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# Benzodiazepine-site pharmacology on GABAA receptors in histaminergic neurons

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## BACKGROUND AND PURPOSE
The histaminergic tuberomamillary nucleus (TMN) of the posterior hypothalamus controls the cognitive aspects of vigilance which is reduced by common sedatives and anxiolytics. The receptors targeted by these drugs in histaminergic neurons are unknown. TMN neurons express nine different subunits of the $GABA_A$ receptor ($GABA_AR$) with three α- (α1, α2 and α5) and two γ- (γ1, γ 2) subunits, which confer different pharmacologies of the benzodiazepine-binding site.

## EXPERIMENTAL APPROACH
We investigated the actions of zolpidem, midazolam, diazepam, chlordiazepoxide, flumazenil (Ro15-1788) and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) in TMN neurons using mouse genetics, electrophysiological and molecular biological methods.

## KEY RESULTS
We find the sensitivity of $GABA_AR$ to zolpidem, midazolam and DMCM significantly reduced in TMN neurons from γ2*F77I* mice, but modulatory activities of diazepam, chlordiazepoxide and flumazenil not affected. Potencies and efficacies of these compounds are in line with the dominance of α2- and α1-subunit containing receptors associated with γ2- or γ1-subunits. Functional expression of the γ1-subunit is supported by siRNA-based knock-down experiments in γ2*F77I* mice.

## CONCLUSIONS AND IMPLICATIONS
$GABA_AR$ of TMN neurons respond to a variety of common sedatives with a high affinity binding site (γ2*F77I*) involved. The γ1-subunit likely contributes to the action of common sedatives in TMN neurons. This study is relevant for understanding the role of neuronal histamine and benzodiazepines in disorders of sleep and metabolism.

## LINKED ARTICLES
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### Abbreviations
Chlordiazepoxide, 7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine-4-oxide; Diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepin-2(3*H*)-one; Div, days in vitro; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; Flumazenil, Ethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxylate; $GABA_AR$, GABA receptor type A; HE, hepatic encephalopathy; KI, knock-in; MEA, microelectrode array; Midazolam, 8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-a][1,4]benzodiazepine; RAMH, R-α-methylhistamine; TMN, tuberomamillary nucleus; WT, wild type; Zolpidem, *N*,*N*-dimethyl-2-(6-methyl-2-*p*-tolylimidazo[1,2-a]pyridin-3-yl)acetamide

## Introduction

Most frequently used drugs for the treatment of insomnia and anxiety are acting at the benzodiazepine-site of $GABA_A$ receptors ($GABA_AR$). These receptors are pentameric assemblies of subunits that form a central ion channel (in mammals: α1–6, β1–3, γ1–3, δ, π, ρ1–3, ε, θ). The GABA-binding pocket is formed at the α/β subunit interface, whereas the modulatory benzodiazepine binding site is located at the α/γ interface (Sigel, 2002) in the subunits

arrangement γ-β-α-β-α (Baumann *et al.*, 2002; Farrant and Nusser, 2005). Nineteen known $GABA_AR$ subunits co-assemble with a restricted number of preferred combinations. For example, the prevailing $GABA_AR$ type in the hypothalamus, striatum and amygdala is composed of α2, β3 and γ2-subunits (Wisden *et al.*, 1992). This molecular structure provides the basis for selective pharmacological modulation of inhibition within and between diverse neuronal networks of the brain. The preference for one of the α-subunit types by some benzodiazepine-site modulators can mark different behavioural actions: sedation and hypnosis [α1 preferring (Anaclet *et al.*, 2012)] or anxiolysis [α2 preferring (Rudolph and Mohler, 2006)]. Benzodiazepine-site ligands at the $GABA_AR$ have a long clinical history for the treatment of insomnia and support a role of GABA in sleep (Wafford and Ebert, 2008). GABA is an evolutionary old and conserved 'sleep' transmitter. Numerous studies in many species describe sleep-active GABAergic neurons inhibiting wake-active neurons during sleep (Saper *et al.*, 2005; Agosto *et al.*, 2008; Parisky *et al.*, 2008; Zimmerman *et al.*, 2008). A small number of neurons maintain an active state in the fruit fly (Parisky *et al.*, 2008), several thousands of aminergic and peptidergic neurons orchestrate waking in vertebrates (Wafford and Ebert, 2008). The histaminergic neurons in the tuberomamillary nucleus (TMN) of the posterior hypothalamus are the only aminergic neurons exhibiting a strict wake-on firing pattern being entirely silent during sleep. Histamine plays an important role for the cognitive aspects of vigilance and supports exploratory activity in a novel environment (Parmentier *et al.*, 2002; Anaclet *et al.*, 2009; Zecharia *et al.*, 2012), last but not least synaptic plasticity and learning are influenced by histamine (Haas and Panula, 2003; Haas *et al.*, 2008). TMN neurons receive a dense innervation from GABAergic sleep-active neurons in the preoptic area (Sherin *et al.*, 1998) and express at least nine different $GABA_AR$ subunits: α1, α2, α5, β1, β2, β3, γ1, γ2 and ε (Sergeeva *et al.*, 2002; 2005; 2010; Yanovsky *et al.*, 2012b,a). The $GABA_AR$ γ2-subunit is enriched at synaptic sites (Essrich *et al.*, 1998; Farrant and Nusser, 2005). Deletion of this subunit in selected types of neurons can lead to behavioural abnormalities as a consequence of the impaired synaptic inhibition (Wulff *et al.*, 2007; 2009). Zecharia *et al.* (2012) generated a novel genetic mouse model with a selective deletion, only in histaminergic neurons, of the γ2-subunit and report a marked reduction of synaptic GABAergic currents in TMN neurons from the knock-out mice compared with littermate controls, but a normal sleep-wake cycle. The authors conclude that the $GABA_AR$ on TMN neurons plays no role for sleep. GABA-currents remaining after γ2-subunit deletion were not analysed in TMN neurons; however, their presence is expected (Gunther *et al.*, 1995; Lorez *et al.*, 2000). Information is needed (i) whether γ2-containing $GABA_AR$ play a dominant role for the pharmacology of all TMN neurons and (ii) how the presence of the three α subunits (α1, α2, α5) affects benzodiazepine-site pharmacology here. Our study aims at providing answers to these questions. Mutation at the γ2-subunit F77I (*γ2F77I*) eliminates high affinity ($IC_{50}$~0.2 μM) negative allosteric modulation by methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) and the modulation of α1- and α2-containing $GABA_AR$ by zolpidem (*N*,*N*-dimethyl-2-(6-methyl-2-*p*-tolylimidazo[1,2-a]pyridin-3-yl)acetamide; Buhr *et al.*, 1997; Wingrove *et al.*, 1997). Zolpidem is inactive at α5-containing $GABA_AR$ (Puia *et al.*, 1991; Ramerstorfer *et al.*, 2010). The benzodiazepine-site pharmacology was investigated now in TMN neurons obtained from wild type (WT) and mutant *γ2F77I* mice generated by W. Wisden (Cope *et al.*, 2004).

## Methods

### *Experimental animals and slice preparation procedures*

Experiments were conducted according to the Animal Protection Law of the Federal Republic of Germany (Tierschutzgesetz BGBI.I, S.1206, revision 2006), European Communities Council directive regarding care and use of animals for experimental procedures (86/609/EEC) and the local guidelines (LANUV FB Tierschutz, Bezirksregierung, Duesseldorf, Germany). All efforts were made to minimize the number of animals and their suffering. Mice were maintained on a 12 h light-12 h dark cycle (light from 0700 h) with food and water available *ad libitum*. Slices were prepared between 0900 and 1200 h from 1 to 6 month old male mice carrying a point mutation on $GABA_AR$ γ2 subunit (γ2F77I) further referred as KI (knock-in) mice and their WT littermates (offspring of heterozygote breeding pairs). Genotyping was performed as previously described (Cope *et al.*, 2004). Coronal brain slices containing the posterior hypothalamus were prepared as described previously (Yanovsky *et al.*, 2012a,b). During preparation, NaCl was replaced by 207 mM sucrose in the ice-cold modified artificial cerebrospinal fluid saturated with carbogen (pH 7.4). Slices containing the TMN region were selected and incubated for 1 h at room temperature in a solution containing (in mM): NaCl 125, KCl 3.7, $CaCl_2$ 1.0, $MgCl_2$ 1.0, $NaH_2PO_4$ 1.3, $NaHCO_3$ 23, D-glucose 10, bubbled with carbogen (pH 7.4). The same solution was used for the patch-clamp recordings from TMN neurons in slices. For the preparation of the acutely isolated neurons, the area including TMN was dissected and incubated with papain in crude form (0.3–0.5 mg $mL^{-1}$) for 10–30 min at 37°C. Dissociation of cells and whole-cell patch-clamp recordings (as in Sergeeva *et al.*, 2010; Yanovsky *et al.*, 2012a,b) were done in a solution with the following composition (in mM): NaCl 150, KCl 3.7, $CaCl_2$ 2.0, $MgCl_2$ 2.0, HEPES 10, glucose 10 (pH 7.4). Sterile electrodes were filled with the following solution (in mM): 140 KCl, 2 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES/KOH (pH 7.2). A fast perfusion technique was used for application of ligands and modulators (Vorobjev *et al.*, 1996). Currents were recorded and analysed with TIDA for Windows software (HEKA, Lambrecht, Germany). The cells were voltage-clamped by an EPC-9 amplifier. The holding potential was –50 mV. Only neurons with no leak current and series resistance lower than 15MΩ were selected for recording. TMN neurons responded to GABA with $EC_{50}$s varying between 3.5 and 100 μM in a gabazine-sensitive way. GABA concentrations of 1–5 μM (below the $EC_{30}$) were chosen for analysis of the modulatory potency and efficacy of benzodiazepine-site ligands. The maximal GABA response (to 500 μM) was determined at the end of each experiment and the relative control response amplitude calculated (ECx). Exact $EC_X$ values are

given where appropriate; a group comparison was done if these values did not differ significantly. For the construction of concentration-response curves for the $GABA_AR$-modulator the control GABA response (ECx) was subtracted from the potentiated responses. All responses were normalized to the maximal potentiation over control, referred to as 'relative potentiation'. Data are given as the mean ± SEM. Statistical analysis was done with the non-parametrical Mann–Whitney *U*-test and Fisher's exact probability test. Significance level was set at $P < 0.05$.

Cell attached recording from TMN neurons in slices was done as previously described (Yanovsky *et al.*, 2012c). Neurons with large somata (major axes >15 μm) and at the typical location (around third ventricle: TMN medial; or at the ventral brain surface: TMN ventral) were approached and the cell-attached configuration (holding potential 0mV) was obtained. Spontaneous action potential currents were recorded (Perkins, 2006). At the end of experiments, the histamine 3 receptor agonist R-α-methylhistamine (RAMH) was applied. Only cells responding to RAMH with significant reduction of firing frequency were considered for the analysis.

Single-cell reverse transcription (RT)-PCR was performed after whole-cell patch-clamp recordings from acutely isolated TMN neurons to identify them *post hoc* through the expression of histidine decarboxylase (HDC), the histamine-producing enzyme, primers and protocols are published in Sergeeva *et al.*, (2002) and Parmentier *et al.* (2009). Transcripts encoding for the $GABA_AR$ subunits were amplified with primers published in Sergeeva *et al.* (2010). For the RT-PCR analysis of γ1- and γ2-subunit expression the following primers were used: γDg Se: 5'-TAT GT(GAT) AAC AGC ATT GG(TA) CC(TA) GT- 3' taken together either with γ1 Ase: 5'-ATC GAA GAG TAT AGA GAA CCC TTC C-3' (PCR product of 262 b.p. size) or with γ2 Ase: 5'-AAC ATC ATT CCA AAT TCT CAG CAT-3' (size of amplimer 234 b.p.). A heat dissociation protocol (PE Biosystems 5700 Software; Applied Biosystems Inc., Darmstadt, Germany) was performed at the end of each PCR amplification. Standard curves were obtained with the sequential dilution of one cDNA sample. From these curves, the linear regression coefficient (r = –0.98) and efficiency (~2.0) were calculated for the amplification of cDNAs encoding the γ1- or γ2-subunit. As previous studies have shown big changes in β–actin as well as other house-keeping genes expression between embryonic, newborn and adult brain (Mauric *et al.*, 2013), we compared γ1–subunit expression with the γ2–subunit (ΔCt = Ct γ1–Ct γ2), as both PCRs showed the same efficiency and the γ2–subunit displayed constant and widespread expression through development (Laurie *et al.*, 1992). The same amount of template (100ng) was taken in all reactions and relative level of γ1 mRNA was estimated by the $2^{-\Delta Ct}$ method.

### *Primary dissociated cultures, electrophysiological recordings and siRNA-based knock-down technique*

Primary dissociated cultures of the posterior hypothalamus were prepared from newborn mice according to the protocols previously described (Sergeeva *et al.*, 2005). Dissociated cells were plated at a density of 1 to 2 × $10^5 \cdot cm^{-2}$ onto polyethylenimine-coated microelectrode arrays (MEAs) in a volume of 100 μL (Multi Channel Systems, Reutlingen, Germany) or on coverslips (for patch-clamp recordings) and cultured in an incubator with 5% $CO_2$, 95% air and 98% relative humidity, at 37 ± 0.5°C. On the second day serum-free neurobasal medium containing supplement B27 (2%) was added to the final volume of 1 mL. Extracellular potentials were recorded on MEAs with a square grid of 60 planar Ti/TiN-microelectrodes (30 μm diameter, 200 μm spacing) at 37°C. Signals from all 60 electrodes were simultaneously sampled at 25 kHz, visualized and stored using the standard software MCRack provided by Multi Channel Systems. Spike detection was performed offline using the software SpAnNer (RESULT Medizinische Analyseverfahren, Tönisvorst, Germany). At the beginning of experiments, the basal medium was replaced by a magnesium-free HEPES-based recording solution (see above) and measurements were started after a 20 min adaptation phase. Every measurement comprised three recordings – control, test substance and washout (second control) – each 2 min long and separated by an intermediate period of 30 s. Whole-cell voltage clamp recordings were performed from non-identified hypothalamic neurons using an application system adapted for adherent cells (Sergeeva *et al.*, 2005). In knock-down experiments, the culture medium was changed on day 10–12 either to transfection medium alone or to transfection medium with four siRNAs (100 μM, Accell SMART pool, Thermo Scientific, Cat# E-059012-00) directed towards the target sequences on the mouse $GABA_AR$ γ1-subunit. A non-targeting siRNA pool or transfection medium alone were used as negative controls. Recordings of neuronal activity were done from day 6 to 28 after plating. For each treatment, cultures of about the same age (days in vitro, Div) were selected (averaged Div is provided as mean ± SEM), the difference in Div between compared groups was not significant (unpaired *t*-test).

### *Drugs and chemicals*

GABA, gabazine (SR95531), diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-1,4-benzodiazepin-2(3*H*)-one), midazolam (8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo [1,5-a][1,4]benzodiazepine), chlordiazepoxide (7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine-4-oxide) and $ZnCl_2$ were obtained from Sigma/RBI (Deisenhofen, Germany); DMCM, flumazenil (Ro15-1788; Ethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4] diazepine-3-carboxylate), RAMH, CGS 20625 and zolpidem from Biotrend (Koeln, Germany).

## Results

Benzodiazepine-site pharmacology demands presence of a γ-subunit in the $GABA_AR$ (Sigel, 2002; Farrant and Nusser, 2005). $GABA_AR$ lacking γ-subunits are blocked by $ZnCl_2$ 10 μM, whereas γ-subunit-containing receptors are weakly or not affected by this concentration (Draguhn *et al.*, 1990; Herb *et al.*, 1992). In contrast to the rat TMN neurons which are all zinc resistant (Sergeeva *et al.*, 2002), we found that, in about 30% of the mouse TMN neurons identified by the expression of HDC, GABA-responses are about halved by $ZnCl_2$ 10 μM (Kletke *et al.*, 2013). In the present study, pyrazolopyridine

CGS 20625, a two to six times more potent positive modulator of α1β2 than of α1β2γ receptors (Khom *et al.*, 2006), confirmed γ-subunit-deficiency of zinc-sensitive TMN neurons [CGS 20625's $EC_{50}$ = 1.1 ± 0.1 μM ($n$ = 5, 28% of total cell number], which is significantly different ($P$ = 0.0055) from the zinc-resistant cells ($EC_{50}$ = 3.4 ± 0.4 μM, $n$ = 13, Supporting Information Figure S1). Histaminergic neurons with the zinc-sensitive $GABA_AR$ were excluded from further analysis.

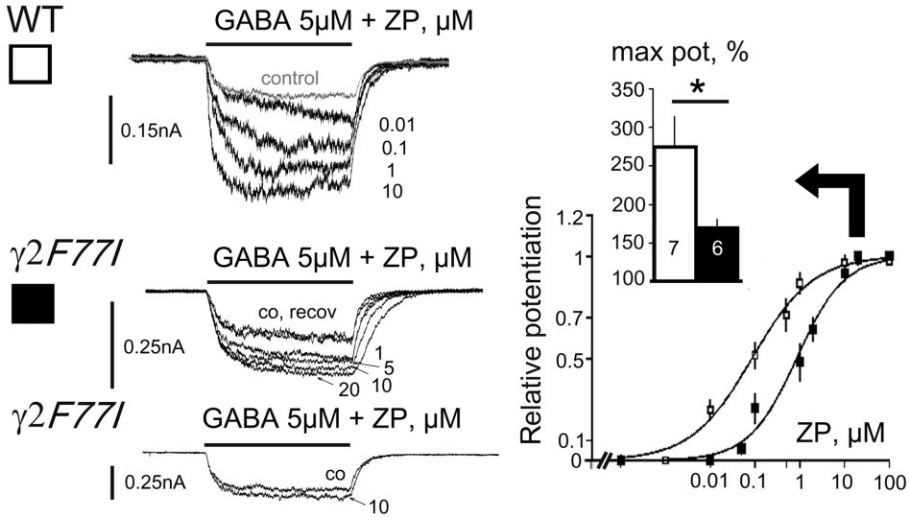
TMN neurons isolated from WT or γ2 *F77I* KI mice showed similar sensitivity to GABA ($EC_{50}$ and nHill calculated in 16 neurons: 14.6 ± 1.1 μM and 1.7 ± 0.2 vs. 13 ± 0.7 μM and 1.5 ± 0.1, respectively). In all TMN neurons from WT mice, zolpidem potentiated GABA-responses with the half-maximal concentration ($EC_{50}$) 0.08 ± 0.01 μM (nHill 0.6 ± 0.05) and maximal modulation 275 ± 40% of control (Figure 1). This indicates that α5-containing $GABA_AR$, which are not modulated by zolpidem, are not dominant in TMN neurons. In mutant γ2 *F77I* mice, a significant potentiation of GABA-evoked responses by zolpidem (1 μM) was only seen in 40% of TMN neurons. In KI neurons responding to zolpidem, the maximal potentiation was significantly smaller compared to WT mice (171 ± 10% of control, $P$ = 0.0165, Figure 1). At 1 μM, zolpidem enhanced GABA-evoked responses to 129 ± 7% of control in KI neurons which was significantly different from the WT neurons (242 ± 39%, $P < 0.005$). The potency of zolpidem was approximately 10 times lower ($P$ = 0.037) in KI mice compared to the WT littermates ($EC_{50}$ = 0.86 ± 0.2 μM, nHill = 0.8 ± 0.12). Thus, zolpidem modulation of GABA-currents in TMN neurons from γ2 *F77I* mice was significantly different from the WT neurons, supporting a functional presence of the γ2-subunit. These experiments indicate expression of another γ-containing receptor population besides the γ2-$GABA_AR$ in 40% of TMN neurons.

Diazepam potentiated GABA-responses with an $EC_{50}$ = 0.1 ± 0.01 μM ($n$ = 6, nHill: 1.7 ± 0.2) in WT and with 0.07 ± 0.003 μM ($n$ = 5, nHill: 1.4 ± 0.2) in γ2 *F77I* mice (no difference between genotypes). The potency of water soluble diazepam analogue, chlordiazepoxide, also did not show difference: GABA-responses were potentiated with an $EC_{50}$ = 0.74 ± 0.15 μM (nHill: 0.98 ± 0.2) in WT and with 0.89 ± 0.14 μM (nHill: 0.8 ± 0.1) in γ2 *F77I* mice (Supporting Information Figure S2B).

The modulatory potency of midazolam in TMN neurons differed significantly ($P$ = 0.018) between WT ($EC_{50}$ = 0.16 ± 0.04 μM, nHill = 0.8 ± 0.1, $n$ = 6) and KI neurons ($EC_{50}$ = 1.0 ± 0.2 μM, nHill = 0.9 ± 0.2, $n$ = 5), whereas maximal potentiation (357 ± 76 vs. 286 ± 62% of control, respectively) did not differ between the two genotypes (Figure 2). The rightward shift in midazolam potency in TMN neurons from γ2 *F77I*- compared to WT-mice can be explained by the presence of γ1-containing receptors (Khom *et al.*, 2006), but contribution of γ3 (Herb *et al.*, 1992) or mutant γ2 *F77I* $GABA_AR$ (Ogris *et al.*, 2004) cannot be excluded.

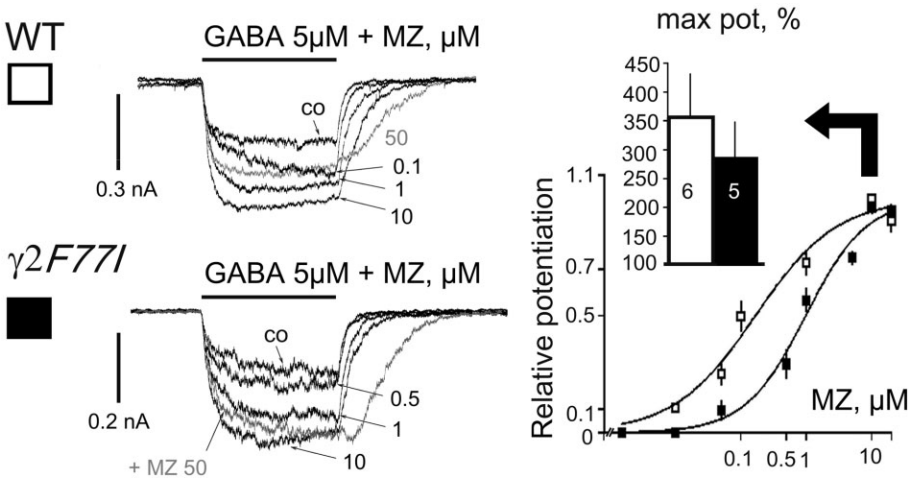
Flumazenil (Ro15-1788) potentiates some types of $GABA_AR$ including those composed of α2,β3,γ2- (Ramerstorfer *et al.*, 2010), α1,β2,γ1- (Khom *et al.*, 2006) or α1,β1- (Malherbe *et al.*, 1990) subunits. Interestingly, potentiation at α2β3γ2 receptors disappears after mutation γ2*F77I* (Ramerstorfer *et al.*, 2010). Flumazenil at concentrations 10 and 100 μM potentiated GABA-evoked currents in TMN neurons to the same extent in WT and KI mice (Figure 3), indicating that low affinity modulation is independent of the γ2*F77*-site in native neurons.

In WT mice, DMCM (from 0.05 to 1 μM) progressively inhibited GABA-responses recorded from histaminergic neurons. In the majority of cells, a further increase in DMCM concentration (up to 100 μM) resulted in an apparent reduc-



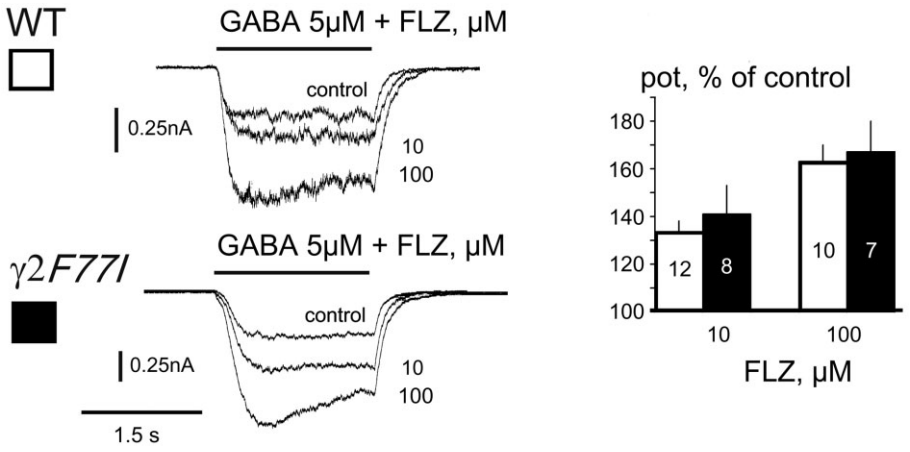

## Figure 1

Modulation of GABA-evoked currents by zolpidem in tuberomamillary nucleus neurons from wild type (WT) and mutant *γ2 F77I* mice. Representative responses to GABA in control and in the presence of zolpidem (ZP) in WT and mutant mice (left) and averaged concentration-response diagrams obtained from neurons with significant ZP modulation (right). Maximal potentiation of control response taken as 100% is shown in insert. Neurons with a modulation smaller than 15% (left, bottom) were excluded from the knock-in group. Size of analysed neuronal groups is shown in columns. GABA taken at $EC_{20}$.




## Figure 2

Modulation of GABA-evoked currents by midazolam (MZ) in mouse tuberomamillary nucleus neurons. Examples of representative recordings (left) and averaged concentration-response diagrams for the relative potentiation of GABA-responses by MZ (right). Number of investigated histaminergic neurons is shown in columns. GABA taken at $EC_{16}$.

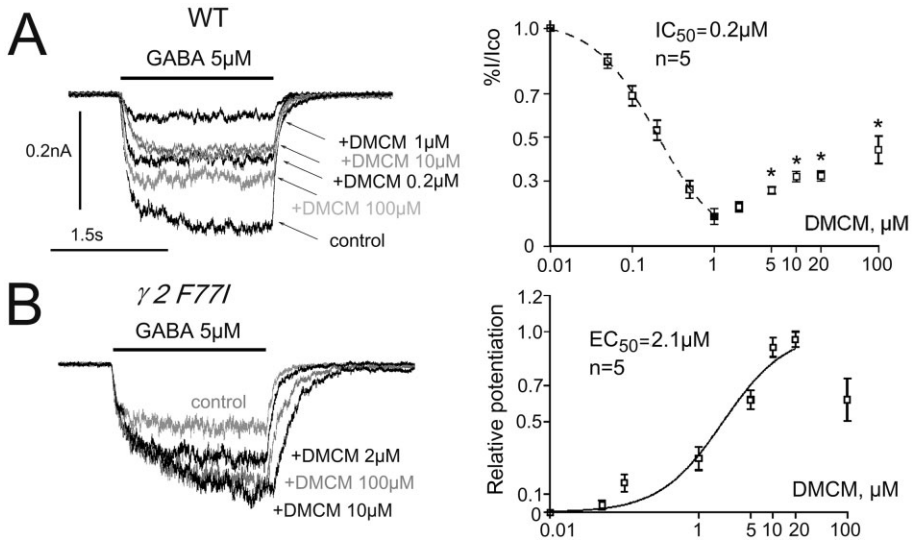



## Figure 3

Flumazenil (FLZ; Ro15-1788) modulates GABA-evoked currents to the same extent in tuberomamillary nucleus neurons from wild type (WT) and knock-in (KI) mice. Representative current traces show recordings from one WT and one KI neuron, which responded to GABA and GABA+ FLZ at indicated concentrations. Averages of potentiated current amplitude relative to the control (% of control) are shown at the right. Number of investigated histaminergic neurons is shown in columns. GABA taken at $EC_{15}$.

tion of the inhibition of the GABA-current, caused by the superimposition of a potentiation (Figure 4A). The inhibitory half-maximal concentration [$IC_{50}$ = 0.2 ± 0.01 μM, Hill coefficient 1.1 ± 0.06 ($n$ = 5)] was obtained when DMCM concentrations below 2 μM were considered for construction of the concentration – response curve. At DMCM concentrations higher than 2 μM, GABA-response modulation was significantly different from the modulation by 1 μM (Figure 4A). DMCM potentiated GABA – evoked responses in γ2 *F77I* mice with an $EC_{50}$ = 2.1 ± 0.5 μM (Hill coefficient 1.0 ± 0.2, $n$ = 5) (Figure 4B) with maximal potentiation achieved in the majority of the cells at 10 μM. In some KI neurons, modulation of GABA-responses by DMCM was negligible and these neurons were excluded from the concentration-response diagram shown in Figure 4B.

Pharmacological analysis of benzodiazepine-site ligands at $GABA_AR$ expressed by mouse histaminergic neurons indicated presence of a further γ-subunit in addition to γ2. In order to test the possibility that the zolpidem and DMCM induced potentiation of GABA-evoked responses involves γ1-containing receptors, a correlation analysis between the expression pattern of $GABA_ARs$ (scRT-PCR) and modulation of GABA-responses by these two compounds was done in KI neurons. Only cells expressing one or two γ-subunits were considered. DMCM potentiated GABA-evoked responses in neurons expressing only the γ2 subunit by 23 ± 6% ($n$ = 10)




## Figure 4

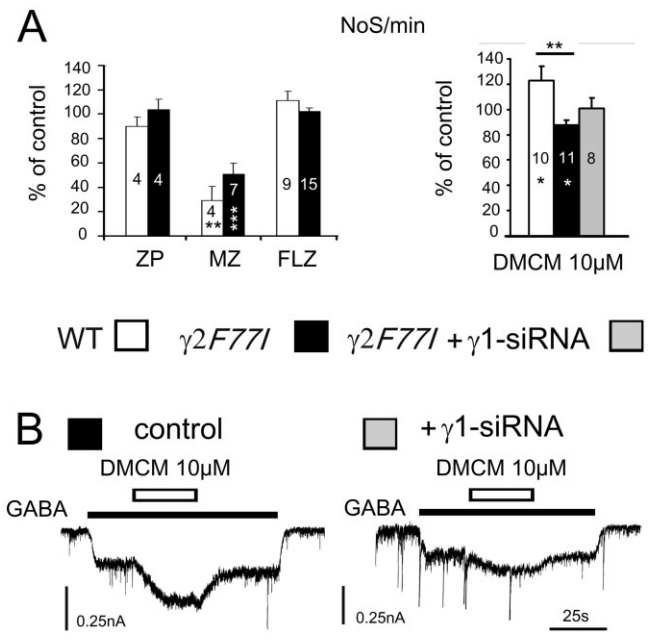
Methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) modulation of GABA-evoked currents in tuberomamillary nucleus neurons. (A) Recordings of DMCM – mediated biphasic modulation of GABA-currents in a wild type neuron (left) and averages from five neurons representing the concentration-dependent action of DMCM on GABA-currents (right). At concentrations upto 1 μM DMCM progressively inhibits GABA-currents, whereas at higher concentrations the reduction of the GABA-current is partially reversed, indicating the involvement of two modulatory sites with high (negative modulation) and low (positive modulation) affinity. Effects of high DMCM concentrations were compared with the modulation by 1 μM DMCM. Significance is indicated by stars (*$P$ < 0.05). (B) Potentiation of GABA-evoked currents by DMCM in histaminergic neuron from a knock-in (KI) mouse. At the right: averaged dose-response curve for the positive DMCM modulation of GABA-responses in KI mice obtained from seven neurons and fitted with logistic equation.

and in neurons expressing γ1 and γ2 subunits by 67 ± 22% ($n$ = 13) ($P$ = 0.009, Mann–Whitney $U$-test). Complete single-cell RT-PCR analysis of $GABA_AR$ expression was successfully performed in 16 WT and 34 KI TMN neurons. Histaminergic cells expressed α1 (25 and 32% of neurons from WT and KI group, respectively), α2 (100 and 94%), α5 (25 and 18%), β1 (31 and 38%), β2 (13 and 15%), β3 (88 and 88%), γ1 (38 and 47%) and γ2 (81 and 79%) subunits (no significant difference in occurrence of any subunit between WT and KI neurons, Fisher's exact probability test). The γ3 subunit was present in positive control (TMN whole) but not detected in individual neurons. Sequencing of selected PCR products confirmed their identity to the known mouse sequences of $GABA_AR$ subunits. We found no difference ($P$ = 0.28) between relative levels of γ1-subunit transcripts in TMN of WT (0.81 ± 0.1, $n$ = 6) and KI (0.99 ± 0.1, $n$ = 7) mice. The γ1-subunit transcripts were only slightly (~20%) less abundant than γ2-transcripts in the TMN region of adult mouse.

Next, we performed knock-down experiments using siRNA technology to test the γ1-subunit function in hypothalamic neurons. We screened $GABA_AR$ modulators (at 10 μM) for effects on firing properties in MEA recordings. Zolpidem and flumazenil did not affect firing rate significantly in hypothalamic cultures derived from WT or KI mice (Figure 5A). Midazolam suppressed spontaneous firing in both WT and γ2 *F77I* groups. Identified TMN neurons recorded in brain slices from adult WT and KI mice showed similar maximal responses to midazolam (10 μM): the firing was inhibited to 58 ± 7% of control in WT (five neurons) and to 70 ± 11% of control in γ2 *F77I* mice (six neurons). Recovery after midazolam withdrawal was delayed in WT compared to KI mice (Supporting Information Figure S3), indicating loss of the high affinity midazolam binding site in KI neurons, which is dependent on the γ2-subunit. Due to the low potency modulation by midazolam of γ3- and γ2 *F77I*-containing $GABA_AR$ (see above), this compound was considered unsuitable for the knock-down experiments.

In cultured hypothalamic neurons, the action of DMCM (10 μM, Figure 5A) on spontaneous firing frequency differed significantly between WT and γ2 *F77I* mice in accordance with $GABA_AR$ block versus positive modulation, respectively. Importantly, this different action on neuronal firing reflected the behavioural action of DMCM: convulsions in WT versus sedation in γ2 *F77I* mice (Leppa *et al.*, 2011). The action of DMCM was further investigated in γ1-knock-down experiments.

Posterior hypothalamic primary dissociated cultures containing TMN neurons were treated for 2–3 days with γ1-siRNA, non-targeting siRNA or incubated in transfection (Accell) medium. Data obtained with two different controls (non-targeting siRNA or vehicle) were pooled, as they were not different. Relative levels of γ1-subunit transcripts dropped from 3 ± 0.8 ($n$ = 4) in control to 0.29 ± 0.05 ($n$ = 4, $P$ = 0.03) in cultures treated with γ1-siRNA. Suppression of neuronal firing by DMCM 10 μM in KI cultures was abolished after γ1-siRNA treatment (Figure 5). Whole-cell patch-clamp recordings showed a significant ($P$ < 0.05) reduction of DMCM-induced positive modulation of GABA-evoked currents after γ1-siRNA treatment in neurons from KI mice (Figure 5B): under control conditions DMCM potentiated




## Figure 5

Microelectrode array (MEA) recordings from posterior hypothalamic cultures reveal functional role of γ1-subunit in methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) action. (A) Firing frequency of hypothalamic neurons (number of spikes recorded from the whole MEA in 1 min: NoS $min^{-1}$) is not significantly affected in the presence of zolpidem (ZP) [Div 11.3 ± 1.4 for the wild type (WT) and 12.5 ± 1 for the knock-in (KI) cultures] and flumazenil (FLZ) (Div 8.7 ± 0.5 for the WT and 9.9 ± 0.5 for the KI), whereas midazolam (MZ) suppressed neuronal activity (Div 8.8 ± 1.1 for the WT and 10.7 ± 0.8 for the KI). Right: DMCM increases firing frequency in WT (Div 13 ± 2) and suppresses it in KI neurons (Div 13.1 ± 1). Treatment with γ1-siRNA abolishes inhibitory activity of DMCM (Div 15.5 ± 1). Significant difference between groups is indicated with stars on top of columns, significant difference from baseline activity within columns. Number of investigated cultures is given within columns. All modulators were used at 10 μM. (B) Whole-cell voltage clamp (-50mV) recordings from two cultured neurons grown in parallel with those on the MEAs shown in A. DMCM modulation is significantly reduced in a neuron treated with γ1-siRNA. Note: preservation of synaptic GABAergic currents in neuron treated with γ1-siRNA (right).

GABA-evoked currents ($EC_{9 \pm 1}$) to 157 ± 7% of control ($n$ = 25), whereas, after γ1-siRNA treatment, potentiation of the same amplitude control currents ($EC_{9 \pm 1}$) amounted to 137 ± 4% of control ($n$ = 26). Zolpidem (10 μM) modulation of the control GABA currents declined after γ1-siRNA treatment from 168 ± 13% of control ($n$ = 5) to 108 ± 3% of control ($n$ = 9, $P$ < 0.005). Spontaneous synaptic GABAergic currents were preserved after γ1-siRNA treatment (Figure 5B). Thus $GABA_A$R-benzodiazepine-site modulators investigated in the present study have a differential impact on GABAergic control of neuronal activity: midazolam and DMCM affect firing frequency of hypothalamic neurons whereas zolpidem and flumazenil are inactive. Location and function of different $GABA_A$R types on TMN neurons might be responsible for this difference.

## Discussion and conclusions

We present a variety of clinically important benzodiazepine-site ligands increasing $GABA_A$ receptor-mediated currents on native hypothalamic TMN neurons, whose activity is thus decreased and expected to lead to a decline in cognitive performance and vigilance. A mutation within the high-affinity benzodiazepine binding site γ2 F77I changed the sensitivity of TMN neurons to zolpidem, midazolam and DMCM, but not to diazepam, chlordiazepoxide and flumazenil. If among the three known γ-subunits only the γ2-subunit would be functionally present in TMN neurons, the mutation γ2 *F77I* should have abolished the allosteric positive modulation of GABA-evoked currents by zolpidem and reduced the efficacy of diazepam. However, an additional site for the high potency zolpidem modulation was detected in KI (γ2 *F77I*) neurons, whose occurrence coincided with the expression of the γ1-subunit and with a strong potentiation of GABA-currents by DMCM. Knock-down of the γ1-subunit in hypothalamic neurons reduced the modulatory activity of DMCM and zolpidem in γ2 *F77I* mutant TMN neurons. Thus multiple types of $GABA_A$R are affected by benzodiazepine-site ligands in TMN neurons.

Our results support a dominant role of α2- or α1-, but not α5- subunits for the benzodiazepine-site pharmacology in mouse TMN neurons. Cerebellar Purkinje neurons expressing the $GABA_A$R α1-subunit display a typical zolpidem potency of about 30nM, whereas striatal neurons expressing the α2-subunit are characterized by a zolpidem potency of about 200nM (Itier *et al.*, 1996). Zolpidem modulation of GABA-responses in TMN neurons occurs at 80nM, indicating the presence of mixed α1, α2-containing receptor populations. In mutant γ2 *F77I* mice, zolpidem enhanced GABA-currents only in 40% of investigated neurons, with lower potency and efficacy than in WT neurons. Mutated γ2-containing $GABA_A$Rs might respond to zolpidem at micromolar concentrations through the anaesthetics-binding site, like it was previously shown for the diazepam modulation of αβ-receptors (Walters *et al.*, 2000). Alternatively, receptors containing the γ1-subunit (Puia *et al.*, 1991; Wafford *et al.*, 1993) or γ3-subunit (Herb *et al.*, 1992) could mediate the response to zolpidem. No potentiation of $GABA_A$R by zolpidem (1 or 10 μM) is observed in oocytes transfected with α1/2, β2/3, γ2*F77I*-subunits (Buhr *et al.*, 1997; Ramerstorfer *et al.*, 2010; Kletke *et al.*, 2013), indicating that the 'anaesthetics-binding site' present on β2 and β3 subunits is not involved. The modulatory potency and efficacy of diazepam and chlordiazepoxide was indifferent between WT and KI neurons in our study, whereas at recombinant α1β2γ2*F77I* receptors the efficacy of diazepam is reduced (Ramerstorfer *et al.*, 2010). Presence of an additional diazepam-responsive receptor population could explain this difference. Receptors composed of α1, α2 or α5, β1 and γ1-subunits are positively modulated by diazepam (Puia *et al.*, 1991) although to a smaller extent than the corresponding γ2-containing receptors. We did not detect γ3-subunit expression in individual TMN neurons of the mouse in this and in our previous studies (Sergeeva *et al.*, 2010; Kletke *et al.*, 2013), whereas the whole TMN region used as positive control contained γ3-subunit transcripts. This subunit confers zinc-resistance to the recombinant $GABA_A$ receptors and benzodiazepine-site

ligands modulate them, although with very low potencies and efficacies (Herb *et al.*, 1992). Therefore a role of this subunit for the benzodiazepine-pharmacology in TMN neurons cannot be excluded.

The structure of the $GABA_AR$ benzodiazepine-binding site is complex, but well characterized. Mutational analysis has identified the amino acid residues H101, Y161, T206 and Y209 on the α1-subunit and F77 and M130 on the γ2-subunit as putative parts forming the benzodiazepine-binding pocket (Buhr *et al.*, 1996; Sigel, 2002). Mutation at the position F77 of the γ2-subunit separates diazepam effects from zolpidem/DMCM effects: the former being unchanged or reduced in efficacy, the latter abolished (Buhr *et al.*, 1996; 1997; Wingrove *et al.*, 1997; Ramerstorfer *et al.*, 2010). The γ1-subunit carries different amino acid residues (I79 and L132) at the positions analogous to F77 and M130 of the γ2-subunit and this may be the reason for the poor modulation by benzodiazepine-site agonists. The α-type subunits influence this modulation and it was reported that association of the γ1-subunit with the α1- and β-subunits results in a greater zolpidem modulation of GABA-evoked currents compared to the α2-containing receptors (Puia *et al.*, 1991). Recombinant receptors composed of α2, β1 and γ1 subunits are not modulated by zolpidem [see Supporting Information Figure S3 in Kletke *et al.*, (2013)]. The α1-subunit thus plays an important role for the zolpidem modulation of GABA-responses in mutant mice. Further studies with α1-preferring benzodiazepine-site modulators (Anaclet *et al.*, 2012) and with mutant *α1H101R* mice (Crestani *et al.*, 2002) are warranted to determine the role of this subunit in TMN neurons.

We used DMCM for the pharmacological identification of γ1-subunit expression in TMN neurons (Puia *et al.*, 1991; Sergeeva *et al.*, 2002). Interpretation of the results is, however, complicated by several DMCM binding sites on $GABA_AR$ (Puia *et al.*, 1991; Stevenson *et al.*, 1995). The mutation *γ2F77I* abolishes the negative modulation (inverse agonism) through the high-affinity binding site, unmasking a low-affinity positive allosteric modulation, which is believed to be mediated either through the γ1- and β1-containing receptors (Puia *et al.*, 1991) or through the β2/3-containing receptors (Stevenson *et al.*, 1995). In the recombinant receptors containing the β-subunit with a mutation in the anaesthetics-binding site (e.g. *β2 N265M*), the loreclezole-like action of DMCM is abolished (Stevenson *et al.*, 1995). In Xenopus Oocytes, $GABA_AR$ composed of α2-, β3- and γ2*F77I*- subunits DMCM at 10 and 100 μM potentiates GABA-responses to 136 and 540% of control, respectively ($n$ = 5) (O. Kletke and O.A. Sergeeva, unpublished), whereas analogous β1-containing receptors are insensitive to DMCM 10 μM. Thus, the prevailing expression of the β1-subunit over the β3-subunit in histaminergic neurons (Sergeeva *et al.*, 2010; Yanovsky *et al.*, 2012a) devoid of the γ1-subunit can explain the lack of positive modulation of GABA-currents by DMCM or zolpidem. Experiments with transient γ1-subunit knock-down performed in hypothalamic cultures revealed that positive modulation by DMCM may be dependent on both sites: α/γ1 or β3N265, as it was not abolished after γ1-siRNA treatment. Further experiments with the mutant β3N265M or double mutant β3*N265M*/γ2*F77I* mice may shed light on the relative participation of each site to DMCM modulation.

Khom *et al.*, (2006) reported that the pyrazolopyridine CGS 20625 is the most efficient positive modulator (when compared to the variety of benzodiazepine-site agonists) of the γ1-containing recombinant α1β2γ1-$GABA_AR$, which enhances GABA responses to more than 600% over control. They did not study $GABA_AR$ containing the β1 subunit, which dominate the pharmacological properties of TMN neurons (Sergeeva *et al.*, 2010; Yanovsky *et al.*, 2012a). Another pyrazolopyridine, tracazolate, modulates β1-containing $GABA_AR$ poorly, in contrast to the receptors composed of α1β3-subunits (Thompson *et al.*, 2002; Kletke *et al.*, 2013). In zinc-resistant TMN neurons, the modulatory potency of CGS 20625 changed from 2.5 μM in WT to 4.4 μM in γ2 *F77I* mice in line with the two times lower potency of this modulator at γ1- than at γ2-containing $GABA_AR$ (Khom *et al.*, 2006). Modulatory efficacy of CGS 20625 in histaminergic neurons was three times lower than in recombinant α1β2γ1 receptors (Khom *et al.*, 2006), which could be due to the different properties of CGS 20625 at β1- versus at β3-containing $GABA_AR$.

Another benzodiazepine-site ligand reported to cause pronounced positive modulation of γ1-containing $GABA_AR$ is flumazenil (Ro15-1788) (Khom *et al.*, 2006). Flumazenil potentiates recombinant α2β3γ2 receptors (but not if α1- or α5- are present) and the mutation γ2 *F77I* abolishes this potentiation (Ramerstorfer *et al.*, 2010). However, α1β1 receptors can be modulated by flumazenil (Malherbe *et al.*, 1990). Flumazenil has a beneficial action in patients suffering from hepatic encephalopathy (HE) (Laccetti *et al.*, 2000; Dursun *et al.*, 2003) with mechanisms poorly understood. Our study revealed that low potency flumazenil modulation does not depend on the benzodiazepine-site formed by the γ2 – subunit. Symptoms of subclinical and advanced HE are worsened by midazolam used for the anaesthesia (Assy *et al.*, 1999; Haq *et al.*, 2012). Potentiating extrasynaptic $GABA_AR$ in hippocampus by midazolam suppresses neuronal firing. In contrast, zolpidem, mainly enhancing phasic (synaptic) inhibition, does not affect neuronal firing rate (Farrant and Nusser, 2005). Our data obtained on hypothalamic neurons are in line with these findings in the hippocampus. We also show that midazolam-suppression of the firing rate of adult histaminergic neurons is not abolished but shortened in γ2 *F77I* mice compared to the WT mice revealing the lack of a high affinity modulatory site. Thus mutant γ2 *F77I* mice could represent a good model for studies on HE neuropathology under midazolam. Hyperammonemia, the major pathogenic factor of HE, triggers brain taurine release, which is neuroprotective through $GABA_A$ and glycine receptors. Our finding that the mutation γ2 *F77I* impairs $GABA_AR$ gating by taurine (Kletke *et al.*, 2013) will complicate interpretation of data obtained from this model. The changed benzodiazepine-site pharmacology in histaminergic neurons from γ2 *F77I* mice highlights a yet unexplored structural complexity of $GABA_AR$, whose modification under pathological conditions is far from being understood (Sergeeva, 2013). Mice with mutated or deleted γ-subunits should provide a clue to the cellular mechanisms underlying changed GABAergic transmission in several neuropsychiatric diseases including HE.

TMN neurons express different $GABA_AR$ types, which respond to the clinically important benzodiazepine-site agonists. Different synaptic pathways impinging on these

neurons may be involved in regulation of different forms of behaviour. We have shown previously that GABAergic axons from sleep-active neurons of the preoptic area form synapses on TMN neurons mainly carrying β1-containing $GABA_AR$, which are not very sensitive to propofol, whereas receptors highly sensitive to propofol contain the β3-subunit and are unlikely to play a role in this pathway (Yanovsky *et al.*, 2012a). Further studies are warranted to delineate the receptor types involved in different forms of behaviour. The recently generated HDC-cre mice (Zecharia *et al.*, 2012; Yanovsky *et al.*, 2012c) allow selective genetic manipulations of $GABA_AR$ in TMN neurons. Deletion of the γ2-subunit only in TMN neurons impairs the habituation to a novel environment, but does not affect sleep-wake patterns with GABAergic synaptic currents strongly compromised (Zecharia *et al.*, 2012). The role of remaining extrasynaptic receptors waits to be analysed. The importance of $GABA_AR$ on TMN neurons controlling wakefulness may become more obvious during pathological states accompanied by reduced vigilance and increased GABAergic tone. High histamine levels during hyperammonemia (Yanovsky *et al.*, 2012c) and loss of coordination among GABAergic inputs likely contribute to the slowed frequencies of oscillatory activity of the brain and cognitive deficits seen in hepatic encephalopathy (Sergeeva, 2013). In conclusion, benzodiazepine-site pharmacology depends not solely on γ2-containing $GABA_AR$ and its understanding is a prerequisite for rational therapeutic interventions in disorders of sleep and metabolism.

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## Conflict of interest

All authors state they have no conflicts of interest.

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## Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1** Modulation of GABA-evoked currents by CGS 20625 in mouse tuberomamillary nucleus neurons. (A) Representative recordings [upper panel shows recordings from zinc-sensitive wild type (WT) neuron and lower panel from zinc-resistant knock-in (KI) neuron]. (B) Averaged concentration-response diagrams for the relative potentiation of GABA-responses by CGS 20625 in pooled KI and WT zinc-sensitive ($EC_{50}$ = 1.1 ± 0.1 μM, $n$ = 5) versus zinc-resistant neurons ($EC_{50}$ = 3.4 ± 0.4 μM, $n$ = 13). Red and blue curves without symbols are fitted data obtained in zinc resistant WT ($EC_{50}$ = 2.5 ± 0.4 μM, $n$ = 6) and KI neurons($EC_{50}$ = 4.4 ± 0.5 μM, $n$ = 7), respectively. Note that maximal potentiation did not differ between different neuronal groups. GABA taken at $EC_{13–16}$.

**Figure S2** Modulation of GABA-evoked currents by Diazepam (DZ) and chlordiazepoxide (CDZ) in mouse histaminergic neurons. (A) Examples of representative recordings (left) and averaged concentration-response diagrams for the relative potentiation of GABA-responses by diazepam (right). Total number of investigated neurons is shown in columns. GABA response amplitude (ECx) normalized to maximal control amplitude in these experiments amounted to 16 ± 4% and 12 ± 2% in wild type (WT) and knock-in (KI) neurons, respectively. (B) Examples of representative recordings (left) and averaged concentration-response diagrams for the relative potentiation of GABA-responses by CDZ (right). Total number of investigated neurons is shown in columns. The GABA ECx in these experiments amounted to 19 ± 5% and 14 ± 2% in WT and KI neurons, respectively.

**Figure S3** Midazolam 10μM inhibits firing of tuberomamillary nucleus neurons identified by the R-α-methylhistamine (RAMH) in mouse brain slices. Averaged time course diagrams show firing frequency normalized to the 7 min control period (cell-attached voltage-clamp mode) in the presence of midazolam (upper plot) or the histamine 3 receptor agonist RAMH 2μM (lower plot) in wild type and knock-in (KI) (γ2F77I) mice. Note: no difference in response to RAMH but faster recovery to control after midazolam withdrawal in KI mice.