca2+ exchange. Our findings can explain the wake-promoting as well as food intake suppressive effects of this peptide.

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. TRH receptors 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. Clinical and experimental reports demonstrated a role of TRH in the modulation of locomotion, cognition, mood and sleep. The tuberomamillary nucleus (TMN) plays a prominent role in sleep-waking regulation (Haas and Panula, 2003). Several sleep related peptides like galanin and the hypocretins affect histaminergic neurons. It was shown previously, that many cells in the rat lateral hypothalamus including a small fraction of TMN neurons show TRH-immunoreactivity (Airaksinen et al., 1992), indicating that TRH may modulate histamine release. On the other hand, histamine stimulates release of TRH from neurosecretory nerve endings in the mediobasal hypothalamus through H2 receptors (Charli et al., 1978). Two TRH receptors are

known, their distribution is not overlapping: TRHR1-expression is restricted to the areas involved in neuroendocrine regulation (mainly found in the hypothalamus), while brain areas expressing TRHR2 provide the anatomical basis for the described effects of TRH on higher cognitive functions as well as its effect on arousal, locomotor activity, and pain perception (Heuer et al., 2000). Biologically stable TRH analogues such as CG3703 and TA0910 increase wakefulness and decrease sleep time in narcoleptic canines (Nishino et al., 1997);(Riehl et al., 2000) without any measurable behavioural side effects, providing possible therapeutic applications for TRH analogs in human sleep disorders. TRH and its analogues have long been known as antiepileptics in animal seizure models (Nillni and Sevarino, 1999) and in clinical use (Kubek and Garg, 2002). While the antiepileptic function of TRH can be explained by its excitatory action on hippocampal interneurons (Deng et al., 2006);(Atzori and Nistri, 1996) cellular mechanisms underlying the promotion of arousal are not fully elucidated. Broberger and McCornick (Broberger and 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. Most hypocretinergic neurons have recently been shown to be excited by TRH (Hara et al., 2007). Lesions or chemical inactivation of the posterior hypothalamus cause hypersomnia (Moruzzi, 1972). Histaminergic cells send widespread projections from the tuberomamillary nucleus to most cerebral regions, including those known to be important in sleep–waking control (Inagaki et al., 1988; Panula et al., 1989; Lin et al., 1996; Lin et al., 1993). In freely moving animals, histaminergic neurons discharge tonically and specifically during waking (Steininger et al., 1999; Vanni-Mercier et al.,2003; Takahashi et al., 2006). Enhancing histaminergic transmission promotes wakefulness (Lin et al., 1988; Monti et al., 1991). Finally, abolition of HA 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 et al., 2002). On the other hand the silencing of TMN is critically involved in the sedation produced by general anesthetics (Nelson et al., 2002). Injection of TRH into the brain antagonizes pentobarbital-, ethanol- and diazepam- induced sleep and sedation (Nillni and Sevarino, 1999), indicating an excitation of

arousal centers. 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 et al., 2007). The aim of the present study was the elucidation of molecular and electrophysiological actions of TRH on histaminergic neurons. We describe theexpression of TRH receptors and demonstrate, that similar to the orexin- and serotonin-mediated excitation (Eriksson et al., 2001a);(Eriksson et al., 2001b), TRHreceptor mediated excitation of TMN neurons demands activation of a Na^{+}/Ca^{2+} exchanger. Parts of this work have been presented in abstract form (Sergeeva et al., 2007).

Materials and methods

Slice preparation.

Coronal brain slices from the posterior hypothalamus (400-450 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 rules of 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 brain transferred to ice-cold modified artificial cerebrospinal fluid (ACSF), saturated with carbogen (95% O2/5%CO2), in which NaCl had been replaced by 207mM 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, CaCl2 2.0, MgSO4 1.3, NaH2PO4 1.24, NaHCO3 25.6, D-glucose 10, phenol red 0.01%, bubbled with carbogen (pH 7.4) for at least 1hour 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 MΩ). According to Ericson et al (Ericson 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 (Axon Instruments, USA), filtered between 0.5-10kHz,

sampled at 20kHz and analyzed with pClamp8 software (Axon Instruments, USA). The frequency of extracellular action potentials was determined online in bins of 15s 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 MΩ. Biocytin (Sigma, Deisenhoffen, 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 Ca2+ 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. 3,4). For the voltage ramp experiments K+acetate 4M intracellular solution was used and CdCl2 (10μM), D-AP5 (50μM), CNQX (20μM), TTX (1μM), bicuculline methiodide (10μM) added to the bath solution. Data were acquired through an Axoclamp-2A amplifier and a Digidata 1200 interface board (Axon Instruments, Foster City, CA, USA). After further amplification, the signal was fed to a chart recorder and to a PC running Clampex 8 software. Neurons were filled with biocytin at the end of experiments by 200 ms-long anodal (depolarizing) current pulses (frequency 1Hz) for at least 20 minutes. Whole cell patch-clamp recordings of GABAergic spontaneous inhibitory postsynaptic potentials (sIPSCs) were made from ventromedial TMN neurons in coronal rat brain slices (350-400 μm thick), were cells were visually identified and approached with the help of infrared differential interference contrast (IR-DIC) . Voltage clamp (at -70mV) recordings of sIPSCs were done either at room temperature (22–240C) or at 32±0.5°C with a flow rate of 2-2.5 ml/min in the presence of AMPA and NMDA receptor blockers: D-AP5 (100μM) and DNQX (10μM), using an EPC9 patch-clamp amplifier (Heka elektronik, Germany). The patch pipette solution contained (mM) 135 KCl, 1 CaCl 2 MgCl, 1 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 (Ih) (Kamondi and Reiner, 1991) and the transient outward current (IA) (Greene et al., 1990). Spontaneous IPSCs were analysed with MiniAnalysis 4.2 (Synaptosoft, Leonia, NJ, USA): Peak amplitude, the 10-90% rise time, τdec (exponential decay time constant in a 100ms 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 (τdec) 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 $p<0.05$.

Immunocytochemistry

Coronal hypothalamic slices containing the TMN region (500μm thick) were fixed in 4% 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide and 0.2% N-hydroxysuccinimide (Sigma) overnight followed by 30min incubation in 4% paraformaldehyde (both prepared in 0.1 M phosphate buffer (PB), pH 7.4) and cryoprotected in PB with 20% sucrose, slices were cryosectioned at 40 μm thickness and mounted on gelatincoated slides. The following primary antibodies were used for the triple immunostaining at dilutions indicated: guinea pig polyclonal antibody to HDC (histidine decarboxylase, Acris, Bad Nauheim, Germany; 1:600), rabbit polyclonal antibody raised against human TRH receptor polypeptide (TRH R1) (1:200; Abcam, Cambridge, UK) and goat anti-rat TRH-R2 (R-20) polyclonal antibody (1:100; Santa Cruz Biotechnology, USA). Double immunostainings were carried out either with HDC immunoreactivity (see above) or a rabbit-anti-histamine antibody (1:1000) (Chemicon International, Germany), and one of the TRH-receptor antibodies raised in goat: goat anti-rat TRH-R1 (N-17) polyclonal antibody (1:100; Santa Cruz Biotechnology, USA) or TRH-R2 (R-20). Detection of the immunoreactivities was carried out via fluorescence-labeled secondary antibodies: Cy3-conjugated donkey anti-guinea pig IgG (1:500; Dianova, Hamburg, Germany); Alexa Fluor 488- labeled donkey anti-rabbit IgG (1:500; Molecular Probes, Eugene, Oregon, USA) and Alexa Fluor 350-labelled donkey anti-goat IgG(1:200; Molecular Probes)(for details on conventional fluorescence microscopy see Korotkova et al (Korotkova et al., 2005).

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 male129/Sv mice (n = 4). Transverse slices (450 μm thick) 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 37oC. 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, MgCl2 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 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 µl) was expelled into an Eppendorf tube, containing 7µl of a mixture prepared according to the protocol of the "first strand cDNA synthesis kit" (Pharmacia Biotech, Freiburg, Germany). After incubation for 1h 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 CTGCAA 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 amplimer 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 R1lo: 5′-TGGCTCTGGAAAACGTGCAGAG-3′ was used in combination with Dgup (amplimer size 201 b.p.) and for the TRHR2-specific amplification R2up: 5′-TGAGAGCACAGACCGTGTGCACTG- 3′ and R2lo: 5′-TCCCCAGCAAGGGTGCAATGAAG-3′ primers were used (amplimer 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 (1-1.5µl), 10x PCR buffer, 10 pM each of sense and antisense primer, 200 µM of each dNTP and 2.5 units Taq polymerase. The final reaction volume was adjusted to 10 µl 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 (Mastercycler, 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 94oC for 48 s, annealing at 53o C for 48 s, and extension at 72oC for 1 min. For the second amplification round 1µl 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.

Drugs and statistical analysis

Drugs used in the present study were: R-α-methyl-histamine, KB-R7943, gabazine (SR-95531), D-AP5 and DNQX from Biotrend (Koeln, Germany). Chlordiazepoxide, tetrodotoxin and TRH were obtained from Sigma/RBI (Deisenhofen, Germany). Drugs were diluted and stored as recommended by the producers. Drugs were perfused for 5-10 min through the recording chamber. Neurons were recorded for at least 15 min to obtain a stable baseline before application of drugs. Maximal effects of the drugs (at maximal concentrations) were achieved within 2-3 min after drug application into perfusion tube. Statistical analysis was performed with the nonparametrical Mann-Whitney U-test. Significance level was set at $p<0.05$. Data are presented as mean ± 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 excusively during intracellular recordings (Eriksson et al., 2001a). 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 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 µM) with an enhancement of firing rate to190 ± 61 % of the control level. In rat slices 10 out of 14 cells (71%) were excited by TRH (2-10µM, 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 1hr (n=4, not shown).

TRH effect is antagonized by chlordiazepoxide

Chlordiazepoxide (CDZ, 10 μM), 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 50μM CDZ, a concentration used also in previous studies for the blockade of TRH receptors (Deng et al., 2006), the TRH effect was abolished (n=6, p=0.0005, Mann-Whitney *U* test, Fig.1D). Interestingly, CDZ (50μM) 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 we found, that CDZ potentiates GABA-evoked whole-cell currents with an EC50=2μM and maximally at 10μM (no further potentiation at 50 μM). Therefore the observed inhibition of firing cannot be attributed to the modulatory action of CDZ at GABAA receptors. Moreover, GABAA receptors do not exhibit a tonic influence on TMN firing in slices, as bicuculline (50μM, n=4) and gabazine (up to 100μM, 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 et al., 1995;Jinsi-Parimoo and Gershengorn, 1997).

Transcriptional and immunohistochemical 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., 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 occurrence of TRH R1 trancripts 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). Triple immunostainings of rat histaminergic neurons were undertaken in the following experiments in order to visualize the pattern of TRH R1 and TRH R2 colocalization. Immunostainings in mice did not give conclusive results for the TRH R2 immunoreaction most likely due to the low effectivity and selectivity of this antibody (on Western Blots performed from the rat posterior hypothalamus and paraventricular nucleus (not shown) many bands of different size were seen indicating weak selectivity, see also high background staining with this antibody in rat sections on Fig.2B and Fig.3). In the first experiments primary antibodies were tested in double stainings. The TRH R1 (Abcam) antibody stained 66% of histidine-decarboxylase (HDC)-positive cells (of total 397 neurons from 4 rats, 41 fields investigated) while the TRH R1 (SantaCruz) antibody stained 51% of HA (histamine)-positive cells (167 neurons, 20 fields). Within the same analyzed fields TRH R1-positive nonhistaminergic cells were seen (Fig.2B), their number was comparable to the HA+TRHR1-cellular group (145 and 84 neurons were counted in a first and second staining paradigm, respectively). TRH R2 antibody (SantaCruz) stained 67 (31.6%) HA-neurons out of 212, 70 cells were found in the same fields (n=16) in close proximity to HA-neurons which were immunopositive only for the TRH R2. TRH R2 and HDC co-staining gave a similar percentage of double stained neurons (40%, 120 HDC+ cells). Thus, HDC or HA –based identification of TMN neurons yielded similar results with TRH R antibodies raised in different species. Triple stainings were performed with guinea-pig HDC, goat TRH R2 and rabbit TRH R (1) antibodies. TRH R1 immunoreactivity was detected in 67% of histaminergic neurons (240 cells analyzed) while TRH R2 was found in 34 % of the cells. Similar to the transcriptional analysis performed with single-cell RT-PCR TRH R2 staining was found within the TRH R1-positive cellular group, and only few TRH R2-positive cells were lacking TRH R1-immunoreactivity.

Intracellular recordings in rat slices

Stable recordings were obtained from 32 neurons with electrophysiological characteristics of TM neurons (Haas and Reiner, 1988). They exhibited spontaneous firing at 3.8 ± 0.2 Hz (n = 29). Under tetrodotoxin, their resting membrane potential was -52.5 ± 0.8 mV (n = 25). Bath application of TRH (1.5μM) increased the spontaneous firing rate of the TM neurons, accompanied by a rapid depolarization and this effect reversed completely 20-40 min after termination of treatment (Fig. 4).

During a washout period of 1 hr, repeated application of TRH resulted in no depolarization or 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. 4). 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 4E). Only the cells which respond 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 μM $BaCl_2$ (n=5; Fig. 4E) or by 3 mM CsCl (n=3). Hereafter, we tested the effects of blocking the Na^+/Ca^{2+} exchanger (NCX). The selective blocker of the NCX, KBR7943 (Iwamoto et al., 1996) at 80μM, 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 μM TRH intersected at -4.3 ± 6.3 mV (n=4, Fig.4B) , a potential close to the predicted one for a mixed cationic conductance.

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 Ih and IA currents (Fig.5A) 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 (2.5μM) for 5 min. The amplitude was not changed significantly under TRH indicating a presynaptic effect. After TRH washout (5-10min) the GABAA receptor antagonist gabazine (10μM) abolished sIPSCs in all cells (n=7, Fig.5B) in accordance with our previous studies (Eriksson et al.,2004);(Sergeeva et al., 2002) showing that spontaneous synaptic currents recorded from somata of TMN neurons are exclusively carried through the postsynaptic GABAA receptors. No postsynaptic inward currents in response to the TRH application were seen in these experiments in accordance with our previous observation that Na^+/Ca^{2+} exchange is functionally inactive at room temperature in TMN neurons (Eriksson et al., 2001b). 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 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 one can only explain 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 5C,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 (group1) in sIPSC frequency from 0.85 ± 0.27 Hz to 1.37± 0.49Hz, in 4 cells (group2) decreases from 0.61 ± 0.27 Hz to 0.43 ± 0.18Hz and in 3 cells (group3) the frequency was not significantly changed (0.87 ± 0.29Hz)(Fig.6). 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 ± 6.2% (n=6); 55.3 ± 18.7% (n=4) and 72.9 ± 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) were seen upon TRH-perfusion 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.6A), 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.

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^+/Ca^{2+} 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 et al., 2006);(Bayliss et al., 1992). However, immature motoneurons displayed activation of a non-selective cationic conductance by TRH (Bayliss et al., 1994). Single cell RT-PCR and immunohistochemistry revealed expression of

one or both TRH receptors in most TMN neurons, whereas a small fraction of neurons (ca 25%) lacks TRH receptor-expression. Both receptor types were found on histaminergic and non-histaminergic neurons in TMN area with double and triple immunofluorescent histochemistry. 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 the posterior hypothalamus (Heuer et al., 2000) and this receptor seems to be more extensively distributed in the brain (Heuer et al., 2000);(O'Dowd et al., 2000), which is in line with our present data. An immunohistochemical analysis by Gotoh and colleagues revealed that TRH-R2, but not TRH-R1, is expressed in the histaminergic TMN neurons of rats (Gotoh et al., 2007). This can be explained by the low specificity of the TRH R2 antibody (see Results section). What is the functional meaning of the TMN neuron heterogeneity with respect to the expression of TRH-receptors? TRH-R1 and TRH-R2 expressing cells may belong to different functional systems (feeding/neuroendocrine vs arousal and cognition) as these receptors are expressed in distinct pathways (Heuer et al., 2000). Such a segregation of TMN neurons is pointed out by several findings. First, early retrograde labelling studies on TMN neurons demonstrated wide-spread projections from this nucleus and lack of somatotopic organization (Kohler et al., 1985): thus cells projecting to distant and distinct regions of the brain can be found in close neighbourhood. In a recent study by Cenni et al (Cenni et al., 2006) injection of the cannabinoid receptor 1 agonist ACEA into the TMN augmented histamine release from lateral hypothalamus, nucleus basalis and striatum but not from cortex while injection of a cannabinoid-uptake inhibitor resulted in histamine release augmentation only in hypothalamus, thus revealing functional heterogeneity of histaminergic neurons. Second, orexin- and TRH- receptors mediate their action through the same effector system (Na^+/Ca^{2+} exchanger), but central injections of these peptides cause opposite effects on food intake. Ishizuka et al (Ishizuka et al., 2006) have shown, that orexin-A increases histamine release and locomotor activity, but does not influence food intake, suggesting that the histaminergic system participates in arousal rather than feeding related effects of orexin-A. Similar to orexin A, intracranial injection of TRH increases locomotor activity (Vogel et al., 1979), in addition TRH suppresses food intake after food deprivation (Gotoh et al., 2007). Serotonin 2C (5-HT2C) receptors are expressed by the majority of TMN neurons (in 70% of TMN neurons mRNA for this receptor can be detected with single-cell RTPCR (Sergeeva et al., 2003)) and their ability to excite these cells depends on the Na^+/Ca^{2+} exchanger (Eriksson et al., 2001b).

Similar to TRH, agonists at 5-HT2C receptors cause hypophagia (Lam et al., 2007;Fone et al., 1998). Different effects of 5-HT2C agonists on locomotor activity were reported: from hyperlocomotion and oral dyskinesia in rabbbits upon acute administration (Simansky et al., 2004) and reduced locomotor activity in a novel environment after long-term treatment (Fone et al., 1998) to the lack of action on locomotion (Lam et al., 2007). Thus, different behavioural effects of serotonin, orexins and TRH, despite of their common effector mechanism (Na^+/Ca^{2+} exchanger) at the cellular level can be explained by the functional integration of different TMN neurons in different systems (cognitive, neuroendocrine, feeding-related). TRH was applied to each slice only once, as no second response was usually observed even after 40-60 min washing. The absence of a second response is in accordance with the previous observations confirming rapid down-regulation of the surface receptor pool due to the agonist-induced internalization of TRH receptors (Gershengorn and Osman, 1996);(Deng et al., 2006). In contrast, changes in receptor expression mediated by changes in gene transcription, mRNA stability or translation are relatively slow effects. High rates of turnover of TRH-Rs have been described in various cell types (Ashworth et al., 1995);(Drmota et al., 1998);(O'Dowd et al., 2000). This rapid agonist induced internalization of TRH-Rs may be responsible for the rapid kinetic observed with the TRH effect in TM cells in our study. This desensitization is rapidly reversible by treatment with chlorodiazepoxide (CDZ), by lowering [Ca2+]i, and down-regulation of PKC (Grimberg et al., 1999). In our study, the competitive TRH-R binding antagonist CDZ dose dependently attenuated and blocked the TRH effect. Interestingly, higher doses of CDZ produce a decrease of TM spontaneous firing. This effect can occur due to a decrease of spontaneous tone by TRH on histaminergic cells or due to lowering constitutive activity of TRH-Rs (Heinflink et al., 1995). TMN neurons are excited via NCX activation that causes depolarization. The NCX is electrogenic, with an exchange ratio of 3 Na^+ in for every Ca^{2+} that is pumped out, and is expressed throughout the brain (Quednau et al., 1997). Recently, we have shown that serotonin and orexin peptides induce depolarization of TMN neurons by activation of the NCX (Eriksson et al., 2001b; Eriksson et al., 2001a). In the same way, H1-receptor mediated depolarization of rat vasopressin neurons in the supraoptic nucleus occurs through the activation of NCX (Smith and Armstrong, 1996). These receptors are all coupled to phospholipase C, and this is also true for TRH-Rs (Gershengorn and Osman, 1996). While the NCX is activated through activation by all these receptors the kinetics are different, perhaps due to different intracellular pathways. The activation of NCX is likely secondary to a surge

in the intracellular Ca2+ concentration, as the activated receptors are coupled to inositol 1,4,5-triphosphate production. In the present study, no change in membrane conductance was seen in association with the NCX activation indicating that Ca^{2+} is most likely released from intracellular stores. Also in previous studies no obvious Ca^{2+}-channel component was seen after NCX activation (Smith and Armstrong, 1996);(Eriksson et al., 2001b);(Eriksson et al., 2001a). In contrast to the hippocampus, where TRH enhances the frequency of sIPSCs in CA1 pyramidal neurons (Atzori and Nistri, 1996; Deng et al., 2006), TRH bi-directionally modulates frequency of sIPSCs in TMN neurons. One possible explanation for that may be a complex interaction with other transmitter systems. As VLPO neurons (the major GABAergic input to the TMN) express both μ- and k-opioid receptors (Mitchell et al., 1997) and agonists of both receptors can suppress sIPSCs in TMN neurons (Eriksson et al., 2004) opioid peptides may suppress GABAergic inputs to the TMN neurons thus supporting TRH-mediated excitation. Dynorphin (kreceptor agonist)-positive fibres are found at high density in TMN region (Lantos et al., 1995). Interestingly, dynorphin is also produced by orexin/hypocretin neurons (Chou et al., 2001), which densely innervate TMN (Eriksson et al., 2001a)). Orexin/ataxin-3 mice with a selective loss of the orexin neurons lack prodynorphin mRNA in the lateral hypothalamic area, confirming that dynorphin expression is restricted to the orexin neurons in this area (Chou et al., 2001). Orexinergic neurons are excited by TRH (Hara 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 dynophin dominates (Eriksson et al., 2001a). Thus, depending on the TRH receptor type or its expression level in orexin neurons one or the other peptide may be preferentially released interacting with GABAergic inputs on the TMN neurons. Although TRH, serotonin and orexin share the same effector system in TMN neurons (activation of the Na^{+}/Ca^{2+} exchanger), they trigger different behavioural responses: while serotonin and TRH suppress feeding, orexin either promotes it or does not affect it. The presynaptic and postsynaptic mechanisms of modulation of TMN excitability are in keeping with the functional heterogeneity of the brain histaminergic system controlling energy homeostasis and wakefulness.

In conclusion, the present findings indicate a role of TMN neurons in TRH induced arousal. As TRH reduces sleeping time in rats and mice and combats excessive sleepiness in canine models of narcolepsy, TRH-mediated excitation of TMN neurons may provide a key factor in controlling attention and arousal.

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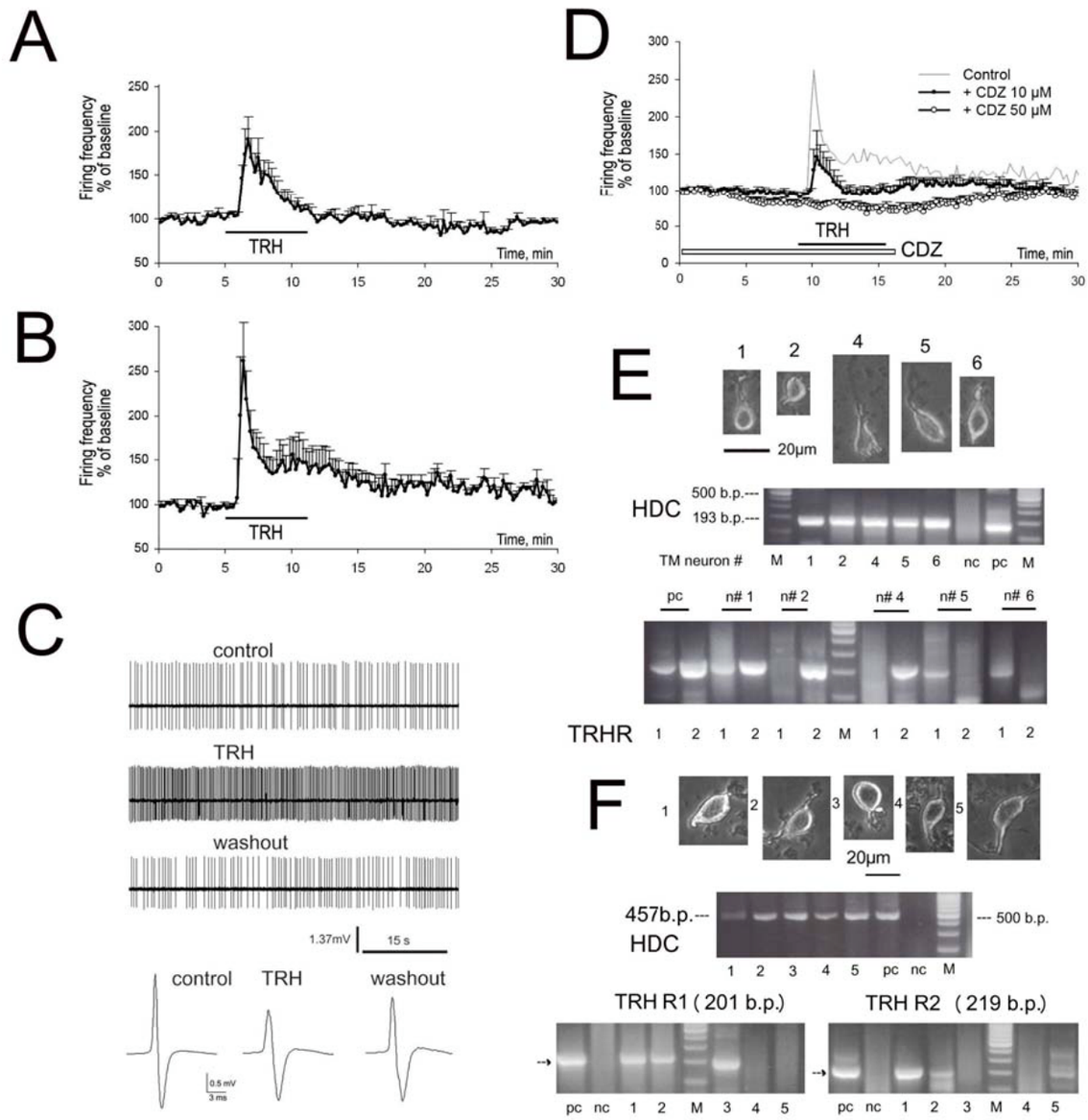


Fig.1.TRH enhances firing rate of TMN neurons. Averaged time course diagrams of firing rates illustrating responses to bath application of TRH in mouse (A, 1.5μM TRH, n=6) and rat (B, 2-10μM TRH, n=10) TMN neurons. Non-responding cells are not included. C. Example of recordings in rat TMN neuron: note regular firing, excitation by TRH 2μM 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), a TRH receptor antagonist, reduces the TRH response in rat TMN neurons at 10 μM (n=4) and abolishes it at 50μM (n=6). E-F. Examples of single-cell RT-PCR analysis of TRH receptor expression in 5 mouse (E) and 5 rat (F) TMN neurons (photographs of corresponding cells are given over the agarose gels). pc-positive control: posterior hypothalamus; nc-negative control; M-DNA size marker: 100b.p. ladder (500 b.p.-most intense line); HDC-histidine decarboxylase.

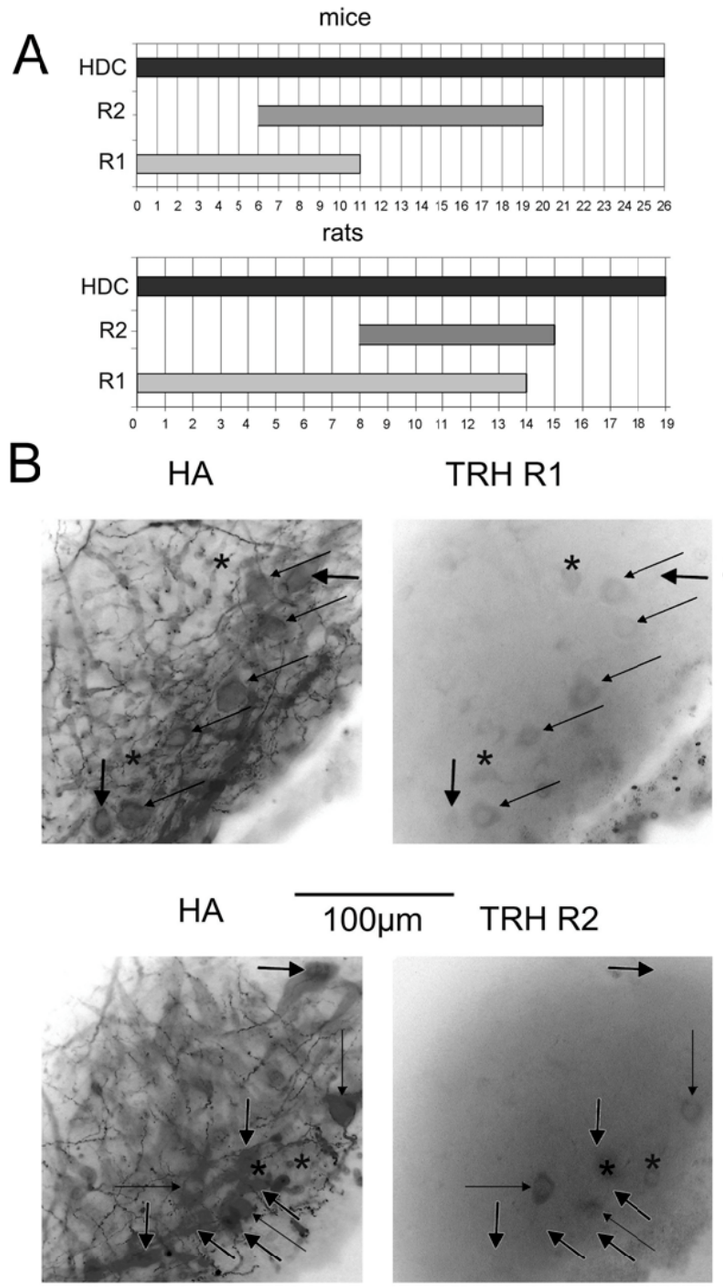


Fig.2 Expression of TRH receptors in rat and mouse TMN neurons. A. Summary of single-cell RT-PCR analysis done in 26 mouse and 19 rat TMN neurons positive for the histamine synthetizing enzyme histidine decarboxylase (HDC). B. Double immunofluorescence stainings for histamine (HA, marked with Alexa Fluor 488) and one of the TRH receptors (marked with Alexa Fluor 350). Thin long arrays show double stained neurons, thick short arrays point to HA+TRH R- cells and asterixs indicate position of TRHR-positive non-histaminergic cells.

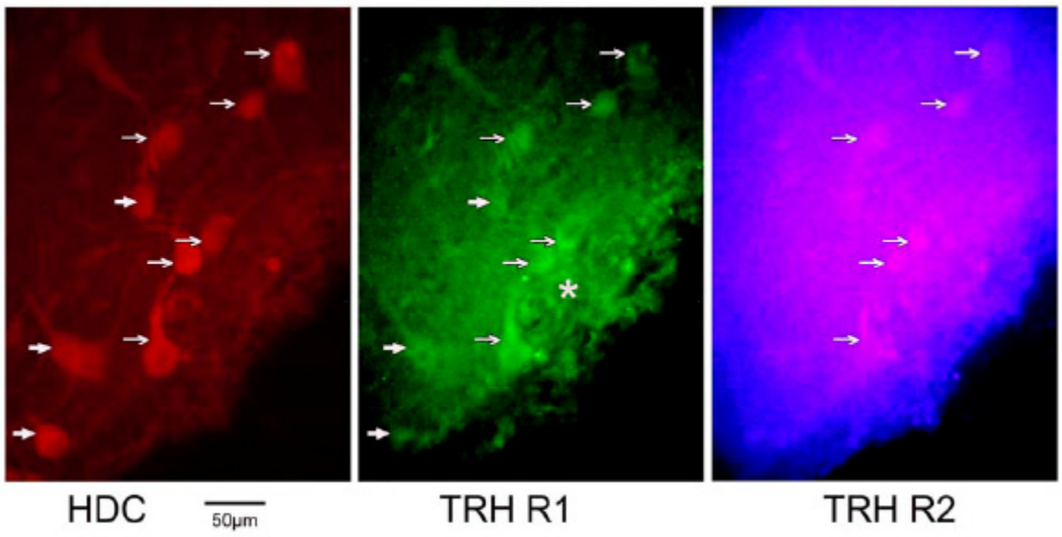
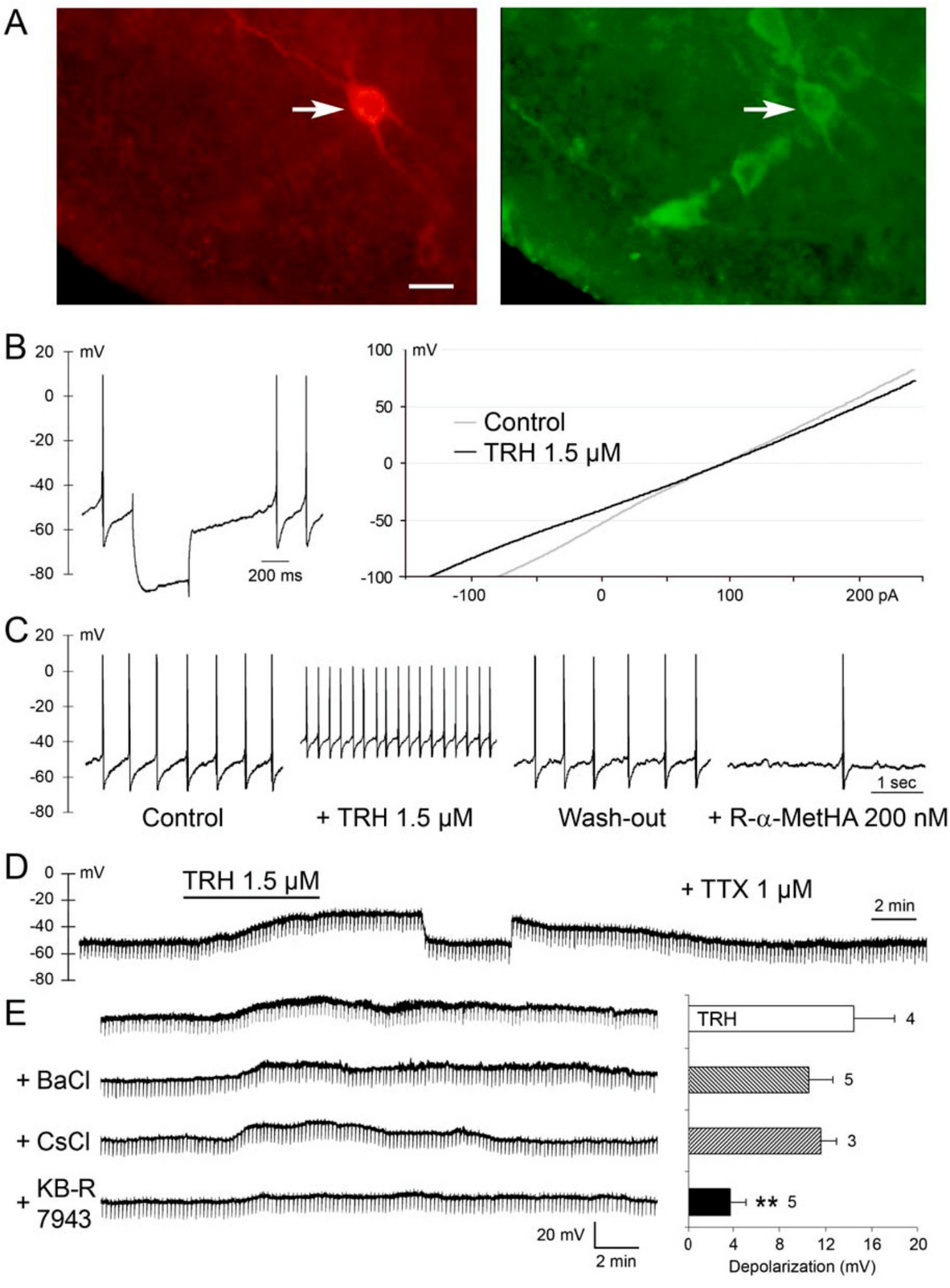


Fig.3 Triple immunostaining for the HDC (Texas Red) and two TRH receptors:
TRHR1 (green, AF488) and TRHR2 (blue, AF350). Long arrays show triple stained neurons, thick short arrays point to HDC+TRHR1+TRHR2- cells and asterix to the TRHR1+ non-histaminergic cell.

Fig.4 Intracellular sharp-electrode recordings from rat TMN neurons. A. TMN neuron stained with both biocytin (red) and HDC (green). Scale bar, 20 µm. B. *Left*, Response to negative current injection: a hyperpolarization-activated inward rectification (owing to the hyperpolarization-activated cationic current) and an outward rectification that delays the return of the membrane potential to the resting potential (owing to A-type K+ currents). *Right*, The voltage–current plots are recorded from 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. C. 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 a markedly reduced firing rate. D. During treatment with 1 µM TTX, TRH strongly depolarizes the neuron and the action is therefore postsynaptic. During the maximal depolarization the membrane potential is manually returned to the resting level. No change in input resistance is noted. E. Depolarization induced by TRH and effects on it by 500 µM BaCl2, 3 mM CsCl or 80µM KB-R7943 (*Right,* representative depolarization under each condition after preincubation with 1 µM TTX, mean + SE for responding cells, numbers beside bars indicate n). While BaCl2 and CsCl do not alter the TRH-evoked depolarization of TMN neurons, the selective blocker of the NCX, KB-R7943 strongly depresses the depolarization. **p<0.01, Student's twotailed *t* test.


A
B
20
mV
0
-20
-40
-60
-80
200 ms
100
mV
50
0
-50
-100
Control
TRH 1.5 μM
-100
0
100
200 pA
C
20
mV
0
-20
-40
-60
-80
1 sec
Control
+ TRH 1.5 μM
Wash-out
+ R-α-MetHA 200 nM
D
0
mV
-20
-40
-60
-80
TRH 1.5 μM
+ TTX 1 μM
2 min
E
+ BaCl
+ CsCl
+ KB-R 7943
20 mV
2 min
TRH
4
5
3
** 5
0
4
8
12
16
20
Depolarization (mV)

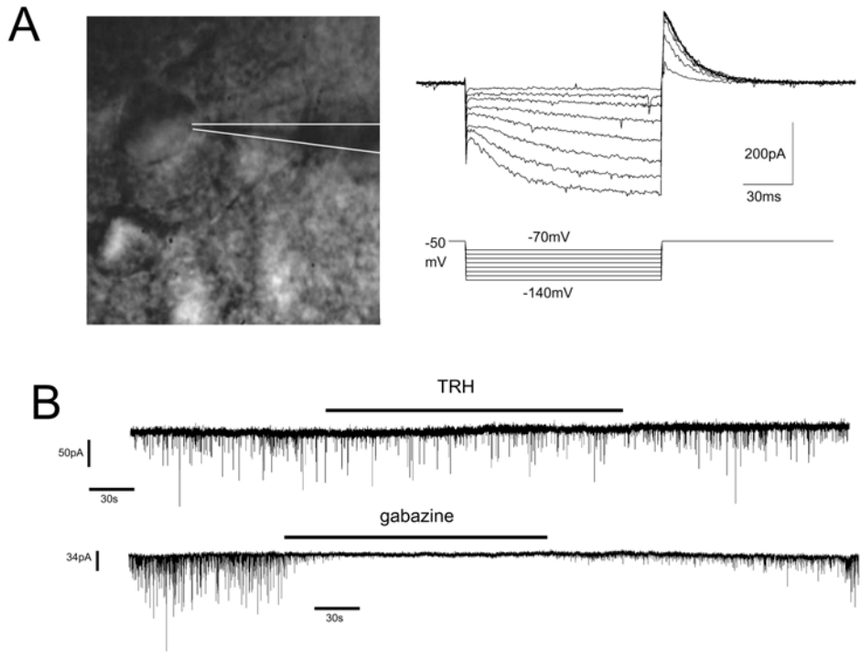
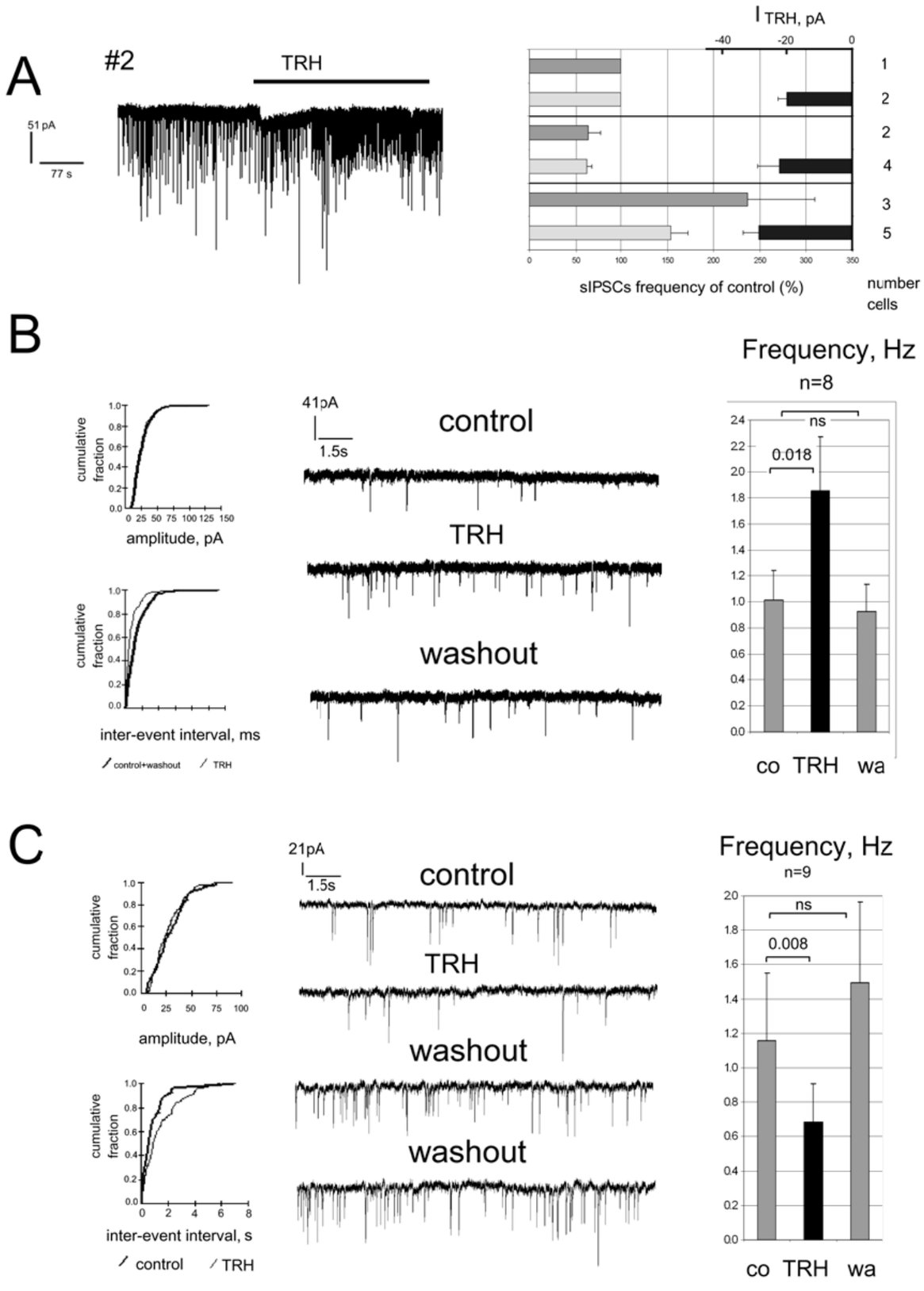

A
200pA
30ms
-50
mV
-70mV
-140mV
B
TRH
50pA
30s
gabazine
34pA
30s
C
control
DNQX + D-AP5
25pA
10s
inter-event interval, s
amplitude, pA
decay time, ms
1.0
0.8
0.6
0.4
0.2
0.0
0.2 0.4 0.6 0.8 1.0
control
DNQX+D-AP5
0 20 40 60 80
x5
D
frequency, Hz
amplitude, pA
decay time, ms
p<0.0001
(n=11)
co
DNQX
D-AP5

Fig.5 Whole-cell voltage clamp sIPSCs recordings from TMN neurons in rat thin slices.
A. TMN neuron is approached under visual control and identified with the stimulation protocol shown at the right side. Hyperpolarization-acivated inward current (Ih) becomes obvious after voltage jumps from -50 to -90mV and to lower values. Maximal outward IA current is seen after return to the holding membrane potential from -120mV. B. Example of TRH (2.5μM)-induced depression of sIPSC frequency (experiment done at room temperature). GABAA receptor antagonist gabazine (10μM) blocks spontaneous synaptic activity. C. AMPA and NMDA receptor antagonists (DNQX and D-AP5, respectively) reduce the frequency of sIPSCs without affecting their kinetics or amplitude. D. Summary from 13 TMN neurons for frequency, amplitude and decay time of sIPSCs during control period and upon DNQX/D-AP5 application.

Fig.6 At physiological temperature TRH induces inward currents and modulates frequency of sIPSCs in TMN neurons recorded in whole-cell voltage-clamp.
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 under TRH was obtained with Fisher´s exact probability test ($p=0.36$) between neurons recorded at room or physiological temperature, all recordings were pooled together. Cells with control sIPSC frequency below 0.1 Hz were not included in summary diagrams.


A
#2
TRH
51 pA
77 s
I TRH, pA
-40
-20
0
1
2
2
4
3
5
0
50
100
150
200
250
300
350
sIPSCs frequency of control (%)
number of cells
B
cumulative fraction
amplitude, pA
inter-event interval, ms
control+washout
TRH
41pA
1.5s
control
TRH
washout
Frequency, Hz
n=8
ns
0.018
co
TRH
wa
C
cumulative fraction
amplitude, pA
inter-event interval, s
control
TRH
21pA
1.5s
control
TRH
washout
washout
Frequency, Hz
n=9
ns
0.008
co
TRH
wa