The EEG effects of THIP (Gaboxadol) on sleep and waking are mediated by the $GABA_A$ δ-subunit containing receptors

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Abstract

THIP (Gaboxadol) is a selective $GABA_A$ agonist, acting *in vitro* with high potency and efficacy at the extrasynaptic $GABA_A$ δ–containing receptors. THIP was suggested to be a potential hypnotic to treat insomnia and it is currently in clinical trial. Here we assessed whether the $GABA_A$ δ-containing receptors mediate *in vivo* the effect of THIP on sleep and the sleep electroencephalogram (EEG). We performed EEG recordings in a mouse model deficient in the $GABA_A$ δ-subunit gene ($\delta^{-/-}$ mice) and in wild-type littermate controls. THIP (4 and 6 mg/kg intraperitoneally) induced an abnormal EEG pattern resulting in dramatic changes in the waking and nonREM sleep EEG spectra in wild-type mice. Indeed, a massive increase in EEG power lasting 2-3 hours occurred in both the frontal and parietal derivation, especially in frequencies below 6 Hz. All effects were more prominent in the frontal EEG. Furthermore, the highest dose of THIP lengthened REM sleep latency and suppressed REM sleep. In contrast, vigilance states and sleep latencies were not affected in $\delta^{-/-}$ mice. Moreover only minor changes were observed in the nonREM sleep EEG spectrum after THIP injection in the δ-subunit deficient mice. The present findings do not indicate a sleep-promoting effect of THIP in mice, which is in accordance with a previous report in this species. Moreover, our results *in vivo* demonstrate that THIP acts preferentially at $GABA_A$ receptors containing the delta subunit.

Introduction

Targeting the central GABA neurotransmitter system has been shown to have therapeutic relevance for specific clinical conditions, including sleep disorders. $GABA_A$ receptors are hetero-pentameric complexes assembled from seven subunit classes (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, θ, ρ_{1-3}). The subunit composition and the regional distribution of the $GABA_A$ receptors are major factors underlying the differentiation of the effects of therapeutic agents (Rudolph & Mohler, 2006). A specific $GABA_A$ receptor agonist, THIP (4,5,6,7-Tetrahydroisoxazolo-[5,4-c]pyridine-3-ol), also named Gaboxadol, is currently under investigation for its potential to treat insomnia (Krogsgaard-Larsen *et al.*, 1977; Ebert *et al.*, 2006). *In vitro* studies using recombinant $GABA_A$ receptors demonstrated that THIP interacts with high potency and efficacy at $\alpha4\beta3$-, $\alpha4\beta3\delta$- and $\alpha6\beta3\delta$-containing receptors (Adkins *et al.*, 2001; Brown *et al.*, 2002; Storustovu & Ebert, 2006). Interestingly, the $\alpha_4\beta_3\delta$ receptor subtype is mainly expressed in regions involved in sleep regulation, such as the ventro-basal nucleus of the thalamus and the neocortex (Pirker *et al.*, 2000; Peng *et al.*, 2002). $GABA_A$ δ-containing receptors show a predominant extrasynaptic localization (Nusser *et al.*, 1998; Sun *et al.*, 2004) and mediate non-desensitizing "tonic" inhibition, in contrast to "phasic" inhibition controlled by synaptic $GABA_A$ receptors (Farrant & Nusser, 2005; Jia *et al.*, 2005). The presence of an extrasynaptic tonic current, sensitive to THIP, was recently demonstrated in the ventro-basal nucleus of the thalamus as well as in cortical layers II-III (Belelli *et al.*, 2005; Jia *et al.*, 2005; Drasbek & Jensen, 2006). In addition, $GABA_A$ $\alpha_4^{-/-}$ mice show a lack of tonic inhibition in the thalamic relay neurons (Chandra *et al.*, 2006). The distinct

electrophysiological properties of extrasynaptic $\alpha_4\beta_3\delta$ receptors may therefore underlie the specific effects of THIP.

Effects of $GABA_A$ receptor agonists on sleep differ substantially from those evoked by $GABA_A$ receptor allosteric modulators such as benzodiazepines or benzodiazepine-like compounds. Rats treated with THIP (4 mg/kg) increased the amount of NREM sleep as well as slow-wave activity (SWA, EEG power in the 0.75-4 Hz frequency range; a measure of sleep intensity) within NREM sleep, independently of circadian time (Lancel & Faulhaber, 1996; Lancel, 1997). In addition, THIP had no effect on REM sleep duration. These results suggested that THIP could be a potential therapeutic compound to treat insomnia. In healthy human subjects, a single oral bed-time dose of THIP promoted slow-wave sleep and enhanced EEG low-frequency activity in NREM sleep (Faulhaber *et al.*, 1997; Lancel *et al.*, 2001; Mathias *et al.*, 2005). However, a recent study showed in mice that THIP elicited an increase in SWA not only in NREM sleep but also in the waking EEG (Vyazovskiy *et al.*, 2005). The increase was due to the recurrent appearance of spike-wave events, their asymmetric shape, as well as an increase in EEG amplitude. This study also demonstrated that the changes induced by THIP were substantially different from those evoked by prolonging wakefulness for several hours.

To further assess THIP's functional selectivity at $\alpha_4\beta_3\delta$ receptors and its mechanism of action *in vivo*, we investigated sleep and the sleep EEG in a mouse model deficient in the δ-subunit gene.

Materials and Methods

Animals

$GABA_A$ delta subunit knockout $\delta^{-/-}$ mice (n=6 males, n=4 females) and their wild-type littermates $\delta^{+/+}$ (n=6 males, n=5 females) were obtained from heterozygous breeding pairs and genotyped by PCR analysis of tail biopsies. A set of oligonucleotides (5′ primer: CTG TGA ATG TGG CGC TTG CCC TAG AG; 3′ primer: GTC CAG CAT GCA CTC CTG CTC ATC CAG) amplified a product (1866 bp and 766 bp for the $\delta^{-/-}$ and $\delta^{+/+}$, respectively) corresponding to the allele of exon 4, using Eppendorf Mastercycler. PCR conditions were: 96°C for 1 min; then 96°C for 15 sec, 51°C for 15 sec, 68°C for 1.5 min, 40 cycles. The mice were maintained on a mixed C57BL/6J x 129Sv/SvJ background (see (Mihalek *et al.*, 1999), for characterization). Mice were housed individually with *ad libitum* access to food and water. The animal facility was maintained on a 12:12 hr light/dark cycle (light on at 9am; ~ 60 lux), at a constant ambient temperature (22-24°C) with a 50 % relative humidity. All experimental procedures were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Cantonal Veterinary Office of Zurich.

Surgery

At surgery, mice were 11-13 weeks-old ($\delta^{-/-}$: males 23.8 ± 0.7 g, females 20.3 ± 0.4 g; $\delta^{+/+}$: males 23.5 ± 0.6 g, females 20.1 ± 0.5 g; no significant differences between $\delta^{-/-}$and $\delta^{+/+}$ mice). For electroencephalogram (EEG) recording, the mice were implanted epidurally under deep anesthesia (ketamine 100 mg/kg-xylasine 20 mg/kg, dose 10 ml/kg intraperitoneally (i.p.)).

Gold-plated miniature screws (diameter 0.9 mm) were positioned on the right hemisphere above the frontal cortex (1.5 mm anterior to bregma and 2 mm lateral to the midline) and the parietal cortex (2 mm posterior to bregma and 3 mm lateral to the midline). A reference electrode was placed above the cerebellum (2 mm posterior to lambda, on the midline). Electrodes were connected to stainless steel wires and fixed to the skull with dental cement. Two gold wires (diameter 0.2 mm) inserted bilaterally in the neck muscle were used to record the electromyogram (EMG). After at least 3 weeks recovery, the mice were placed in experimental cages for at least 3 days to adapt to the recording conditions.

Experimental protocol

The mice were injected i.p. with either 4 mg/kg THIP (4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride; Tocris Bioscience, Bristol, UK) or saline (0.9 % NaCl) 3 hr after light onset, following a crossover design with at least one week between treatments. A third injection (THIP 6 mg/kg) was administered at least 7 days after the crossover treatment. The dose was chosen based on previous studies showing that THIP (2 mg/kg) did not induce significant changes in the EEG in C57BL/6 mice, and only minor changes on the EEG power spectrum in rats (Lancel & Faulhaber, 1996; Lancel, 1997; Vyazovskiy *et al.*, 2005). At 3 h after light onset, EEG SWA in NREM sleep has dissipated considerably (Franken *et al.*, 1999; Huber *et al.*, 2000; Franken *et al.*, 2001). Therefore, we chose this time point for injection as previously (Vyazovskiy *et al.*, 2005) to test potential hypnotic effects of THIP by ensuring a possible increase of SWA in NREM sleep following THIP injection. Mice were observed by the investigator for 50-120 min after each injection and behavior was systematically monitored. In addition, motor activity was continuously recorded by an infra-red (IR) sensor

placed above the cage. Continuous EEG-EMG recordings were obtained throughout the 12 hr light periods.

Data acquisition

The EEG and EMG signals were amplified (amplification factor approx. 2000), conditioned by analogue filters (high-pass filter: -3dB at 0.016 Hz; low-pass filter: -3 dB at 40 Hz, less than -35 dB at 128 Hz.) sampled with 256 Hz, digitally filtered (EEG: low-pass FIR filter 25 Hz; EMG: band-pass FIR 20-50 Hz) and stored with a resolution of 128 Hz. EEG power spectra were computed for consecutive 4 sec epochs by a Fast Fourier Transform routine within the frequency range of 0.25-25 Hz. Between 0.25 and 5 Hz, the 0.25 Hz bins were added to yield 0.5 Hz bins, and between 5.25 and 25 Hz to yield 1 Hz bins.

Data analysis

Based on the raw parietal and frontal EEG, the corresponding slow-wave activity values (SWA, EEG power between 0.75-4 Hz) as well as the raw and integrated EMG, the three vigilance states NREM sleep (low EMG and high EEG amplitude, high slow-wave activity), REM sleep (low EMG and low EEG amplitude, high theta activity – EEG power between 6.25-9 Hz) and waking (high EMG and low EEG amplitude and high theta activity concomitant with highest EMG values) were visually scored for 4 sec epochs (Tobler *et al.*, 1997). In the first 90 min after THIP injection, the waking and NREM sleep EEG were often indistinguishable. Determination of vigilance states was then based on the EMG, IR activity, and the behavioral observations. Epochs containing EEG artifacts were identified and excluded from spectral analysis.

NREM sleep latency after treatment was defined, as previously (Kopp *et al.*, 2004; Vyazovskiy *et al.*, 2005), as time elapsed between the injection and the first NREM sleep episode lasting at least 1 min (and not interrupted by more than 6 consecutive 4 sec epochs, or by a cumulated total of 32 sec not scored as NREM sleep). In order to assess the effect of THIP on REM sleep latency, we determined the time elapsed between the beginning of the first NREM sleep episode and the occurrence of a REM sleep epoch. The 9 hr following THIP or vehicle injection were subdivided into 1.5 hr intervals. EEG power spectra were computed between 0.25-25 Hz for each vigilance state and expressed as percentage of the individual mean power density in the corresponding interval of saline injection, starting at light onset for the 12 hr light period. The software package MATLAB (The MathWorks, Inc., Natick, MA, USA) was used for data and signal analysis.

Activity counts derived from the infra-red sensor were stored in 1 min epochs and 12 h mean activity profiles were computed (Stanford Software System, Chronobiology Kit). The effect of THIP on motor activity was investigated for the first three hours after injection of THIP and compared with saline control.

Statistics

For a given treatment, each mouse was referred to its own control represented by the data obtained after injection of saline. The effects of THIP and saline on EEG spectra were compared by two-way ANOVA factors ‘treatment’ (THIP and saline) x ‘1.5 hr interval’, followed by post-hoc paired *t*-test when significance was reached. Regional differences of EEG power in waking and NREM sleep between the frontal and parietal derivation after THIP (4 or 6 mg/kg i.p.) were analyzed for the three first 1.5 hr intervals after treatment

injection by two-way ANOVA factors ‘derivation’ (frontal and parietal) x ‘1.5 hr interval’, followed by post-hoc paired *t*-test when significance was reached.

Vigilance states and sleep latency values are expressed as mean ± SEM (data were normally distributed as tested with the procedure Univariate; SAS). The effects of THIP were compared with the corresponding saline control, within each genotype, by one-way ANOVA factor ‘treatment’ for each 1.5 hr interval. In case of significance, the ANOVA was followed by the Dunnett test for mean comparison. Subsequently, a two-way ANOVA factors ‘treatment’ x ‘interval: mean int 1-2; 3’ was performed to compare the effect of THIP (i.e., first 1.5 hr interval after injection *versus (vs)* mean of the two 1.5 hr intervals prior to pharmacological treatment). To compare genotypes, statistical analyses were performed using two-way ANOVA for factors ‘treatment’ and ‘genotype’ followed by a post-hoc Dunnett test when significance was reached.

Motor activity was analyzed within genotype by one-way ANOVA factor ‘treatment’. Genotypes were compared by two-way ANOVA factor ‘treatment’ x ‘genotype’.

SAS package was used for statistical analysis (SAS Institute Inc., Cary, NC, USA).

Results

We performed EEG recordings in $GABA_A$ receptor δ-subunit knockout mice and their $\delta^{+/+}$ littermates to investigate the effects of two doses of THIP (4 and 6 mg/kg i.p.) in comparison with saline control. No difference between the $\delta^{+/+}$ and $\delta^{-/-}$ mice was found in the vigilance states scored for the 3 hr before treatments (not shown), as well as after saline injection (Table 1).

Sleep latency

For both genotypes, NREM sleep latency and REM sleep latency showed a large variability after injection of THIP and saline (Table 1). REM sleep latency, after occurrence of the first NREM episode, was significantly lengthened by 6 mg/kg THIP in $\delta^{+/+}$ mice (one-way ANOVA factor 'treatment': $p=0.0256$). NREM sleep and REM sleep latencies did not differ in $\delta^{-/-}$ mice after THIP injection compared to saline. Sleep latency after saline did not differ between the genotypes (Table 1).

EEG spectra

In $\delta^{+/+}$ mice, a massive increase in EEG power lasting 2-3 hours was observed in the waking period following THIP injection, as well as in the subsequent NREM sleep, in the frequencies below 5 Hz, corresponding to slow wave activity (Fig. 1 and 2). These effects were seen in both the parietal and frontal EEG derivation, being more prominent in the frontal EEG (two-way ANOVA interaction 'derivation' x '1.5 hr interval' was significant in waking at 1 Hz and 2.5-18 Hz after 4 mg/kg THIP and at 1 Hz and 2.5-21 Hz after 6 mg/kg THIP; $p<0.05$ post-hoc paired *t*-test; significant in NREM sleep at 2.5-3 Hz after 4 mg/kg THIP, at 1 Hz and 2-4

Hz after 6 mg/kg THIP; $p<0.05$ post-hoc paired *t*-test). In addition, the changes in the EEG spectrum showed a dose response both in waking and NREM sleep (Fig. 1 and 2).

EEG spectra in waking. Specifically, in the waking EEG obtained from the frontal derivation, THIP induced a significant increase in EEG power in a broad frequency range comprising between 0.75-21 Hz (two-way ANOVA interaction 'treatment' x '1.5 hr interval' was significant after 4 and 6 mg/kg THIP; $p<0.05$ post-hoc paired *t*-test). This effect dissipated within 3-4 hr after injection (Fig. 1). In the parietal derivation, THIP induced a significant increase in frequencies between 0.75-6 Hz (two-way ANOVA interaction 'treatment' x '1.5 hr interval' was significant after 4 and 6 mg/kg THIP; $p<0.05$ post-hoc paired *t*-test; not shown). The surge of SWA (0.75-4 Hz band) occurred during the first 90 minutes in both EEG derivations. The magnitude of the increase was dose-dependent (during the first 1.5 hr interval, in the frontal derivation, the maximal increase in the slow wave band was in waking 600-fold and 670-fold above the corresponding saline after 4 and 6 mg/kg, respectively). In contrast, THIP induced only a slight, non significant, increase in the waking spectra of the $\delta^{-/-}$ mice (p values ranging from 0.1162-0.9958 for the SWA frequency range after two-way ANOVA interaction 'treatment' x '1.5 hr interval') (Fig. 1).

EEG spectra in NREM sleep. In NREM sleep, EEG power was increased in the lower frequencies after both the 4 and 6 mg/kg THIP dose (two-way ANOVA interaction 'treatment' x '1.5 hr interval' was significant between 0.75-5 Hz and 0.75-4 Hz in the frontal and parietal derivation respectively; $p<0.05$ post-hoc paired *t*-test) (Fig. 2). This effect lasted 3-4 hr after injection and dissipated thereafter. THIP also induced an EEG power decrease at frequencies between 10-19 Hz during the first 1.5 hr after injection in the frontal EEG (two-way ANOVA interaction 'treatment' x '1.5 hr interval' was significant after 4 and 6 mg/kg

THIP; $p<0.05$ post-hoc paired *t*-test; Fig. 2). In $\delta^{-/-}$ mice, a minor increase in NREM sleep EEG power was observed after injecting THIP 4 or 6 mg/kg for frequencies below 4 Hz (two-way ANOVA interaction 'treatment' x '1.5 hr interval' was significant for the 3.5 Hz frequency in the frontal derivation; $p<0.05$ post-hoc paired *t*-test; p values after ANOVA showed a trend for 2.5-3 Hz; $p=0.0607$ and 0.0698) (Fig. 2).

Regarding the effect of THIP observed in $\delta^{+/+}$ mice in the low EEG frequencies (i.e., SWA), caution is warranted. Indeed, THIP consistently elicited an abnormal EEG pattern characterized by recurring spike-wave events both in waking and NREM sleep (Fig. 3). These spike-wave events, recurring at a frequency of approximately 0.5-1 Hz, largely contributed to the SWA increase in the spectrum. This abnormal EEG pattern was previously reported in rat (Lancel & Faulhaber, 1996; Lancel, 1997) and analyzed in detail in the mouse (Vyazovskiy *et al.*, 2005).

Vigilance states

After 4 and 6 mg/kg THIP injection in $\delta^{+/+}$ mice, waking and NREM sleep were not affected significantly. However, waking tended to increase with a concomitant reduction in NREM sleep (Table1: one-way ANOVA factor 'treatment': $p=0.0531$ and $p=0.0823$ for waking and NREM sleep, respectively). REM sleep was significantly suppressed by 6 mg/kg THIP (one-way ANOVA factor 'treatment': $p=0.0070$; post-hoc Dunnett test $p<0.05$ for THIP 6 mg/kg *vs* saline). The effects of THIP on vigilance states dissipated after the first 1.5 hr interval. We further compared the effects of THIP during the first 1.5 hr after injection with the three first hr of the light period preceding injection. THIP enhanced the time spent in waking with a concomitant decrease in NREM sleep and REM sleep (2-way ANOVA factors 'treatment' x

'interval: mean int 1-2; 3': p=0.0111; p=0.0182 and p=0.0059 for waking, NREM sleep and REM sleep, respectively). In contrast, the vigilance states were not affected significantly by THIP in $\delta^{-/-}$ mice (Table 1: two-way ANOVA factors 'treatment' x 'interval: mean int 1-2; 3' not significant).

Motor activity

The analysis of infra-red activity for the first three hours after injection showed no difference in mean counts per hour between saline and THIP treatment (mean counts per hr ± SEM: WT saline, THIP 4 mg/kg, 6 mg/kg: 379.9 ± 27.2, 366.4 ± 47.9, 294.4 ± 28.6; KO saline, THIP 4 mg/kg, 6 mg/kg: 392.5 ± 27.1, 358.7.4 ± 33.4, 337.2 ± 30.2; one-way ANOVA factor 'treatment' and two-way ANOVA factors 'genotype' x 'treatment' and interaction were not significant).

Discussion

This is the first study demonstrating that the effects of THIP on the EEG and on sleep were virtually eliminated by genetic inactivation of the $GABA_A$ δ–subunit containing receptor. The present results show that THIP elicited only minor changes in $GABA_A$ $\delta^{-/-}$ mice, in contrast to their $\delta^{+/+}$ littermates which displayed dramatic alterations in their cortical EEG after THIP. Our results suggest that *in vivo* THIP acts preferentially via $GABA_A$ receptors containing the δ subunit. This finding is in accordance with a recent study showing that THIP (30 mg/kg s.c.) induced a 50% loss of righting reflex in $GABA_A$ $\delta^{-/-}$ mice (Boehm *et al.*, 2006). $GABA_A$ $\alpha_4^{-/-}$ mice also displayed a greatly reduced sensitivity to THIP (10 and 15 mg/kg, i.p.) as assessed by behavioral paradigms including rotarod, tail flick assay and open field assay (Chandra *et al.*, 2006). Interestingly, neuroanatomical data showed a decreased expression of the α4 subunit in the $GABA_A$ $\delta^{-/-}$ mice (Peng *et al.*, 2002). Reciprocally, preliminary studies suggested a decreased expression of the δ subunit in $\alpha_4^{-/-}$ mice (Chandra *et al.*, 2006).

The time course of THIP effects we observed in the $\delta^{+/+}$ mice is consistent with pharmacokinetic studies showing that THIP can cross the blood brain barrier, the highest concentration of drug being observed in the brain 30 min after administration. Its half-life was shown to be 1.4 hours in mice (Schultz *et al.*, 1981). In addition, THIP is a poor substrate for reuptake into neurons and glia, and therefore likely to produce a more widespread tonic activation of $GABA_A$ receptors (Ebert *et al.*, 2002). Therefore, THIP administration might elicit a sustained increase in tonic inhibition.

Consistent with previous reports, we observed that THIP induced recurring spike-wave events (Lancel & Faulhaber, 1996; Lancel, 1997; Vyazovskiy *et al.*, 2005). THIP modified

dramatically the patterns of electrical activity and thus the biochemical states of neurons. It was shown that relatively minor changes in chloride concentrations in post-synaptic neurons can lead to a switch from inhibitory to excitatory activity (Marty & Llano, 2005). In addition, tonic inhibition in the thalamic and cortical slices was shown to be highly sensitive to THIP (Porcello *et al.*, 2003; Belelli *et al.*, 2005; Jia *et al.*, 2005; Drasbek & Jensen, 2006). Therefore, the effect of THIP may result in quite profound alterations in properties of networks involved in sleep regulation. Interestingly, the general anesthetic etomidate, which potentiates $GABA_A$ receptor activity, elicited similar high amplitude, recurrent spike-like events (Reynolds *et al.*, 2003). However, the origin of such EEG waveforms is still unknown.

Our results do not provide evidence for a sleep-inducing action of THIP (4 and 6 mg/kg, i.p.) in mice. THIP did not shorten sleep latency but rather tended to increase the time spent in waking after injection. NREM sleep and REM sleep latencies were prolonged after 6 mg/kg THIP. Thus, in $\delta^{+/+}$ mice the effects of THIP were more evident in the waking EEG spectrum. In contrast, sleep latencies and time spent in the different vigilance states were not modified by THIP administration in $\delta^{-/-}$ mice. Thus, in $\delta^{-/-}$ mice the minor effects of THIP were observed already in the NREM sleep spectrum, while in the $\delta^{+/+}$ mice the main effects were seen in waking and dissipated in the subsequent NREM sleep due to the induction of waking after THIP injection.

Interestingly, we observed a regional effect of THIP on the sleep EEG. In the frontal EEG, THIP enhanced the power spectrum in a broad frequency range, both in waking (0.75-21 Hz) and NREM sleep (encompassing the SWA band, as well as frequencies between 10-19 Hz), while in the parietal derivation its effects were restricted mainly to the SWA range. A

topographical difference on the sleep EEG was previously reported in mice after administration of diazepam (Kopp *et al.*, 2003).

Other drugs have been shown to increase SWA, including Tiagabine, a GABA uptake inhibitor which results in elevating the synaptic GABA levels and therefore may enhance tonic inhibition. Tiagabine partially mimics the effect of THIP: it elicited recurrent episodes of hypersynchronous EEG signals in both waking and NREM sleep in rat. Tiagabine elevated EEG activity in frequencies between 1-8 Hz and 11-16 Hz in NREM sleep in rats (Lancel *et al.*, 1998). In healthy elderly subjects, slow-wave sleep, SWA and sleep efficiency were increased after Tiagabine (Mathias *et al.*, 2001; Walsh *et al.*, 2005). Furthermore, recent studies evaluated the impact of these drugs on sustained attention and cognitive performance. In healthy human subjects, Tiagabine counteracted the effects of sleep restriction on sustained vigilance attention (Walsh *et al.*, 2006), and elderly subjects did not show any impairment of next day attention and memory function after three consecutive treatment nights with THIP (Mathias *et al.*, 2005). We observed massive effects of THIP on cortical EEG power density. The potential consequences of these changes on performance (i.e., improvement or impairment of learning, memory consolidation and retrieval) need to be further investigated.

In summary, our findings suggest that $GABA_A$ $\alpha_4\beta_3\delta$ receptors mediate the effect of THIP on the cortical waking and NREM sleep EEG. $GABA_A$ $\alpha_4\beta_3\delta$ receptors have been shown to mediate tonic currents which are highly sensitive to THIP in thalamic relay neurons. Thus, the lack of response of the $\delta^{-/-}$ mice indicates the importance of tonic inhibition in the regulation of fast oscillatory EEG activities occurring during the wake state, as well as the low frequency rhythms typical for NREM sleep.

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Abbreviations

EEG: electroencephalogram; EMG: electromyogram; i.p.: intraperitoneally; NREM: non rapid-eye movement sleep; SWA: slow-wave activity.

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Table 1. Effect of THIP on vigilance states and sleep latency

Data are mean values (min ± SEM) after saline or THIP (4 and 6 mg/kg). * $p<0.05$, post-hoc Dunnett test when significance was reached for one-way ANOVA factor 'treatment'. Comparison between genotypes was performed by two-way ANOVA factors 'genotype' x 'treatment', followed by post-hoc Dunnett test († $p<0.05$).

	$\delta^{+/+}$			$\delta^{-/-}$		
	Saline	THIP 4 mg/kg (n=9)	THIP 6 mg/kg (n=10)	Saline	THIP 4 mg/kg (n=9)	THIP 6 mg/kg (n=9)
Waking	54.5 ± 5.3	68.5 ± 6.2	73.2 ± 5.1	53.8 ± 4.9	51.8 ± 2.6	49.0 ± 3.6
NREM sleep	31.7 ± 4.5	19.6 ± 5.7	16.3 ± 5.0	32.0 ± 4.4	34.6 ± 2.3	37.0 ± 3.1
REM sleep	3.8 ± 0.9	1.9 ± 0.7	0.5 ± 0.2*	4.2 ± 0.8	3.6 ± 0.6	3.9 ± 0.8
Latency to NREM sleep	43.2 ± 7.1	32.4 ± 7.6	67.6 ± 12.8	41.1 ± 6.8	37.3 ± 3.8	31.0 ± 3.2
Latency to REM sleep (after onset of first NREM sleep episode)	14.1 ± 2.1	23.9 ± 5.7	34.9 ± 7.0*	21.9 ± 4.6	24.0 ± 5.1	30.4 ± 5.1
Latency to REM sleep (after injection)	57.2 ± 6.9	86.3 ± 7.6*, †	102.5 ± 7.1*, †	63.0 ± 8.8	61.3 ± 3.3	61.4 ± 5.1

Figure legends

Figure 1. EEG power density in the frontal derivation in waking for the first three 1.5 hr intervals after THIP (4 and 6 mg/kg) for knockout $\delta^{-/-}$ mice and wild-type $\delta^{+/+}$ littermates. Mean values of relative EEG power density after THIP (4 mg/kg: n=9 for both genotypes; 6 mg/kg: n=9 and n=10 for $\delta^{-/-}$ and $\delta^{+/+}$, respectively) expressed as percentage of EEG power of the same bin in the corresponding interval after saline injection (=100 %). Note different y-axis scaling for $\delta^{-/-}$ and $\delta^{+/+}$mice. Values are plotted at the upper limit of each bin. Horizontal lines above the curves indicate frequency bins that differed significantly from the corresponding bins after saline (two-way ANOVA factor 'treatment' x '1.5 hr interval'; $p<0.05$ post-hoc paired *t*-test).

Figure 2. EEG power density in NREM sleep (frontal derivation) for the first three 1.5 hr intervals after THIP (4 and 6 mg/kg) for knockout $\delta^{-/-}$ mice and wild-type $\delta^{+/+}$ littermates. Mean values of relative EEG power density after THIP (4 mg/kg: n=9 for both genotypes; 6 mg/kg: n=9 and n=10 for $\delta^{-/-}$ and $\delta^{+/+}$, respectively) expressed as percentage of EEG power of the same bin in the corresponding interval after saline injection (=100 %). Note different y-axis scaling for $\delta^{-/-}$ and $\delta^{+/+}$mice. Horizontal lines above the curves indicate frequency bins that differed significantly from the corresponding bins after saline (two-way ANOVA factor 'treatment' x '1.5 hr interval'; $p<0.05$ post-hoc paired *t*-test).

Figure 3: Representative examples of 12 sec raw EEG (frontal derivation) and EMG traces in waking and NREM sleep, after saline or THIP (4 mg/kg) in $\delta^{+/+}$ control mice.

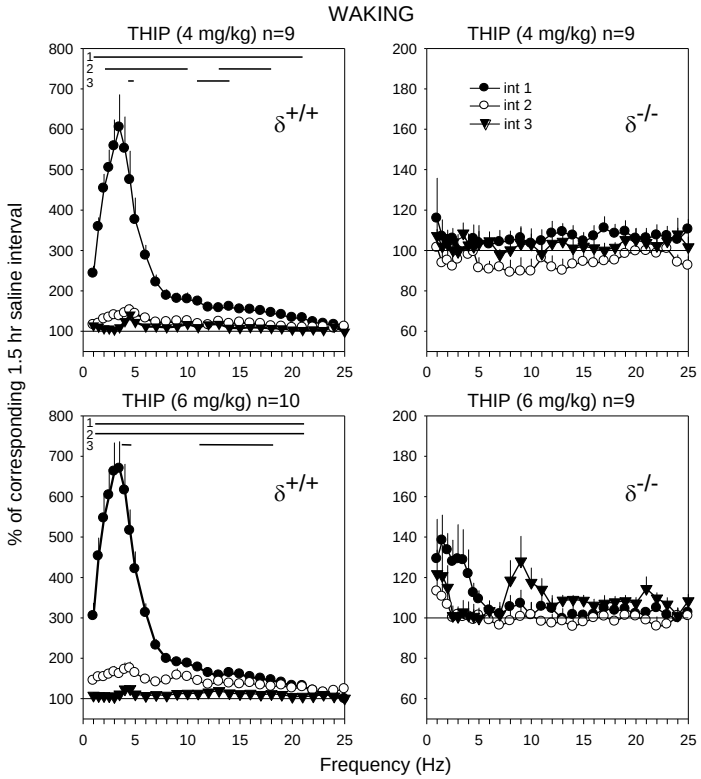
WAKING
THIP (4 mg/kg) n=9
THIP (4 mg/kg) n=9
THIP (6 mg/kg) n=10
THIP (6 mg/kg) n=9
δ+/+
δ-/-
int 1
int 2
int 3
% of corresponding 1.5 hr saline interval
Frequency (Hz)

Fig. 1

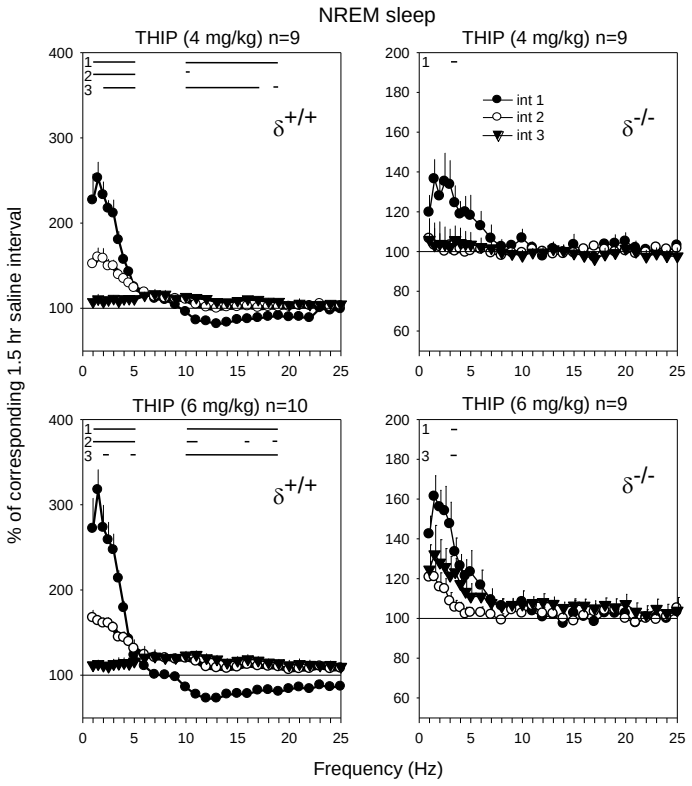
NREM sleep
THIP (4 mg/kg) n=9
THIP (4 mg/kg) n=9
THIP (6 mg/kg) n=10
THIP (6 mg/kg) n=9
δ+/+
δ-/-
δ+/+
δ-/-
int 1
int 2
int 3
% of corresponding 1.5 hr saline interval
Frequency (Hz)

Fig. 2

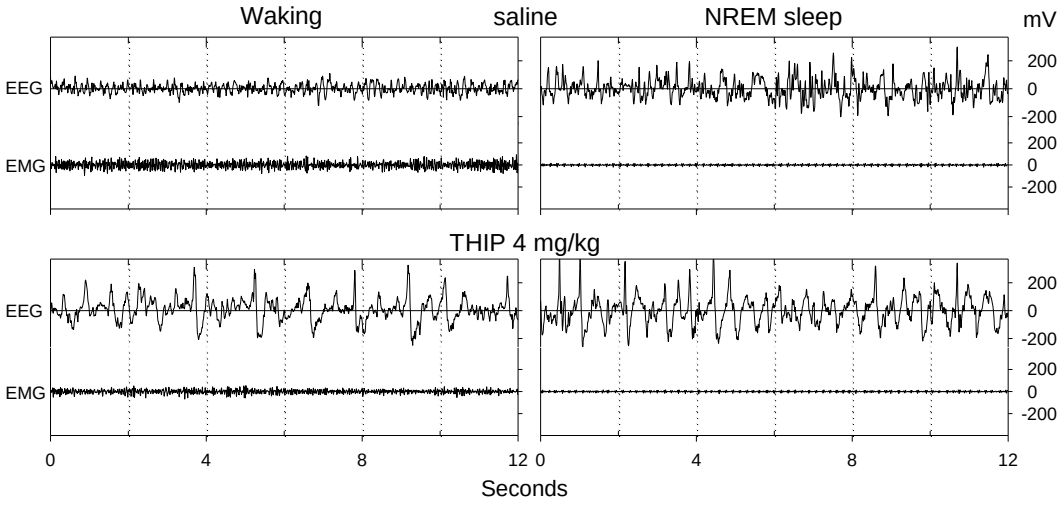
Waking
saline
NREM sleep
mV
EEG
EMG
200
0
-200
200
0
-200
THIP 4 mg/kg
EEG
EMG
200
0
-200
200
0
-200
0
4
8
12
0
4
8
12
Seconds

Fig. 3