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Effect of microdialysis perfusion of THIP in the perifornical hypothalamus on sleep-wakefulness: Role of δ -subunit containing extrasynaptic GABA_A receptors

Mahesh M. Thakkar¹, Stuart Winston², and Robert W. McCarley²

¹Harry Truman Memorial Veteran's Hospital & Dept. of Neurology, University of Missouri, Columbia, MO, 65201

²Boston VA Healthcare System, & Dept. of Psychiatry, Harvard Medical School, Brockton, MA, 02301

Abstract

Gaboxadol or 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol (THIP) is a selective agonist for the δ -subunit containing extrasynaptic GABA_A receptors that will soon enter the U.S. market as a sleep aid (Winsky-Sommerer et al., 2007). Numerous studies have shown that systemic administration of THIP reduces wakefulness and increases sleep both in humans and rats (Lancel and Langebartels, 2000; Walsh et al., 2007). However, it is yet unclear where in the brain THIP acts to promote sleep. Since the perifornical lateral hypothalamus (PFH) contains orexin neurons and orexin neurons are critical for maintenance of arousal (McCarley, 2007), we hypothesized that THIP may act on PFH neurons to promote sleep. To test our hypothesis, we used reverse microdialysis to perfuse THIP unilaterally into the PFH and studied its effects on sleep-wakefulness during the light period in freely behaving rats.

Microdialysis perfusion of THIP (100 μ M) into the PFH produced a significant reduction in wakefulness with a concomitant increase in nonREM sleep as compared to ACSF perfusion. REM sleep was unaffected.

This is the first study implicating the δ -subunit containing extrasynaptic GABA_A receptors in PFH in control of sleep-wakefulness in freely behaving rats.

Keywords

Orexin/hypocretin; perifornical hypothalamus; THIP; reverse microdialysis

The γ -aminobutyric acid (GABA) system is closely linked with the regulation of sleep-wakefulness. Thus, it is not surprising that pharmacological landscape for treatment of various sleep disorders including insomnia have been dominated by agents that activate GABA_A receptors (Wafford and Ebert, 2006). Classical synaptic GABA transmission results in phasic inhibition that is mainly mediated by γ 2 subunits containing postsynaptic GABA_A receptors (Rudolph and Mohler, 2006; Ebert et al., 2006; Olsen et al., 2007). In contrast, tonic inhibition is mainly mediated by δ subunit containing "extrasynaptic" GABA_A receptors (Olsen et al.,

Correspondence: Mahesh Thakkar, Harry Truman Memorial Veteran's Hospital, Columbia, MO., Department of Neurology, University of Missouri, 800 Hospital Drive, Columbia, MO, 65201, Ph. (573) 814 6000 ext 53697, Email: thakkarm@health.missouri.edu.

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2007). These "extrasynaptic" GABA_A receptors have a higher affinity for GABA and slower rates of desensitization and deactivation than do the classical synaptic receptors.

The GABA_A agonist THIP selectively activates extrasynaptic GABA_A receptors (Winsky-Sommerer et al., 2007). Systemic administration of THIP induces sleep in rats and humans (Faulhaber et al., 1997; Lancel, 1997). However it is yet unknown where in the brain does THIP act to induce sleep.

There is strong evidence indicating that the PFH is critical for wakefulness. Although the PFH contains several cell types, including the orexin/hypocretin and the melanin concentrating hormone containing neurons, there is compelling and consistent evidence implicating the orexin neurons in the control of wakefulness (McCarley, 2007). For example, local administration of orexin in various brain regions produced wakefulness (Bourgin et al., 2000; Thakkar et al., 2001; Xi et al., 2001; Methippara et al., 2000). In contrast, a deficiency or reduction of orexinergic neurotransmission resulted in a reduction in wakefulness and cataplexy like episodes in rodents (Lin et al., 1999; Chen et al., 2006; Gerashchenko et al., 2001; Chemelli et al., 1999; Thakkar et al., 1999) and narcolepsy in humans (Thannickal et al., 2000; Mignot, 2004). Single unit recording studies suggest that the orexin neurons exhibited the Wake-On/REM–Off discharge pattern (W>nonREM<REM) (Alam et al., 2002; Lee et al., 2005; Mileykovskiy et al., 2005). The δ -subunit-containing GABA_A receptors are present in the PFH (Pirker et al., 2000) and the role of PFH GABA_A receptors in sleep induction has been previously shown (Alam et al., 2004). However, it is yet unclear whether extrasynaptic GABA_A receptors in the PFH have any role in control of sleep-wakefulness. To evaluate the role of δ -subunit containing extrasynaptic GABA_A receptors in the PFH and its influences on sleep-wakefulness, we examined the effects of THIP locally administered into the PFH on spontaneous bouts of sleep-wakefulness in freely behaving, naturally sleeping rats.

METHODS

Animals & Surgery

Adult male Sprague-Dawley rats were housed under constant temperature, with ad libitum access to food and water, and with 12 h light (0700h to 1900h) and dark (1900h to 0700h) cycle at least 10 days before surgery.

Under sterile conditions and using the standard surgical protocol (for details see Thakkar et al., 2001. Thakkar et al, 2008), the animals were implanted with electrodes for recording electroencephalogram and electromyogram for determination of behavioral state. Intracerebral guide cannulas (CMA/Microdialysis, Acton, MA; for lateral insertion of the microdialysis probes) were implanted at 90 degree angle above the target site in the orexinergic zone of the PFH. The target coordinates (Paxinos and Watson, 1998) for the tip of the microdialysis probe were: AP -3.3 , ML ± 1.5 , DV -8.5 , relative to bregma and skull surface at bregma. Every effort was made to minimize animal suffering and to reduce the number of animals used. All animals were treated in accordance with the American Association for Accreditation of Laboratory Animal Care's policy on care and use of laboratory animals. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Research Committee of the Boston VA Healthcare System.

THIP-hydrochloride—4,5,6,7-tetrahydroisoxazolo-pyridin-3-ol, a selective agonist for δ -subunit-containing extrasynaptic GABA_A receptors (Winsky-Sommerer et al., 2007) was purchased from Tocris Biosciences Ellisville, Missouri and dissolved in artificial cerebrospinal fluid (ACSF = NaCl 147 mM, KCl 3 mM, CaCl₂ 1.2 mM, MgCl₂ 1.0 mM, pH 7.2) to make a stock solution of 1 mM. Three concentrations, (1, 10 and 100 μ M) were used in this study to provide an effective concentration of 0.1, 1 and 10 μ M at the probe tip (Thakkar et al. 1998).

Post-operative Recovery, habituation and sleep-wakefulness recordings of sleep-wakefulness

Experiments were conducted in sound-attenuated chambers with food and water available ad libitum and lights on from 0700 to 1900 hr; After 3 days of post-operative recovery, the rats were tethered to a light weight recording cable and habituated to the recording setup for at least 7 days before the experiment was begun. The animals remained tethered until the end of the experiment except during probe insertion.

Unilateral microdialysis perfusion of THIP coupled with sleep-W recordings in freely behaving rats

A unilateral microdialysis probe was implanted. The following protocol for probe insertion was used: The rat was disconnected from the recording cable and gently swaddled in a towel. After removing the stylus, a microdialysis probe (CMA 11, 1 mm membrane length, 0.24 mm O.D; CMA/Microdialysis, Acton, MA) was gently inserted into the guide cannula. The flow was checked and the rat was reconnected. After allowing 12 hr for recovery from probe-insertion and for equilibrium at the probe tip, the experiment was begun. The experimental protocol is described in Table 1. ACSF and/or THIP were perfused @ 2 μ l/min. Most previous studies have performed systemic administration of THIP during the light (inactive) period (Lancel and Faulhaber, 1996; Vyazovskiy et al., 2005; Winsky-Sommerer et al., 2007) and found subsequent effects on sleep. Since a key purpose of this study was to define brain region where THIP might its sleep inducing effects in rats; in this initial study, we decided to perform microdialysis perfusion of THIP during the light period.

Localization of the injection site

On completion of the experiment, the animals were euthanized under deep Phenobarbital anesthesia and perfused with 0.9% saline followed by perfusion of 10% formalin. The brains were isolated, blocked and processed for orexin-A immunohistochemistry (Chen et. al., 2006) to localize the injection site in the PFH.

Analysis of Behavioral States

Behavioral state data was acquired and digitized using the Harmonie software (Stellate Systems, Montreal, Canada and sleep-wakefulness was visually scored in 10 sec epochs as (1) Wakefulness, (which included both active and quiet wakefulness) determined by the presence of low amplitude, high frequency desynchronized EEG with the concomitant presence of active muscle tone; (2) non-REM sleep; determined by the presence of low frequency, high amplitude, synchronized EEG with low EMG tone; and (3) REM sleep, determined by complete absence of muscle tone along with desynchronized EEG (Thakkar et al, 2003). The effect of THIP on the sleep-wakefulness was analyzed by repeated measure ANOVA followed by Bonferoni's test (EZAnalyze Ver 3.0. <http://www.ezanalyze.com>).

RESULTS

Only those animals with microdialysis probe tips (N=7) in the PFH as revealed by orexin-A immunohistochemistry were included in data analysis. A representative photomicrograph illustrating the perfusion site in the midst of orexin neurons is shown in Figure 1

The behavioral state data during two ACSF perfusions were comparable. Therefore behavioral state data during ACSF perfusion were pooled together. As described in Table 2 and illustrated in Figure 2, there was significant decrease in wakefulness ([F=3.25; DF=27; p<0.05, N=7, One way RM ANOVA] with a concomitant increase in nonREM sleep [F= 3.54; DF=27; p<0.05, N=7, One way RM ANOVA] during three hours of unilateral THIP perfusion into the PFH as

compared with during ACSF perfusion. Post-hoc Bonferroni tests revealed that the 100 μM dose of THIP significantly reduced wakefulness (Mean $T=3.9$; $p<0.05$) and increased nonREM sleep ($T=4.1$; $p<0.05$). Although REM sleep was decreased during THIP perfusion, the effect did not reach significance [$F=0.71$; $DF=27$; $p=0.559$, $N=7$, One way RM ANOVA] (see Table 2 and Figure 2). The changes in the behavioral states returned to baseline during 90 min of post-THIP ACSF perfusion (data not shown)

DISCUSSION

Local unilateral administration of THIP, a selective agonist for δ -subunit containing extrasynaptic GABA_A receptors in the PFH produced a significant increase in nonREM sleep with a concomitant reduction in wakefulness during the light period in freely behaving rats. To our knowledge, this report is the first implicating the δ -subunit containing extrasynaptic GABA_A receptors in the PFH in the regulation of sleep-wakefulness in freely behaving rats.

The use of a microdialysis probe to apply drugs locally in specific regions of the brain provides precise control over the concentration and duration of the drug administration and offers several advantages over other techniques including the ability to deliver low and constant concentrations of drugs without disturbing the animal (Thakkar et al., 1998). In addition, the drug delivered through microdialysis probe does not diffuse more than 1 mm from the probe membrane (Hocht et al., 2007; Westerink and De Vries, 2001). One limitation associated with reverse microdialysis is that the brain concentration of the delivered drug needs to be estimated from *in vitro* probe experiments. Previous studies done in our lab suggest that ~10% of the drug diffuses out of the probe (Portas et al., 1996). Thus, perfusion of 100 μM of THIP will deliver 10 μM concentration of THIP outside the probe.

Numerous studies have shown that THIP selectively activates the δ -subunit containing extrasynaptic GABA_A receptors in the brain (Krogsgaard-Larsen et al., 2004; Winsky-Sommerer et al., 2007; Wafford and Ebert, 2006) and systemic administration of THIP, in rats and humans, increases nonREM sleep and reduces wakefulness without affecting REM sleep (Lancel and Faulhaber, 1996; Lancel and Langebartels, 2000). Although, *in vitro* studies conducted in mice suggest that the ventrobasal thalamus may be critical for THIP induced sleep promotion (Belelli et al., 2005; Jia et al., 2005), recent *in vivo* studies have shown that systemic THIP administration does not promote sleep in mice (Vyazovskiy et al., 2005; Winsky-Sommerer et al., 2007).

Our study suggests that unilateral administration of 100 μM THIP into the PFH increased nonREM sleep and reduced wakefulness as compared to ACSF perfusion. This effect may be due to THIP induced inhibition of orexin neurons because Alam et al. (2005) have shown that orexinergic neurons are under GABAergic control during sleep although, THIP induced inhibition of other no-orexinergic neurons cannot be ruled out.

Unilateral perfusion of THIP in the PFH did not produce any significant effect on REM sleep, most likely because the critical REM sleep promoting neurons are in the brainstem (Datta, 2007).

In conclusion, while further studies especially bilateral infusion of THIP in the PFH and other wakefulness centers (Datta and Maclean, 2007) and monitoring the effects of THIP during the dark period are necessary; our initial study suggests that unilateral perfusion of THIP, a selective extrasynaptic GABA_A receptor agonist, into the orexinergic PFH increased nonREM sleep and reduced wakefulness during the light period in freely behaving, naturally sleeping rats. This is the first study to implicate extrasynaptic GABA_A receptors in the orexinergic PFH in the control of sleep-wakefulness and the first to localize the effects of THIP to a specific brain region in freely behaving rats.

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Glossary

ACSF, Artificial cerebrospinal fluid; GABA, γ -aminobutyric acid; nonREM, non-rapid eye movement or slow wave sleep.; PFH, perifornical lateral hypothalamus; REM, Rapid Eye Movement; THIP, 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol.

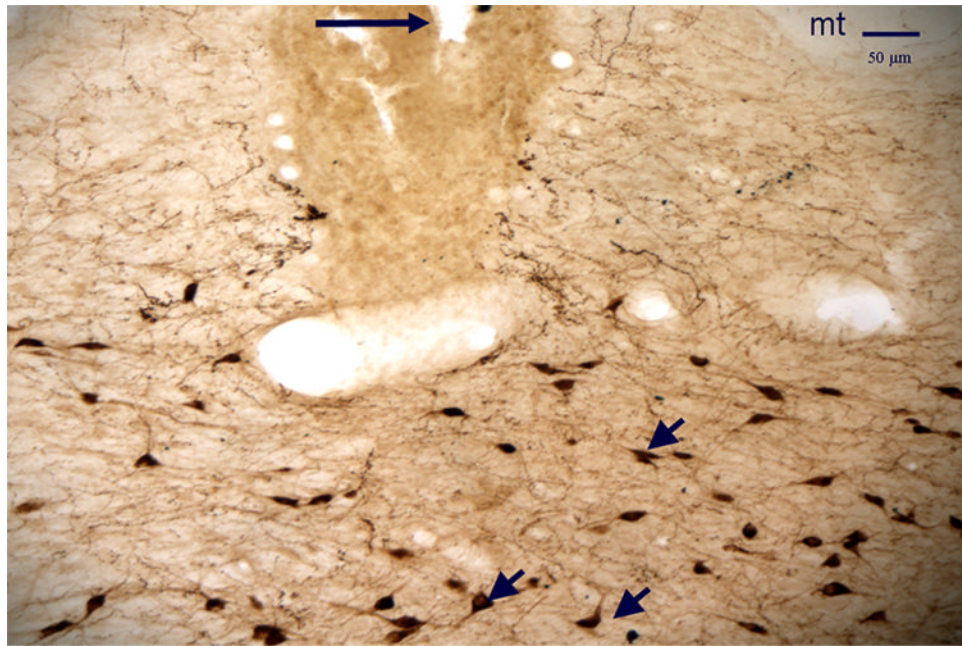


Figure 1.

A representative photomicrograph illustrating the localization of the microdialysis probe tip (black arrow) in the midst of orexin neurons (black arrowheads) in the orexinergic PFH is shown. All the probe tips (N=7) were localized within the orexinergic PFH. Abbreviations: mt = mammillothalamic tract. Calibration bar = 50 µm

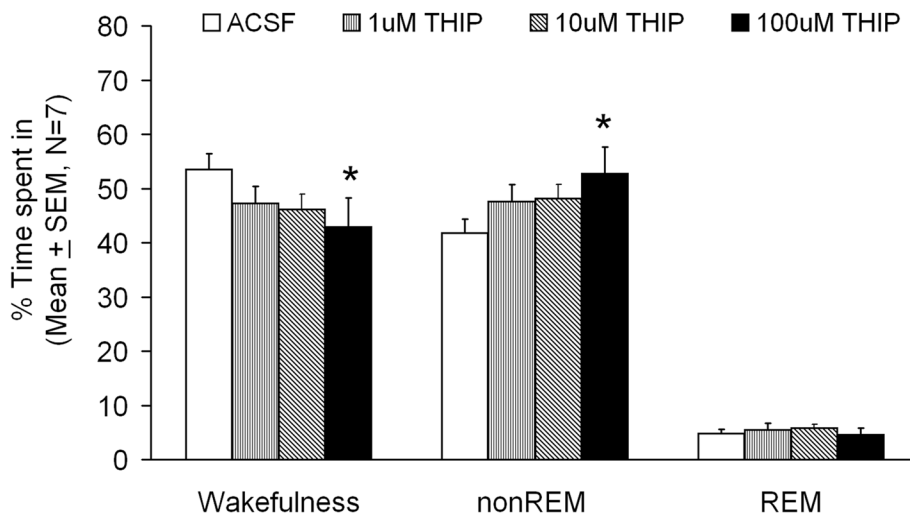


Figure 2.

Unilateral microdialysis perfusion (3 hr) of 4,5,6,7-tetrahydroisoxazolo-[5,4-c]pyridine-3-ol (THIP; also known as Gaboxadol), a selective agonist for δ -subunit containing extrasynaptic GABAA, into the orexin-rich perifornical hypothalamus increased the amount of time (Mean \pm SEM; N=7) spent in nonREM and decreased the amount of time spent in wakefulness during light period compared to ACSF perfusion. REM sleep was not affected. One way repeated measures ANOVA followed by Bonferroni's post hoc test revealed that the maximum effect was produced by the highest (100 μ M; effective concentration at the probe tip = 10 μ M) dose of THIP. * = $p < 0.05$; level of significance (see text for details).

Table 1

Experimental Protocol.

ACSF perfusion	Day 1	ACSF perfusion begins at 09.30 – 15.30 hr with two syringe changes to match syringe changes on THIP perfusion days
THIP (1 μ M) Perfusion	Day 2	ACSF perfusion:-09.30 to 11.00 ; THIP perfusion (1 μ M):-11.00 to 14.00 ACSF: - 14.00 to 15.30 hr.
ACSF perfusion	Day 3	Same as described for Day 1
THIP (10 μ M) Perfusion	Day 4	Same as described for Day 2 except 10 μ M of THIP will be used
ACSF perfusion	Day 5	Same as described for Day 1
THIP (100 μ M) Perfusion	Day 6	Same as described for Day 2 except 100 μ M of THIP will be used
ACSF perfusion	Day 7	Same as described for Day 1

Table 2Percent time (Mean \pm SEM, N=7) spent in during 3 hr of THIP perfusion.

	ACSF	THIP		
		1 μ M	10 μ M	100 μ M
Wakefulness	53.51 \pm 2.9	47.21 \pm 3.2	46.20 \pm 2.8	42.87 \pm 5.4
nonREM	41.83 \pm 2.5	47.54 \pm 3.1	48.21 \pm 2.6	52.69 \pm 4.9
REM	4.84 \pm 0.7	5.43 \pm 1.2	5.78 \pm 0.7	4.63 \pm 1.1