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NEUROSCIENCE

$GABA_A$ receptors involved in sleep and anaesthesia: β1- versus β3-containing assemblies

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Abstract The histaminergic neurons of the posterior hypothalamus (tuberomamillary nucleus—TMN) control wakefulness, and their silencing through activation of $GABA_A$ receptors ($GABA_AR$) induces sleep and is thought to mediate sedation under propofol anaesthesia. We have previously shown that the β1 subunit preferring fragrant dioxane derivatives (FDD) are highly potent modulators of $GABA_AR$ in TMN neurons. In recombinant receptors containing the *β3N265M* subunit, FDD action is abolished and GABA potency is reduced. Using rat, wild-type and *β3N265M* mice, FDD and propofol, we explored the relative contributions of β1- and β3-containing $GABA_AR$ to synaptic transmission from the GABAergic sleep-on ventrolateral preoptic area neurons to TMN. In *β3N265M* mice, GABA potency remained unchanged in TMN neurons, but it was decreased in cultured posterior hypothalamic neurons with impaired modulation of $GABA_AR$ by propofol. Spontaneous and evoked GABAergic synaptic currents (IPSC) showed β1-type pharmacology, with the same effects achieved by 3 μM propofol and 10 μM PI24513. Propofol and the FDD PI24513 suppressed neuronal firing in the majority of neurons at 5 and 100 μM, and in all cells at 10 and 250 μM, respectively. FDD given systemically in mice induced sedation but not anaesthesia. Propofol-induced currents were abolished (1–6 μM) or significantly reduced (12 μM) in *β3N265M* mice, whereas gating and modulation of $GABA_AR$ by PI24513 as well as modulation by propofol were unchanged. In conclusion, β1-containing (FDD-sensitive) $GABA_AR$ represent the major receptor pool in TMN neurons responding to GABA, while β3-containing (FDD-insensitive) receptors are gated by low micromolar doses of propofol. Thus, sleep and anaesthesia depend on different $GABA_AR$ types.

Keywords Hypothalamus · Histamine · Sleep · GABA · Patch clamp

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Introduction

Propofol is the most frequently used intravenous anaesthetic. The essential goal of anaesthesia is immobility and hypnosis (unconsciousness) without awareness or memory about surgical procedures. Mild sedation and impairment of memory occurs at a propofol concentration 3% of that needed to induce immobility (reviewed in [27]). Different parts of the CNS need to be affected by propofol in order to achieve immobility (depression of spinal neurons) or amnesia and hypnosis (suppression of neuronal activity in the brain) [27]. The specific regions targeted by anaesthetics to produce hypnosis, measured by the loss of righting reflex in rodents, are not well defined. Thalamic, hypothalamic and brainstem areas were suggested to be involved [1, 36].

Different receptors and channels are targeted by the general anaesthetic propofol [5, 10, 12, 22, 25] with the γ-aminobutyric acid type A receptor ($GABA_AR$) being one of the most sensitive and important targets [14, 19, 27]. Replacing the asparagine at position 265 with serine in the β2 subunit [26] or with methionine in the β3 subunit [14] renders $GABA_AR$ containing these subunits insensitive to etomidate in vitro. In mice with the mutation *β2N265S*, the sedative action of etomidate (reduced locomotor activity and rotarod performance) was lost but immobilisation achieved by high doses of this anaesthetic (measured as loss of hind limb withdrawal reflex) was largely preserved [26]. In contrast, in mice with the mutation *β3N265M*, the immobilising action of etomidate (which is the result of complex interactions of spinal and supraspinal inhibition) is absent [14]. Potencies in the induction of hypnosis (loss of righting reflex) were similarly reduced in *β2N265S* mice for etomidate and in *β3N265M* mice for etomidate and propofol [14]. Recombinant $GABA_AR$ containing the *β3N265M* subunit are less sensitive to GABA compared to wild-type (WT) receptors [33]. This 3-fold reduction in sensitivity to agonist could contribute to functional impairment of the low-dose propofol action in vivo if this action is mediated by the high affinity $GABA_AR$ sensing ambient GABA. Unfortunately, GABA sensitivity of native mutated receptors was not yet investigated. The localisation of brain $GABA_AR$, incorporating β2 or β3 subunits, which are responsible for the reduced potency of general anaesthetics in mutant mice, is still under debate. These $GABA_AR$ (1) may represent an extrasynaptic receptor pool mediating a tonic inhibition, which regulates neuronal firing and shows high sensitivity to GABA, anaesthetics and neurosteroids [1]; (2) may be prominently expressed in specific brain circuits, mediating the action of anaesthetics, as hypothesised for the histaminergic tuberomamillary nucleus, TMN [4, 21, 42], which lacks tonic inhibition in mice [31, 42], but not in rats [29].

The histaminergic tuberomamillary nucleus (TMN) plays a prominent role in sleep–waking regulation [8, 9]. In freely moving animals, histaminergic neurons discharge tonically and specifically during waking but are silent during sleep [34, 38, 39]. Injection of a $GABA_AR$ antagonist into TMN during sleep [18] or during propofol anaesthesia [21] produces waking. Spontaneous GABAergic IPSCs are prolonged by propofol in rat and mouse TMN neurons [29, 42]. Nelson et al. [21] showed reduced cFos expression in the TMN region of rats under propofol anaesthesia. During natural sleep, TMN neurons are silenced by GABA released from VLPO neurons [6, 17, 20, 23, 35]. It is not known whether this endogenous sleep pathway, VLPO–TMN, predominantly meets the β3-type $GABA_AR$, which are particularly sensitive to anaesthetics.

We have now investigated the role of $GABA_AR$ β1 and β3 subunits in the synaptic transmission from VLPO to TMN. Using the β1-selective modulator PI 24513 [31] and propofol in rat and *β3N265M* mouse, we show now that synaptic β1-containing (FDD-sensitive) $GABA_AR$ represent the largest receptor pool in TMN neurons responding to GABA, whereas β3-containing (FDD-insensitive) receptors are gated by low micromolar propofol concentrations.

Materials and methods

Electrophysiology in native neurons, slices from rat hypothalamus

Recordings from histaminergic neurons in male Wistar rat (22–26 days old) hypothalamic slices were performed as previously described [41]. In the beginning of whole-cell voltage clamp recordings (holding potential −70 mV), TMN neurons were identified by the presence of I_A and I_H currents (for details see [24]). Inhibitory postsynaptic currents (IPSCs) were evoked by local stimulation through a bipolar stainless-steel stimulating electrode (50 μm; WPI, UK). The stimulation point was found in each slice by testing several electrode positions along the dotted line in Fig. 2a with maximal strength to get the optimal stimulation point. Stimulation strength was then reduced to evoke IPSCs with a 1/3 maximal amplitude. Visual identification of histaminergic neurons recorded in cell-attached configuration (see [41]) was confirmed at the end of experiment by application of the histamine 3 (H3) receptor agonist R-α-methylhistamine (0.2 μM): it reduced the firing rate of all investigated neurons to 65±9% of control (n=13).

Electrophysiology in native neurons, isolated from slices of mouse

Three- to eight-week-old mice carrying the point mutation β3N265M as well as their WT littermates were bred and genotyped as previously described [14]. Transverse posterior hypothalamic slices (450 μm thick) containing tuberomamillary nucleus (TMN) were prepared from adult male mice and incubated for 1 h 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 and D-glucose 10, bubbled with carbogen (pH 7.4). The TMN was dissected after incubation with papain in crude form (0.3–0.5 mg/ml) for 40 min at 37°C. After rinsing, the tissue was placed in a small volume of recording solution with the following composition (mM): NaCl 150, KCl 3.7, $CaCl_2$ 2.0, $MgCl_2$ 2.0, HEPES 10 and glucose 10 (pH 7.4). Cells were separated by gentle pipetting and placed in the recording chamber. For the sIPSC recordings, TMN neurons were isolated in the recording chamber with the help of a vibrodissociation device [40] from slices briefly (5–10 min) pre-incubated

with papain. TMN neurons were recorded and identified as previously described [30, 31]. Briefly, patch electrodes were sterilised by autoclaving and filled with the following solution (mM): 140 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA and 10 HEPES/KOH, adjusted to pH 7.2. The cells were voltage-clamped by an EPC-9 amplifier. The holding potential was −50 mV. Experiments were conducted and analysed with commercially available software (TIDA for Windows; HEKA, Lambrecht, Germany). An acutely isolated cell was lifted into the major chute of the application system, where it was continuously perfused with the sterile control bath solution. Substances were applied through a glass capillary (application tube) 0.08 mm in diameter. 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. Data were considered for the analysis if the control GABA-evoked response (5 μM in acutely isolated neurons, 1–2 μM in cultured neurons) represented 4–30% of the maximal GABA (0.5 mM)-evoked response. Averaged percentage of maximal response (ECx) was calculated and compared between groups (no significant difference observed) within each experimental session. Fitting of data points in experiments where different concentrations of positive modulator were co-applied with GABA 5 μM was performed with the equation: $R=R_{max}/\{1+(EC_{50}/[modulator])^n\}$, where R is the relative potentiation (control GABA responses were subtracted from the potentiated responses) as a fraction of maximal potentiation R_{max} over control, EC_{50} is the modulator concentration producing a half-maximal potentiation of the control response, [modulator] is modulator (propofol or PI 24513) concentration and n is the Hill slope. Potencies of GABA, propofol or FDD for the $GABA_AR$ gating were determined as previously described [30] with R_{max}: maximal GABA-induced current. Data are presented as the mean±SEM.

Peak amplitude, the time to peak (rise time), time to decay, area and frequency of spontaneous IPSCs were recorded and analysed with MiniAnalysis 4.2 (Synaptosoft, Leonia, NJ, USA) as previously described [29, 31]. The detection threshold was set to a current of 5 pA amplitude and 20 pA×ms (fC) area. The frequency, amplitude and time to decay (to 30% of peak in a 100-ms window) of sIPSCs was determined from all automatically detected events after false positives were removed during visual inspection of the recording traces. Values obtained from sIPSCs collected in this way were plotted as cumulative histograms and compared with the Kolmogorov–Smirnov two-sample test in each cell between control (before and washout periods together) conditions versus presence of modulator (each of three testing periods lasted 60–90s). The significance level was set at $p<0.05$. Data presented in Fig. 3 were obtained after 'curve fitting' of the 'control' or '+modulator' events collected within each period, after their alignment followed by their averaging; decay time constants were obtained in this case by fitting a double exponential to the falling phase of the averaged events.

$GABA_AR$ expression analysis in native cells (single-cell RT–PCR)

Amplification of cDNAs encoding for histidine decarboxylase and $GABA_AR$ subunits was performed as previously described [30, 31]. Thin-walled PCR tubes contained a mixture of first strand cDNA template (1 μl), 10× PCR buffer, 10 pM each of sense and antisense primer, 200 μM of each dNTP and 2.5 U Taq polymerase. The final reaction volume was adjusted to 10 μl with nuclease-free water (Promega, Mannheim, Germany). The magnesium was taken at 2.5 mM. The Taq enzyme, PCR buffer, Mg^{2+} solution and four dNTPs were all purchased from Qiagen (Erkrath, Germany). All oligonucleotides were synthesised by MWG-Biotech (Ebersberg, Germany). Products were visualised by staining with ethidium bromide and analysed by electrophoresis in 2% agarose gels. PCR products after $GABA_AR$ subunit-specific amplification from positive control (posterior hypothalamus) and TMN neurons were the following: α1 (227 bp), α2 (234 bp), α3 (231 bp), α5 (230 bp), β1 (397 bp), β2 (254 bp), β3 (527 bp), γ1 (262 bp), γ2 (234 bp) or epsilon (406 bp). Randomly selected PCR products were purified in water and sequenced. Comparison with corresponding GenBank sequences revealed no mismatches.

Electrophysiology and immunohistochemistry in primary dissociated cultures

Posterior hypothalamic primary dissociated cultures were prepared from newborn mice as previously described [29, 31]. Briefly, animals were anaesthetised with isofluran and decapitated, and coronal slices were cut in phosphate-buffered saline. The posterior hypothalamus was dissected and triturated after trypsinisation (20 min) and washed in nutrient medium consisting of fetal calf serum (10%), minimal essential medium (Eagle, 89%), glucose (0.8%), glutamine (2 mM), insulin (0.1 U/ml) and HEPES (10 mM). Dissociated cells were plated in a density of 1 to $2\times10^5/cm^2$ onto polyethylenimine-coated coverslips in a volume of 30 μl and cultured in an incubator with 5% CO_2, 95% air and 98% relative humidity, at 35.5±0.5°C. On the second day, serum-free neurobasal medium containing supplement B27 (2%) was added to the final volume of 1 ml. Whole-cell voltage clamp recordings were performed from non-identified posterior hypothalamic neurons on days 10–21 after plating using an application system

adapted for adherent cells [29]. Cultures (10–21 days after plating) were fixed in EDAC buffer [4% 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide and 0.2% *N*-hydroxysuccinimide (Sigma) prepared in 0.1 M phosphate buffer (PB), pH 7.4] overnight and post-fixed for 30 min in paraformaldehyde (4% in PB). The following primary antibodies were used for the immunostainings at dilutions indicated: rabbit anti-histamine antibody (1:1,000) (Chemicon International, Germany) and mouse monoclonal affinity purified anti-β3 antibody (1:500, Davies University of California/NIH NeuroMab Facility, USA; antibody production was supported by NIH grant U24NS050606). Detection of the immunoreactivities was carried out via fluorescence-labelled secondary antibodies: donkey anti-rabbit Texas Red IgG (1:200; Dianova, Hamburg, Germany) and Alexa Fluor 488-labelled donkey anti-mouse IgG (1:500; Molecular Probes, Eugene, OR, USA). Fixation of rat brain slices, prepared in the same way as for the recordings, was done as described above for the cultures but followed by cryoprotection and cryosectioning in 25 μm (for details see [41]). Confocal laser scanning microscopy was performed using a Zeiss LSM-510META (Zeiss, Jena, Germany). Denoised Z-stacks (ImageJ, 3d Hybrid Median Filter) were utilised for three dimensional volume reconstruction using the Volocity-4 software (Improvision, Lexington, USA). In order to selectively visualise expression of the $GABA_AR$ β3 subunit in histaminergic neurons, co-localised fluorescence was extracted from Z-stacks using ImageJ software (Wayne Rusband, NIH) before volume reconstruction.

Behavioural analysis

Six-week-old C57BL/6J mice (from the Janvier Laboratory, France) were maintained in a climate-controlled animal colony with a 12 h day/night cycle (lights on at 7:00 AM) with food and water provided ad libitum. Propofol or PI24513 were dissolved in PEG 400 and administered i.p. at a volume of 5 ml/kg of body weight before behavioural testing. The open field test was used to assess animals' exploratory activity and reactions to novel environment. A digital video camera (TSE, Bad Homburg, Germany) was mounted above the enclosures to monitor the movements. The horizontal travel distances of the animals were recorded and analysed as a parameter of exploratory locomotor activity. Distance moved was calculated for periods of 30 min. The loss of the righting reflex (LORR) was used as a measure of the hypnotic component of anaesthesia, defined as the inability of an animal to right itself when placed on its back or side. Following anaesthetic administration, LORR was assessed every 30 s. Injections of propofol and PI 24513 were performed at 11 AM.

Drugs and statistical analysis

Propofol, gabazine (SR 95531 hydrobromide) and CNQX (6-cyano-7-nitroquinoxaline-2,3-dione) were purchased from Tocris-Biotrend (Köln, Germany). PI 24513 was generously donated by Dr. Panten from Symrise GmbH & Co. KG (Holzminden, Germany). All other chemicals were obtained from Sigma-Aldrich (Taufkirchen, Germany). Drugs were diluted and stored as recommended. Neurons were recorded for at least 10 min to obtain a stable baseline before perfusion of drugs. Statistical analysis was performed with the non-parametrical Mann–Whitney *U* test if not indicated otherwise. Significance level was set at $p < 0.05$. Data are presented as mean±standard error of the mean (SEM).

Results

Propofol and *PI 24513*: gating and modulation of $GABA_AR$ in TMN neurons from WT and *β3*N265M knock-in (KI) mice

We recorded whole-cell current responses to GABA, propofol and *PI 24513* [31] in TMN neurons acutely isolated either from *β3N265M* or WT littermates in parallel experiments; at least two neurons from a WT and two neurons from a knock-in mouse were studied on the same day with the same drug dilutions in a random way. Single-cell RT–PCR was performed from these recorded neurons in order to (1) identify them—only histidine decarboxylase (HDC)-positive cells were considered for the further analysis—and (2) to determine their $GABA_AR$ expression (Fig. 1a). Fifteen KI neurons and 16 WT neurons were identified as histaminergic. Sensitivity to GABA did not differ between WT and KI neurons [half-maximal effective concentrations (EC_{50}s) were 14.2±0.9 μM (range from 12.3 to 21.6 μM) vs 15.7±0.5 μM (range from 8.4 to 25.2 μM) in WT vs KI neurons, respectively].

Propofol (12 μM) modulation (percent of control) of GABA 5 μM responses in WT (460±79%, $n=9$) did not differ significantly from KI (506±68%, $n=9$) neurons, identified as histaminergic with single-cell RT–PCR. In the presence of propofol, control GABA responses reached 68.1±3.6% (WT) and 73.8±3.1% (KI) of the maximal amplitude. Potentiation of control GABA-evoked currents by propofol 1.2 μM was 219±45% and 186±19% in WT and KI neurons, respectively ($p=0.96$). EC_{50}s for propofol modulation were also not different: 4.46±0.8 μM vs 3.9±0.5 μM for WT and *β3N265M*, respectively (Fig. 1b, right plot). TMN neurons recorded from *β3N265M* mice showed, similar to WT, modulation of their whole-cell GABA-evoked currents by PI 24513 [EC_{50}s 21.4±2.5 μM vs 22.0±3.4 μM in WT ($n=9$) vs KI ($n=9$), respectively]. PI 24513 (100 μM) modulation (percent of control) of GABA 5 μM responses in these

neurons did not differ significantly between WT (474±72.5%, n=9) and KI (506±68%, n=9) mice. In the presence of PI 24513, control GABA responses reached 65.4±6.2% (WT) and 76.2±3.4% (KI) of the maximal amplitude. The closely investigated WT (n=9) and KI (n=9) neurons did not differ in their $GABA_AR$ expression: the α2 subunit was detected in nine WT neurons and in eight KI neurons, the γ2 subunit in four WT and six KI neurons, and an identical number of cells were positive for the α1 (33.3%), α5 (11%), β1 (55.5%), β3 (89%) and γ 1 (44%) cDNAs. PCR products derived from β3-subunit amplification (n=16) were sequenced and the mutation was clearly recognised in KI neurons from sequencing chromatograms (Fig. 1a). The epsilon (ε) subunit was detected in one WT neuron.

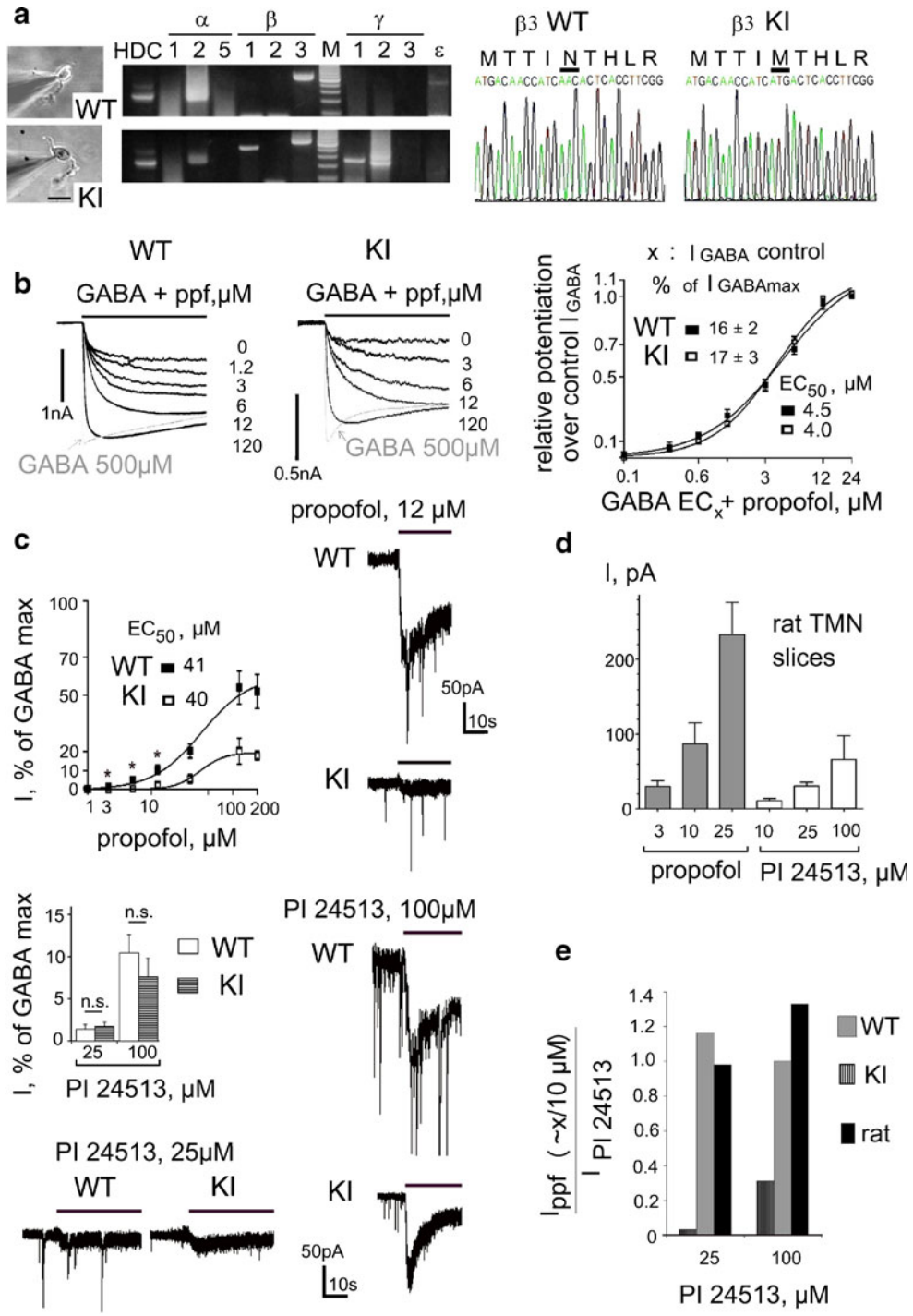
Propofol-induced currents were significantly impaired in *β3N265M* mice, when concentrations below 30 μM were used (Fig. 1c, upper plot and traces). All TMN neurons obtained from WT mice responded to propofol 6 μM (n=7), the majority of WT neurons (seven of 10) responded to propofol 3 μM, while 1.2 μM propofol induced measurable currents only in two out of 10 tested WT neurons. One of these neurons expressed the α5 subunit in addition to α2, β3, γ1 and γ2 subunits whereas in another neuron the PCR analysis of $GABA_AR$ expression was not successful. In *β3N265M* mice, none of the TMN neurons (n=14) responded to propofol 1.2 μM and only one neuron responded to propofol 3 and 6 μM. The difference in occurrence of propofol (6 or 12 μM)-induced currents in WT vs KI group was significant (Fisher's exact probability test, $p<0.05$). Dose–response curves for the GABA-mimetic action of propofol yielded EC_{50}s and gating efficacies (percent of maximal GABA response) of 41.4±12.5 μM and 60±7% vs 39.6±9.7 μM and 19.6±1.7% in WT vs KI neurons (Fig. 1c). TMN neurons recorded in rat brain slices (Fig. 1d) showed the same sensitivity to propofol as isolated mouse neurons with a concentration of 3 μM inducing currents of 30.3± 7.4 pA (n=5) in the majority of investigated neurons (83%, one neuron did not respond).

PI 24513 induced currents in rat TMN neurons in slices starting from 10 μM (10.7±2.9 pA, n=4) and in isolated mouse TMN neurons from 25 μM. In contrast to propofol, PI 24513-induced currents were preserved in *β3N265M* mice (Fig. 1c, lower plot and traces). For this reason, the ratio of averaged current amplitudes: propofol 3 μM/PI 24513 25 μM and propofol 10 μM/PI 24513 100 μM, being nearly 1.0 in WT mice and in rat TMN neurons, was less than 0.3 in *β3N265M* mice (Fig. 1e).

Evoked and spontaneous IPSCs recorded from TMN neurons are highly sensitive to PI 24513

GABA released from axons leaving the VLPO suppresses the activity of TMN neurons during sleep [32]. In keeping with this retrograde tracing study, we found that stimulation around the place indicated by the dotted line on the schematic drawing of a hypothalamic slice in Fig. 2a evokes pure GABAergic IPSCs, which are abolished by gabazine (10 μM) (n=5). Propofol (3 μM) and PI 24513 (10 μM) significantly increased the decay time constant of control eIPSCs, obtained by stimulation at 0.25 Hz, without affecting their amplitudes (Fig. 2a, b). Similarly, actions of propofol at 25 μM and PI 24513 at 100 μM were indistinguishable. Thus, there was a less than 4-fold difference in potencies between these two modulators.

Stimulation at a frequency, comparable to the natural firing rate of VLPO neurons during slow wave sleep (10 Hz [37]) caused neither summation of eIPSCs nor increased modulation by propofol in rat TMN neurons recorded in slices (Fig. 2c). Propofol modulation of eIPSCs elicited by five trains of 10 pulses at 10 Hz was similar to the modulation of low-frequency stimulation-induced eIPSCs (Fig. 2a). In some experiments, spontaneous and evoked IPSCs were simultaneously recorded and analysed. Exponential decay time constants and amplitudes under control conditions were 16.2±1.6 ms and 72.8±6.1 pA (nine neurons) vs 29 ±1.7 ms and 326± 15 pA (24 neurons) for the sIPSCs vs eIPSCs, respectively. Both displayed the same increases of decay kinetics by propofol 3 μM (Fig. 2c) and PI 24513 10 μM**.** A significant increase in the amplitudes of sIPSCs, but not eIPSCs, was observed with the two modulators. In the presence of 3 μM propofol or 10 μM PI 24513, the amplitudes and decay kinetics of sIPSC were increased to 144±11% and 159±18% (n=6) or 131±10% and 160± 28% (n=4), respectively. The same tendency for the increase in sIPSC amplitudes by high propofol and PI 24513 concentrations (Fig. 3) was seen in some isolated mouse WT neurons. The change in amplitude of sIPSCs in these experiments reached significance level only for the 12 μM of propofol in WT neurons [85±42% over control (n=7) vs 20±15% over control (n=9) in KI neurons, p= 0.03]. Effects of PI 24513 (5 and 25 μM) on sIPSCs amplitude were indistinguishable between WT and KI neurons [29±13% (n=15) over control vs 19±17% (n=9) over control (p=0.3)]. Propofol modulation of sIPSC kinetics in TMN neurons isolated from *β3N265M* mice was significantly smaller than in cells obtained from WT mice at concentrations of 1.2 and 3 μM. On average, decay time constant values of sIPSCs in control and washout (22±2 ms) and in the presence of 1.2 μM propofol (32±4 ms) recorded from the acutely isolated KI neurons were similar to the values reported by Zecharia et al. [42] for the TMN neurons recorded from slices of KI and WT mice [in control 25 to 36 ms in different experimental sessions versus 37 ms (KI) and 54 ms



(WT) under 1.5 μM of propofol]. In our study, the difference between the two animal groups (WT and KI) in prolongation of decay kinetics by propofol did not reach significance level for the propofol concentrations 6 μM and higher (Fig. 3a). Prolongation of sIPSC decay time by PI 24513 did not differ between KI and WT neurons on the whole concentration range (Fig. 3b).

Suppression of spontaneous firing of TMN neurons by propofol and PI 24513

Propofol, applied for 7 min at 5 μM, significantly reduced the spontaneous firing of three out of five rat TMN neurons (on average to 90±9% of control). PI 24513 significantly inhibited the firing of six out of seven TMN neurons at

◀ **Fig. 1** GABA-modulating and GABA-mimetic actions of propofol and PI 24513 in TMN neurons from mice and rats. **a** Photographs of two representative neurons (individual traces shown in **b** are obtained from these two neurons) and gels with PCR products amplified from mRNA harvested from these cells (*middle*). At the *right*: fragment of sequencing chromatograms for β3-subunit cDNAs of these cells. **b** Whole-cell responses to GABA and their modulation by different concentrations of propofol (*ppf*). At the *right*: concentration–response plot for the potentiation of GABA currents by propofol in WT and *β3N265M* knock-in (*KI*) HDC (histidine decarboxylase)-positive TMN neurons (nine neurons in each group). **c** Concentration–response plot for the GABA-mimetic action (as percent of maximal GABA response) of propofol (4–14 neurons analysed with different concentrations) and averaged PI 24513 (5–10 neurons)-induced currents as *bar histograms* in *β3N265M* knock-in (*KI*) and WT (wild-type) neurons. *$p<0.05$. Next to the plots: representative traces of propofol- and PI 24513-induced responses in WT compared to the KI TMN neurons with the similar amplitudes of maximal GABA-evoked currents (about 2 nA). **d** GABA-mimetic action of propofol and PI 24513 in rat TMN neurons recorded in slices. **e** Ratios of propofol- to PI 24513-induced currents with concentrations of propofol 10 times lower than indicated PI 24513 concentrations. Note that currents of comparable amplitude (ratios are close to 1.0) are evoked by 3 μM ppf and 25 μM PI24513 or by 10–12 μM ppf and 100 μM PI 24513 in rat TMN neurons as well as in WT mouse TMN neurons. Due to the impaired propofol—but not PI 24513—gating in *β3N265M* mice, the ratio is dramatically reduced

100 μM (on average to 83±6%), an effect not significantly different from that of 10 μM propofol (to 62±18%, $n=5$, Fig. 4). Thus, PI 24513 was more than 10 times less potent than propofol in the suppression of spontaneous firing of rat TMN neurons. Preserved direct and modulatory actions of FDD in TMN neurons from *β3N265M* mice, described in the present study, suggested that $GABA_A$Rs different from those containing the β3 subunit respond to PI 24513.

Co-localisation of histamine and β3-subunit immunoreactivities in posterior hypothalamic neurons

Immunostainings in rat brain slices were done in order to visualise β3-subunit distribution around and within the TMN nucleus (Fig. 5a). Not all histaminergic cells showed clear co-localisation of histamine and the β3-subunit protein. Strong neuropil staining did not allow clear background subtraction. Therefore, we performed the same staining in dissociated cultures from the posterior hypothalamus.

A three-dimensional volume reconstruction was performed for $GABA_A$R β3-subunit- and histamine-co-localised fluorescence. In 10 fields such as shown in Fig. 5b, 10 histaminergic neurons displayed the double fluorescence in the soma; in eight neurons somatic β3-subunit immunoreactivity was absent. In some of those cells, however, β3-subunit-positive synaptic clusters were seen on remote dendrites (Fig. 5b). In fields containing histaminergic neurons, the number of β3-subunit-positive/histamine-negative cells ($n=121$) exceeded the number of histaminergic neurons ($n=18$). These cells were smaller than the histaminergic neurons (Fig. 5b).

Detection of 'propofol-resistant' and 'propofol-sensitive' neurons in cultured posterior hypothalamic neurons

We recorded GABA-mediated currents from cultured neurons of 10–15 μm soma size in control and in the presence of propofol (1.2 μM). The potentiation of GABA responses by 1.2 μM propofol varied from 164% to 590% in WT neurons ($n=22$); therefore, we divided all neurons recorded from KI mice in two groups: propofol-sensitive cells and propofol-resistant cells with a modulation larger or smaller than 160% of control (Fig. 5c). Propofol-resistant cells were significantly less sensitive to GABA (EC_{50}=22.5±8 μM, $n=5$) when compared either to the WT (EC_{50}=5.8±2.2 μM, $n=7$) or to the propofol-sensitive neurons from *β3N265M* mice (EC_{50}=7.8±2.5 μM, $n=4$) (Fig. 5d). This is in keeping with the previous observation on heterologously expressed *α2β3N265Mγ2* receptors being ca. three times less sensitive to GABA (EC_{50} 122 μM) compared to the WT (EC_{50} 47 μM) receptors [33]. Thus, the mutation *β3N265M* can be characterised in some native neurons by reduced GABA sensitivity and reduced modulation by propofol, but this is not the case in TMN neurons.

PI 24513 is sedative but not hypnotic in mice

Locomotion of C57BL/6J mice was measured for 30 min in an open-field paradigm. Following an injection of PI 24513 (i.p., 0.5 or 0.2 g/kg), the activity significantly decreased by 87% ($p<0.001$; *U* test) and 75% ($p<0.01$; *U* test), respectively, compared to animals injected with the vehicle (Fig. 6). A similar behavioural reaction was shown for benzodiazepines [28]. Propofol diluted and injected in a similar way as PI 24513 produced the LORR at doses of 0.1 g/kg and 0.05 g/kg for 37.0±5.3 min and 9.5±2.1 min, respectively, while at 0.025 g/kg propofol induced sedation but not LORR (four mice tested for each dose). The LORR was never observed with PI 24513 at 0.5 g/kg (note that this dose is eight times higher than the propofol dose inducing LORR, as the molecular weights are 178 g/mol and 221 g/mol for propofol and PI24513, respectively).

Discussion

We found that β1-containing (FDD-sensitive) $GABA_A$R mediate synaptic transmission in the endogenous sleep pathway, ventrolateral preoptic area–TMN, and determine the pharmacology of GABA-evoked currents in TMN neurons (GABA potency, modulation by propofol and PI 24513), whereas β3-containing (FDD-insensitive) $GABA_A$R are targets for low micromolar concentrations of propofol which fail to gate $GABA_A$R in TMN neurons of *β3N265M* mice. As the modulatory potency of propofol

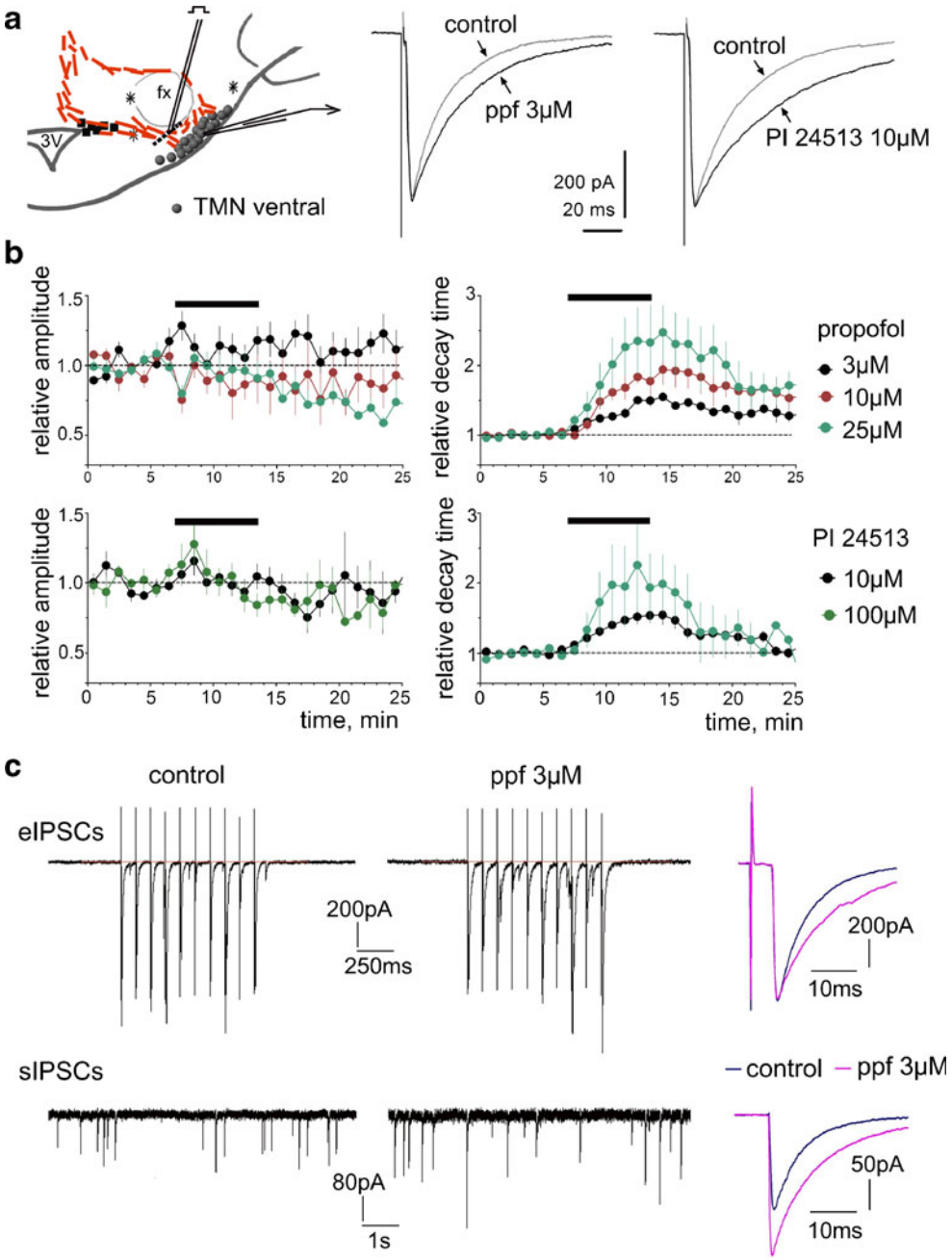


Fig. 2 TMN GABAergic IPSCs (eIPSCs) evoked by stimulation of ventrolateral preoptic area (VLPO) input are prolonged by propofol (*ppf*) and PI 24513. **a** Position of stimulating and recording electrodes shown on schematic drawing of coronal hypothalamic slice (ventral TMN). *fx* fornix, *3V* third ventricle. At the *right*: representative recordings (each trace is an average of 75 consecutive responses taken at the end of the control period and at the end of a 7-min bath perfusion with the modulator). **b** Dose-dependent action of propofol (*ppf*) on relative amplitude and decay time of eIPSCs relative to control. *Below*: dose-dependent action of PI 24513 on relative amplitude and decay time. Each concentration of each modulator was tested in separate slices. Each *point* represents the average from three to five neurons. Note that a comparable increase in decay time is caused by PI 24513 10 μM and ppf 3 μM or by PI24513 100 μM and ppf 25 μM. None of modulators changed the amplitude of eIPSCs. **c** *Upper traces*: evoked IPSCs (eIPSCs) at 10 Hz are modulated by propofol in a way similar to those evoked by single stimuli (in **a**). *Right*: averaged eIPSCs in control and under propofol (n=40 each) recorded from the same neuron. *Below*: spontaneous IPSCs (sIPSCs) in control and under propofol. *Right*: averaged sIPSCs (control, n=228; propofol, n=245)

measured for macroscopic GABA-evoked currents did not differ between WT and *β3N265M* TMN neurons, we can explain the difference in sIPSC modulation by low but not high propofol doses by the contribution of presumably extrasynaptic β3-containing receptors to the 'tail' kinetics of synaptic currents. Evoked and spontaneous IPSCs in rat brain slices and sIPSCs in acutely isolated mouse TMN neurons are highly sensitive to the β1-selective $GABA_AR$

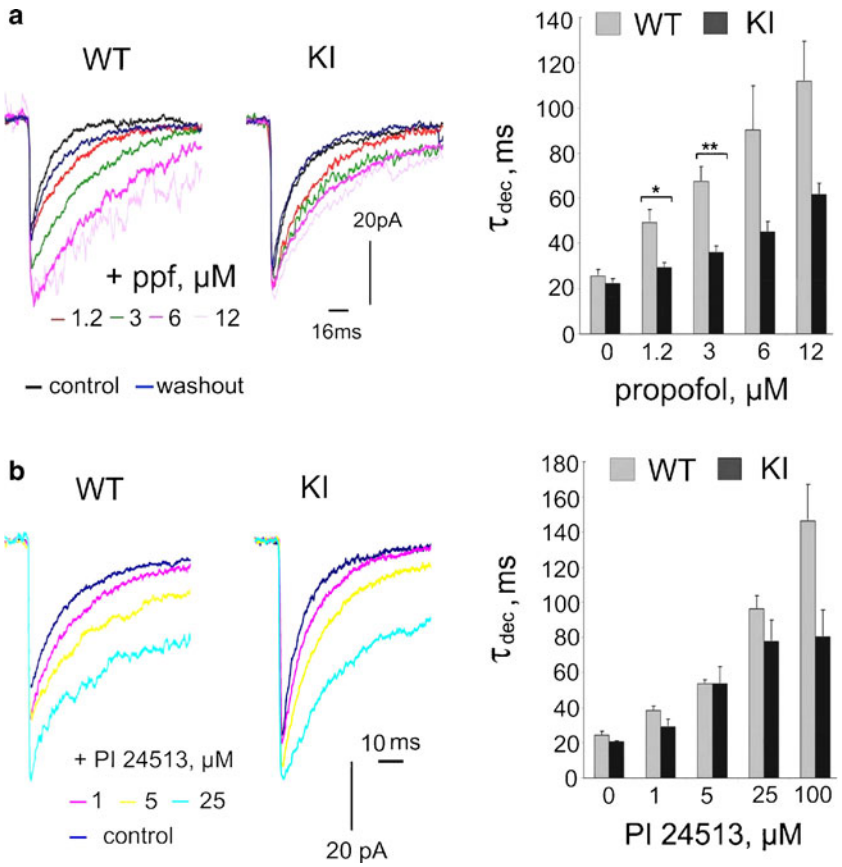


Fig. 3 Spontaneous IPSCs recorded from adult mouse TMN neurons after acute isolation from slices. **a** Averages of eight to 45 individual sIPSCs obtained from WT (*left*) and *β3N265M* KI neurons (*right*) in control and under different concentrations of propofol. At the *right*: averaged values for the exponential decay time constant of sIPSCs (obtained from individual averages shown at the *left*) in control (no drug) and under different concentrations of modulators in nine WT and nine KI neurons. **b** Averages of 13 to 49 individual sIPSCs obtained from WT and *β3N265M* TMN neurons in control and under different concentrations of PI 24513. At the *right*: averaged values of exponential decay time constant calculated from averaged traces (as shown at the *left*) from eight WT and nine KI neurons are represented in the form of *bar histograms*

modulator PI 24513, which shows an about four times lower potency when compared to propofol. Silencing of TMN neurons occurred at gating concentrations of both compounds at 250 μM for PI 24513 and at 25 μM for propofol. The threshold concentrations reducing the firing rate of TMN neurons induced inward currents of larger than 50 pA amplitude. For propofol, this concentration (5 μM) exceeded 10 times the clinically relevant dose [42]. In *β3N265M* mice, the gating efficacy of propofol but not of FDD was reduced to 33% of the WT value. Low micromolar concentrations of propofol, which induced currents in some (at 1.2 and 3 μM) or in all (at 6 μM) TMN neurons from WT mice, became ineffective in *β3N265M* mice. The maintenance of responses to 3 and 6 μM of propofol in one out of 14 KI neurons can be explained by the undetected expression of the β2 subunit, which shows similarities with the β3 subunit in responses to propofol and etomidate and is present in 16% of rat TMN neurons [29]. Further pharmacological analysis with etomidate, which gates β2- and β3- but not β1-containing $GABA_AR$, will be necessary to determine the functional expression rate of β2 subunits in TMN neurons from *β3N265M* mice.

Gating and modulatory activities of PI 24513 remained unchanged in *β3N265M* mice, indicating the recruitment of $GABA_ARs$ other than those containing β3. We have shown recently that knock-down of the β1 subunit with siRNA changes the potency of FDD for the suppression of firing of cultured hypothalamic neurons [31], but does not affect the propofol action. Thus, the suppression of firing by FDD totally relies on β1-containing $GABA_ARs$, whereas propofol below 12 μM affects mainly β3-containing receptors. Higher propofol doses most likely affect both β1- and β3-containing $GABA_ARs$ which act synergistically in suppressing neuronal activity. Due to the low efficacy and potency of FDD in gating of $GABA_AR$, it seemed rather unlikely that this compound will show anaesthetic-like activity unless synaptic β1-containing $GABA_AR$ play a prominent role. As expected, FDD produced sedation but not hypnosis or immobilisation in mice, indicating that β1-

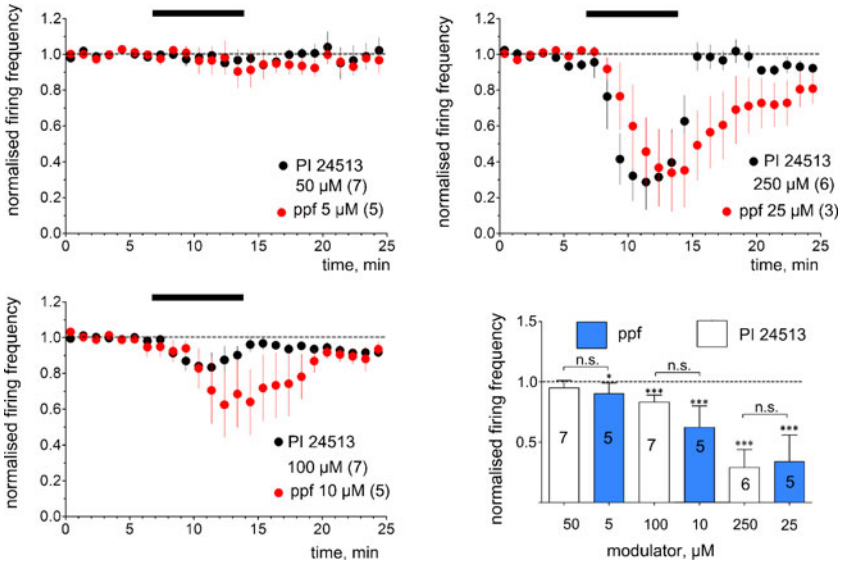


Fig. 4 Comparison of propofol (*ppf*) and PI 24513 actions on the firing frequency of rat TMN neurons recorded in slices in cell-attached mode, drug application period indicated by *black bar*. Averaged values normalised to the control frequency are presented as *columns*. *Columns* are arranged to show that similar effects are achieved by both drugs when PI 24513 was taken at a nearly 10 times higher concentration than propofol. Number of investigated neurons is given within *bars*. Each averaged value was compared with the baseline value (*t* test; **p*<0.05, ***p*<0.01, ****p*<0.005). Comparison between groups was done with the unpaired Mann–Whitney test

subunit-containing $GABA_A$Rs are not expressed in the endogenous pathways mediating the action of general anaesthetics. A previous study on *β2N265S* mutant mice showed abolished sedation and reduced hypnosis (loss of righting reflex) under etomidate [26] supporting, together with the present study, the unique role of β3-containing $GABA_A$Rs in the immobilisation under general anaesthetics [14], which most likely relies on spinal β3 $GABA_A$Rs [27].

Are hypothalamic β3-containing $GABA_A$Rs playing a critical role in hypnosis and immobilisation under propofol? Silencing of TMN neurons by propofol occurs at 50 times higher than clinical concentrations. After noise subtraction and extraction of histamine/β3 co-localisation points β3-containing $GABA_A$R were absent from the somata of many TMN neurons, some neurons displayed β3-immunoreactive clusters on their dendrites. The mRNA encoding for this subunit was detected with single-cell RT–PCR in 89% of acutely isolated neurons. Low somatic expression of the β3 subunit explains why GABA sensitivity, which is decreased in the recombinant *β3N265M*-containing $GABA_A$ receptors [33], was unchanged in identified histaminergic neurons isolated from KI mice. Thus, β1-subunit-containing (FDD-sensitive) receptors represent the largest receptor pool in TMN neurons responding to GABA, modulated by FDD and propofol, and it seems unlikely that TMN neurons represent a primary target of propofol action. Reduced propofol modulation of GABA currents and lower GABA sensitivity compared to the WT was detected in some unidentified hypothalamic neurons from *β3N265M* mice where β3-containing $GABA_A$R play a dominant role for the somatic currents. Their identity and potential role in anaesthesia awaits elucidation.

What is the receptor composition of the β3-containing $GABA_A$R super-sensitive to propofol gating? If widely expressed through the posterior hypothalamus, these receptors may indeed play an important role in the systemic response to propofol. It was shown previously that α subunits influence channel gating by propofol with the α6β3γ2 receptors being more effectively gated by 10 μM propofol compared to the α1β3γ2 receptors [15]. On the other hand, exchanging the β subunit in $GABA_A$R does not affect its gating by propofol [11]. Thus, the impaired propofol gating of $GABA_A$R in the *β3N265M* mutation can be attributed to the impairment of a particular $GABA_A$R population, containing an α subunit highly sensitive to propofol but not PI 24513. Data on recombinant $GABA_A$R exclude α1- and α2-containing populations [16]. Although α2 $GABA_A$R are gated by propofol more efficiently than α1-containing receptors [16], 10 μM propofol induced nearly five times larger currents in TMN neurons compared to the α2β2γ2L receptors [16]. Our previous studies on rat and mouse TMN neurons showed variable expression of nine different $GABA_A$R subunits in individual cells, where functional presence of ε, γ2, γ1 and β1 subunits can be proven by pharmacology [29–31]. In addition to the major type, α2, TMN neurons express α1 (33% of all neurons) and α5 (11% of neurons) subunits. In hippocampal neurons, α5-containing $GABA_A$R are never found at synapses. They represent an extrasynaptic receptor pool responsible for the tonic inhibitory currents and are

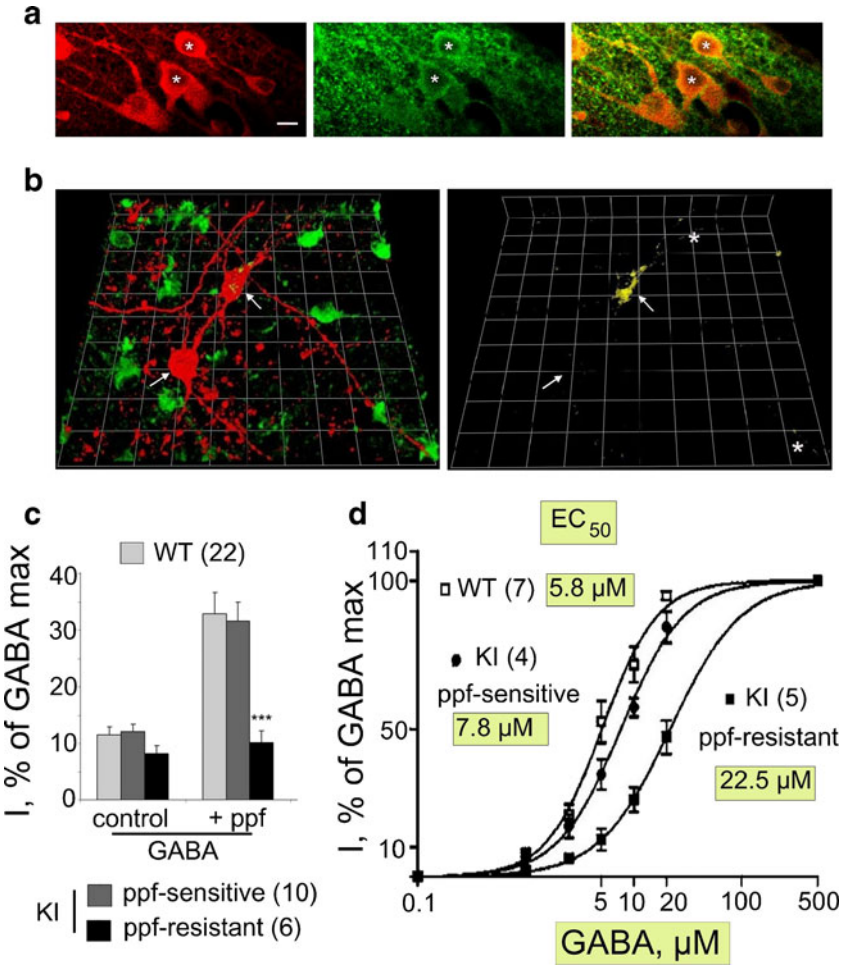


Fig. 5 Functional evidence of β3 subunit expression in posterior hypothalamus. **a** Co-localisation of β3-subunit/histamine immunoreactivities in rat brain slice containing ventral TMN. *Asterisks* indicate clearly double-stained neurons. *Scale bar*= 10 µm. **b** Volume reconstruction image of co-localisation of histamine (in *red*) and β3 subunit of $GABA_AR$ immunoreactivities in cultured rat posterior hypothalamic neurons. Extracted double-stained dots are *yellow* in the *right image*. Grid unit size=14.6 µm. *Arrows* indicate somata of histaminergic cells. *Asterisks* indicate double-stained stretches on dendrites of upper cell. **c** Modulation by propofol (*ppf*) of GABA-evoked currents in WT and KI neurons (number of investigated neurons is given in *brackets*). KI neurons were assigned as ppf resistant if modulation by ppf was below 160% of control response (GABA 1–2 µM) amplitude. **d** Concentration–response plots for GABA in three types of cells (same as in **b**). Averaged GABA concentrations evoking half-maximal response are *framed*

most frequently co-assembled with β3 subunits [13]. Taking into account the low detection rate of this subunit in TMN neurons, studies on recombinant $GABA_AR$ are warranted in order to determine activation and modulation of α5-containing receptor types by propofol vs FDD. Recombinant α5β3γ2L receptors expressed in HEK 293 cells show three times lower efficacy of gating by PI 24513 when compared to the α2β3γ2L receptors (Sergeeva and Gisselmann, unpublished observation). When excluding the ε (epsilon) subunit [29], α5-containing receptors are most favourable candidates for the tonic inhibition in rat TMN neurons. However, the question remains why mouse TMN neurons expressing this subunit at the same rate as rats do not show tonic inhibition [31]. Many previous studies have demonstrated that the tonic $GABA_AR$-mediated inhibition plays the major role for the action of anaesthetics as charge transfer through the persistent current has much more impact on neuronal firing than the fast transient synaptic currents [1]. The action of the hypnotic etomidate in α5 knockout mice is not impaired [2], questioning a major role of α5β3γ2 receptors for anaesthesia. However, this study should be considered with care, as the δ-subunit compensates for the loss of α5-containing receptors [7]. In addition, other types of α subunits may replace α5 at the extrasynaptic sites. Are the β3-containing receptors of the hypothalamus involved in sleep control? Knockout animals lacking the β3 subunit have a disturbed rest–activity cycle [3], whereas *β3N265M* mice do not differ from WT littermates in the amount of motor activities [14]. We

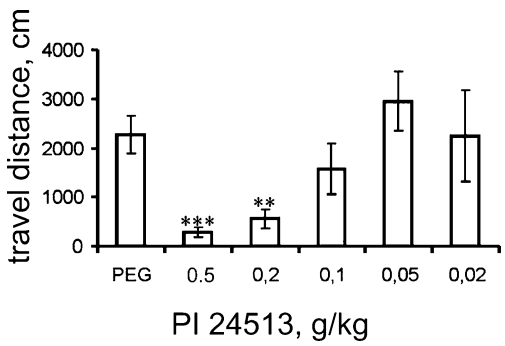


Fig. 6 PI 24513 (starting from 0.2 g/kg) significantly reduced the locomotor activity of C57BL/6J mice (by 87%). Total amount of horizontal movements in 30 min was measured starting 5 min after i.p. injection of PI 24513. Each dose was tested in a separate group of mice (n=4–6 for each dose). All values were compared with the control (vehicle, PEG 400)

demonstrate that the mutation *β3N265M* renders a reduced GABA sensitivity to native posterior hypothalamic neurons, which are inhibited by GABA during sleep [18, 20, 23, 35]. Further studies employing sleep EEG recordings from conditional, cell-type-specific knockout for β1 or β3 subunit or mutant *β3N265M* mice are warranted to determine particular roles of these and other associated subunits in the endogenous pathways for the sleep and/or anaesthesia.

In conclusion, we demonstrate that the endogenous sleep pathway in the hypothalamus operates through the β1-containing (FDD-sensitive) $GABA_AR$ whereas inhibition of neuronal firing by low propofol doses relies on β3-containing (FDD-insensitive) $GABA_AR$. Thus, sleep and anaesthesia depend on different $GABA_AR$ types.

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