Pharmacological Properties of GABA_A Receptors in Rat Hypothalamic Neurons Expressing the ϵ -Subunit

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The pharmacological properties and functional role of native GABA_A receptors (GABA_ARs) were investigated in rat hypothalamic neurons expressing the ϵ -subunit with the help of whole-cell patch-clamp recording and single-cell reverse transcription-PCR. Two cell groups were identified: histaminergic tuberomammillary and orexinergic/hypocretinergic neurons. Approximately 25% of histaminergic and 70% of orexinergic neurons contained mRNA encoding for the ϵ -subunit. Double-immunofluorescence staining revealed a somatic localization of this protein in these two neuronal groups. Constitutive activity, diazepam modulation, fast desensitization of maximal currents, and activation by propofol (6–98 μ M) of GABA_ARs did not correlate with ϵ -subunit expression. Propofol at 3–12 μ M potentiated GABA-mediated currents similarly in all neurons. However, noise variance analysis of GABA-mediated currents enhanced by propofol revealed a significant difference between ϵ -positive and ϵ -negative neurons. The former displayed no difference between control and potentiated responses, and, in the latter, noise was decreased in the presence of propofol. Spontaneous IPSCs recorded in cultured hypothalamic neurons were prolonged in the presence of propofol in all ϵ -negative neurons, whereas propofol-resistant IPSCs were recorded in ϵ -positive cells. The infrequent expression of the ϵ -subunit may be a key factor in the recently discovered central role of the tuberomammillary nucleus in anesthesia.

Key words: histamine; orexin; single-cell RT-PCR; primary cultures; propofol; patch-clamp

Introduction

GABA_A receptors (GABA_ARs) are heteropentameric structures composed of 5 of 16 different subunits grouped in several classes (Bonnert et al., 1999; Korpi et al., 2002). Properties of native GABA_ARs depend on their subunit combination; for example, they can be allosterically modulated by many drugs in wide clinical use, such as benzodiazepines and anesthetics (Fritschy and Brunig, 2003). A gene encoding the ϵ -subunit of the GABA_AR was first reported by three independent groups in 1997 (Davies et al., 1997; Garret et al., 1997; Whiting et al., 1997). Expression of the ϵ -subunit gene is restricted to a few brain regions: it was found in primate hippocampal dentate gyrus and hypothalamus (Whiting et al., 1997), as well as in rat hypothalamus and aminergic nuclei (Moragues et al., 2000, 2002). During heterologous expression, this subunit has been shown to confer several properties to the GABA_ARs: Whiting et al. (1997) reported a higher rate of desensitization of maximal GABA responses in oocytes and human embryonic kidney HEK 293 cells, whereas Neelands et al. (1999) did not detect fast desensitization but described constitutive activity of ϵ -containing GABA_ARs. The same spontane-

ous activity was reported by another group (Davies et al., 2002). Controversial data were obtained by different groups regarding GABA_AR modulation by anesthetics, most probably attributable to the different expression levels of the ϵ -subunit in different artificial systems (Thompson et al., 2002): Davies et al. (1997, 2002) found ϵ -containing GABA_ARs resistant to anesthetics; Thompson et al. (1998) did not. In an elegant study by Irnaten et al. (2002), cardiac parasympathetic neurons were transfected with the ϵ -subunit, which changed the pentobarbital modulation of spontaneous IPSCs (sIPSCs) recorded from these cells: the decay time constants (τ_{dec}) of IPSCs, normally prolonged by anesthetic, were unaffected after transfection.

The properties of native ϵ -containing GABA_ARs are unknown. We showed (Sergeeva et al., 2002) that a fraction of histaminergic neurons from the tuberomammillary (TM) nucleus of the hypothalamus express the ϵ -subunit; however, functional features of this expression and the exact location of the ϵ -subunit protein in this region are unknown. The low percentage of ϵ -positive cells in the TM nucleus has prompted our investigation of another neuronal group in the posterior hypothalamus, the orexin neurons, in which the ϵ -subunit mRNA is found in all neurons with *in situ* hybridization (Moragues et al., 2003). We also studied the functional consequences of the presence of this subunit in cultured hypothalamic neurons, in which intact cells can be recorded. We addressed the localization of the ϵ -subunit protein in two identified hypothalamic neuron groups, TM and orexinergic (OX), and the related functional properties that are at variance with those found in artificially expressed receptors.

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Materials and Methods

Primary dissociated cultures of hypothalamic neurons were prepared from newborn Wistar rats. The dissected posterior hypothalamus was collected into glucose-supplemented PBS. After trypsinization and washing, cells were suspended in a 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) (Andreeva et al., 1991). After mild centrifugation (1000 rpm, 1 min), the cells were resuspended in the required volume of the nutrient medium. Then 0.03 ml of cell suspension was applied to polyethylenimine-coated glass coverslips (diameter of 12 mm; three per 35-mm-diameter Petri dish with $\sim 2 \times 10^5$ cells). Plated cells were placed in a CO₂ incubator (5% CO₂–95% air and 98% relative humidity, $35.5 \pm 0.5^\circ\text{C}$) overnight. On the next day, equal amounts of nutrient medium and neurobasal medium containing supplement B27 (2%) were added (0.4 ml of each per dish). To inhibit the proliferation of non-neuronal cells, 10 μM cytosine arabinoside was added to all cultures on *in vitro* day 6. Cultures were maintained 14–15 d before recording. Some cultures were fixed with paraformaldehyde for immunohistochemistry.

Acutely isolated hypothalamic neurons were prepared from the brains of 3- to 4-week-old male Wistar rats ($n = 14$). Transverse slices (450 μm thick) containing the TM region ("TM slice," caudal) or the perifornical area (containing orexinergic neurons, "OX slice") (see Fig. 1A, rostral) were cut and incubated for 1 hr in a solution containing the following: 125 mM NaCl, 3.7 mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 1.3 mM NaH₂PO₄, 23 mM NaHCO₃, 10 mM D-glucose, and 0.01% phenol red, bubbled with carbogen, pH 7.4. Areas indicated in gray in Figure 1A were dissected and incubated 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 (in mM): 150 NaCl, 3.7 KCl, 2.0 CaCl₂, 2.0 MgCl₂, and 10 HEPES, pH adjusted to 7.4 with NaOH. Cells were separated by gentle pipetting and placed in the recording chamber. Whole-cell patch-clamp recordings in voltage-clamp mode, fast drug application, and single-cell reverse transcription (RT)-PCR procedures were performed as described previously (Sergeeva et al., 2002). Briefly, patch electrodes were sterilized by autoclaving and filled with the following solution (in mM): 140 CsCl, 2 MgCl₂, 0.5 CaCl₂, 5 EGTA, and 10 HEPES/CsOH, adjusted to pH 7.2. The cells were voltage clamped by an EPC-9 amplifier. The holding potential was -50 mV. An acutely isolated cell was lifted into the major chute of the application system, in which it was continuously perfused with the sterile control bath solution. The substances were applied through two glass capillaries (application tubes), 0.2 mm in diameter. All solutions flowed continuously, gravity driven, at the same speed and lateral movements of the capillaries exposed a cell to either control or test solutions. Hypothalamic neurons display a high cell-to-cell variability in sensitivity to GABA (Sergeeva et al., 2002). Therefore, solutions containing propofol or diazepam plus four different GABA concentrations (2.5, 5, 10, and 20 μM) were prepared. A GABA dose–response curve was obtained for the recorded neuron, and then solutions based on a GABA concentration below the EC₅₀ (as close as possible to the EC₂₀) were taken. At the end of the experiment, the GABA concentration delivering a maximal current was again applied, and, if a rundown of the current was observed, the EC₅₀ was corrected. Cells with a rundown of $>15\%$ were excluded from the study. The exact EC value was calculated for each cell, and those tested with a GABA concentration below EC₁₀ or higher than EC₅₀ were not considered. EC values were compared with the Wilcoxon test between ϵ -negative and ϵ -positive neurons, and they were found not to be different in all cases. Averages are given in Results.

Cells in cultures selected for recording were placed under an application system, modified from Vorobjev et al. (1996) by featuring longer application tubes standing out of the major chute filled with recording solution. Experiments were conducted and analyzed with commercially available software (TIDA for Windows; HEKA Elektronik, Lambrecht, Germany). Data are given as the mean $\pm$ SEM. Statistical analysis was done with the nonparametrical Wilcoxon test and Fisher's exact probability test. The significance level was set at $p < 0.05$. Variance analysis of current fluctuations was done with MiniAnalysis 4.2 (Synaptosoft, Le-

nia, NJ); steady-state currents were computed at 128 msec bins, and at least four values were averaged from each response.

Pre-pro orexin peptide (OX) cDNA was amplified in the first amplification round with the following primers: OX up, 5'-ATT TGG ACC ACT GCA CCG AAG AT-3' and OX lo, 5'-ACA GGG ATA GAA GAC GGG TTC A-3'. In the second amplification round, OX up primer was taken in combination with OX lo2, 5'-GAG TGA GGA TGC CCG CGG-3', yielding an amplification product of 256 bp size. Amplification of histidine decarboxylase (HDC), the ϵ -subunits, and nine additional GABA_AR subunits was performed as described previously (Sergeeva et al., 2002). Primers for the ϵ -subunit span the region between exons 4 and 7 (expected size of PCR product, 406 bp) and therefore can amplify equally well the short and large (with an insert in exon 2) rat isoforms (GenBank accession numbers AF255387 and AF255385, respectively). Thin-walled PCR tubes contained a mixture of first-strand cDNA template (2–5 μl), 10 $\times$ PCR buffer (5 μl), 10 pM each of sense and antisense primer, a 200 μM concentration of each deoxyNTP (dNTP), and 2.5 U of *Taq* polymerase. The final reaction volume was adjusted to 10 μl with nuclease-free water (Promega, Mannheim, Germany). The magnesium concentration was varied for different PCR reactions: 2 mM for the GABA_AR subunit and OX amplifications and 1.5 mM for HDC-cDNA amplifications. The *Taq* enzyme, PCR buffer, Mg²⁺ solution, and four dNTPs were all purchased from Qiagen (Erkrath, Germany). All oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Amplification was performed on a thermal cycler (Mastercycler; Eppendorf, Hamburg, Germany). A two-round amplification strategy was used in each protocol. In each round, 35 cycles of the following thermal programs were used: denaturation at 94°C for 48 sec, annealing at 53°C for 48 sec, and extension at 72°C for 1 min. For the second amplification round, 1 μl of the product of the first PCR was used as a template. Products were visualized by staining with ethidium bromide and analyzed by electrophoresis in 2% agarose gels.

Immunocytochemistry. Hypothalamic slices (400–500 μm thick) containing the TM region (see Fig. 1, TM slice) or perifornical area (see Fig. 1, OX slice) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 6–8 hr, cryoprotected in PB with 20% sucrose, and cryosectioned at 25 μm thickness. Sections were mounted on gelatin-coated slides, dried, and stained according to the immunofluorescence staining protocol. The sections were first washed in PBS with 0.25% Triton X-100 (PBS-T) for 5 min and then preincubated with 1% normal donkey serum in PBS-T for 30 min at room temperature. This solution was also used to dilute a guinea pig polyclonal antibody to HDC (Acris, Bad Nauheim, Germany) at 1:1000, affinity-purified goat antiserum against orexin-A (Santa Cruz Biotechnology, Heidelberg, Germany) at 1:1000, and rabbit anti-rat ϵ -subunit antiserum (Moragues et al., 2000) at 1:500. The antibody solution was applied to the sections for 12–16 hr at 4°C . After washing, sections were incubated with Alexa Fluor 488-labeled goat anti-guinea pig IgG (1:500; Molecular Probes, Eugene, OR) to reveal the HDC immunoreactivity, with Alexa Fluor 488-labeled donkey anti-goat IgG (1:500; Molecular Probes) to reveal the OX-A immunoreactivity and Texas Red donkey anti-rabbit IgG (1:200; Dianova, Hamburg, Germany) to reveal the ϵ immunoreactivity, for 90 min at room temperature. Two negative controls (single stainings) were performed in each experiment, in which one of the primary antibodies was replaced with the normal serum, and further incubation with both secondary antibodies was performed as usual.

Stainings were analyzed with conventional fluorescence microscopy. Bandpass filters were XF22, with excitation at 485 ± 11 nm and emission at 530 ± 15 nm, for Alexa Fluor 488 and XF43 (580 ± 13.5 nm excitation and 630 ± 15 nm emission) for Texas Red (Omega Optical, Brattleboro, VT). We detected no leakage of signal through the incorrect filter for either fluorochrome, even at very strong fluorescence.

Results

ϵ -Subunit protein in TM and orexin neurons

We demonstrated previously (Sergeeva et al., 2002) that $\sim 30\%$ of TM neurons express mRNA encoding for the GABA_AR ϵ -subunit. Because we did not find any specific features of ϵ -positive neurons, two questions arose. (1) Is this protein local-

Figure 1. Two identified types of hypothalamic neurons are positive for the ϵ -subunit protein. Schematic drawings of hypothalamic slices containing histaminergic (red) and orexinergic (green) neurons (*A*) and double-immunofluorescence stainings performed on corresponding slices (*B*) illustrating the localization of the ϵ -subunit of the GABA_A receptor are shown. Dissected areas for cell dissociation and neuronal cultures used in the following experiments are indicated in gray (*A*). mt, Mammillo-thalamic tract; fx, fornix; V3, third ventricle; V3m, mammillary recess of third ventricle; cp, cerebellar pedunculi. In *B*, arrows indicate double-stained neurons in a TM slice (tuberomammillary) for HDC and the ϵ -subunit and in an OX slice (containing the perifornical area) for orexin A and the ϵ -subunit. Asterisks indicate neurons stained only for ϵ -subunit but neither HDC nor orexin. Scale bar, 20 μ m.

ized on the soma, in which current recordings are done? (2) Can one identify ϵ -positive neurons pharmacologically? The first question was addressed by double-immunofluorescence stainings and regular fluorescence microscopy. We investigated the presence of the ϵ -subunit protein in HDC- and orexin-positive neurons. Double staining was found in less than half of the HDC-positive neurons in posterior hypothalamic TM slices (Fig. 1*B*). In the OX slice (Fig. 1*A,B*, bottom), orexin-positive neurons were frequently stained by the ϵ -specific antibody; however, strong neuropil staining for the ϵ -subunit in these slices did not allow a firm conclusion whether all or only the majority were double-stained neurons. Interestingly, in this type of slices, neurons with strong single staining for the ϵ -subunit were occasionally found (Fig. 1*B*, marked with asterisks). We call these neither orexin- nor HDC-positive cells non-identified (NI) neurons. Thus, we investigate the properties of GABA-mediated responses in three cellular groups: histaminergic cells (TM), obtained from rostral and caudal slices (OX and TM, respectively), and orexinergic (OX) and non-identified (NI) neurons, both obtained from the rostral, OX slices.

We also recorded from hypothalamic neurons with intact processes and synaptic inputs in primary cultures from the posterior hypothalamus. Double stainings were performed for HDC and the ϵ -subunit or for orexin A and the ϵ -subunit. In all neuronal cultures, double-stained neurons were found. The specificity of the labeling was controlled by omitting the anti- ϵ primary serum (Fig. 2).

GABA-mediated responses and GABA_AR expression in acutely isolated neurons

All neurons obtained for the recordings from the OX and TM slices ($n = 68$) responded to GABA in a gabazine (10–20 μ M)-

Figure 2. Somatic localization of the ϵ -subunit protein in cultured hypothalamic neurons. Double-immunofluorescence stainings (top and bottom panels) are as in Figure 1. Arrows indicate double-stained neurons. Scale bar is valid for all images. Single staining (for HDC) demonstrates that the secondary antibody (donkey anti-rabbit Texas Red) has no inherent affinity to the HDC antibody (substitution of ϵ antibody with the normal serum and incubation with secondary antibodies as for double staining results in no detectable fluorescence signal).

sensitive way, indicating activation of GABA_ARs. Single-cell RT-PCR yielded amplification products of at least two of nine amplified GABA_AR subunit cDNAs in 51 neurons; and 24 of them were isolated from TM slices (among them, 23 cells were HDC positive), and 27 neurons were isolated from OX slices (among them, 13 HDC- and 7 OX-positive neurons). The soma size of HDC-positive neurons measured at major axes was $22.1 \pm 0.6 \mu$ m ($n = 21$; range of 17–27 μ m; TM slices) and $23 \pm 1.1 \mu$ m ($n = 12$; range of 17–30 μ m; OX slices). The size of OX-positive neurons was not significantly different from HDC-positive cells ($p = 0.11$): $20 \pm 2 \mu$ m ($n = 7$; range of 13–28 μ m; OX slices). NI cells ($n = 5$) from the OX slice varied in soma size from 10 to 35 μ m ($20.4 \pm 4 \mu$ m).

GABA was applied at six different concentrations in most cells to construct dose–response plots. In line with our previous findings (Sergeeva et al., 2002), EC₅₀ values of 15–25 μ M were obtained from TM cells in which single-cell RT-PCR failed to detect any GABA_AR γ -subunit. The highest GABA sensitivity (EC₅₀ values between 3 and 11 μ M) was found in TM cells, which were γ 1- and γ 2-subunit positive. Neurons expressing only the γ 2-subunit responded to GABA with an EC₅₀ higher than 25 μ M. The maximal GABA-evoked currents were not different (Wilcoxon test; $p > 0.11$) between neurons with highest (2.1 ± 0.2 nA; $n = 21$), intermediate ($EC_{50} > 11$ and $< 25 \mu$ M; 2.4 ± 0.4 nA; $n = 10$), and lowest (2.7 ± 0.6 nA; $n = 7$) GABA sensitivities. The most frequently detected GABA_AR subunits were α 2 and β 3 (expressed in >86% of neurons) (Fig. 3*A*), in accordance with *in situ* hybridization data obtained in hypothalamus (Wisden et al., 1992). Occurrence of each GABA_AR subunit mRNA (besides ϵ) in individual neurons was not different between orexinergic and hista-

Figure 3. Structure–function relationship for GABA_ARs in hypothalamic neurons. *A*, Example of GABA-mediated responses in an acutely isolated hypothalamic neuron from the perifornical area (OX slice) and single-cell RT-PCR analysis of GABA_AR expression in the same neuron. All investigated gene products are present on the bottom gel (positive control), demonstrating the expected amplicon sizes: 256 bp (orexin), 457 bp (HDC), 406 bp (ϵ), 410 bp (α 1), 434 bp (α 2), 467 bp (α 5), 397 bp (β 1), 254 bp (β 2), 527 bp (β 3), 515 bp (γ 1), and 482 bp (γ 2). As a marker for the DNA size, a 100 bp ladder (Promega) was used. The histograms summarize the GABA_AR subunit expressions in three neuronal groups; the occurrence was compared with Fisher's exact probability test and was significantly different only for the ϵ -subunit ($p < 0.05$). *B*, Photographs and corresponding whole-cell recordings (at the right) of two acutely isolated neurons (from an OX slice), demonstrating a shift of baseline current by gabazine (gz) in ϵ -negative as well as in ϵ -positive neurons.

minergic neuronal groups. The sensitivity to GABA of OX neurons (positive for the pre-pro orexin mRNA) from OX slices was not different from that of histaminergic neurons. EC_{50} values ($\pm$ SD) were $8.5 \pm 4.3 \mu$ M ($n = 12$) and $8.4 \pm 7.5 \mu$ M ($n = 7$) in HDC- and OX-positive cells, respectively. Both were not different from the values obtained for histaminergic neurons in TM slices ($8.8 \pm 7.5 \mu$ M; $n = 16$). In contrast, NI cells ($n = 4$) demonstrated significantly different ($p = 0.018$) EC_{50} values: $18.8 \pm 2.5 \mu$ M.

The maximal GABA-evoked currents in histaminergic cells obtained from the two different slice types were not different ($p = 0.18$): 2.8 ± 0.5 nA ($n = 14$) in caudal (TM slices) versus 1.8 ± 0.3 nA ($n = 12$) in OX slices. Maximal GABA-evoked

currents in OX neurons as well as in NI cells in OX slices were not different from those obtained in HDC-positive cells: 3.1 ± 0.3 nA ($n = 7$) and 2.9 ± 0.5 nA ($n = 4$), respectively. The desensitization time constant of maximal responses was not different ($p = 0.72$; Wilcoxon test) between ϵ -positive (2.8 ± 0.7 sec; $n = 4$) and ϵ -negative (2.6 ± 0.3 sec; $n = 9$) neurons.

Constitutive activity of GABA_ARs in hypothalamic neurons

In our previous study, we did not detect any constitutive activity of GABA_ARs in four tested ϵ -positive TM neurons (Sergeeva et al., 2002), expecting to recognize it as a shift in baseline current in response to gabazine (10 μ M) application. The reason for that could be an insufficient concentration of antagonist. Now we applied gabazine at 20 μ M, and, in addition, we tested two other GABA_AR antagonists, bicuculline and picrotoxin. In 10 of 17 ϵ -negative acutely isolated neurons (59%) obtained from OX and TM slices, gabazine induced a shift of baseline current (19.2 ± 4.3 pA) (Fig. 3*B*). In ϵ -positive neurons, an effect of gabazine was seen in 5 of 10 cells (shift of baseline current, 11.5 ± 2.9 pA). The difference between ϵ -positive and -negative neurons in occurrence of this phenomenon was not significant ($p = 0.28$; Fisher's exact probability test). Thus, the effect of gabazine (20 μ M) on baseline current was detected in 56% of neurons; among them, 19% were ϵ -subunit positive, and 37% lacked ϵ -subunit expression.

An effect of picrotoxin (100 μ M) on baseline current also occurred similarly in ϵ -positive and ϵ -negative cells ($p = 0.34$; Fisher's test). It was observed in 89% of ϵ -negative neurons and represented 31.3 ± 10.3 pA ($n = 8$) and in 71% of ϵ -positives in which the amplitude was 24.4 ± 18.9 pA ($n = 5$). The amplitude of a picrotoxin-induced shift in baseline current was not different from gabazine-induced changes when ϵ -negative and ϵ -positive acutely isolated hypothalamic neurons were considered together (28.6 ± 9.2 , $n = 13$ vs 16.7 ± 3.2 , $n = 15$, respectively). Picrotoxin was effective in a larger fraction of cells compared with gabazine; however, this difference did not reach significance level ($p = 0.064$; Fisher's exact probability test). Thus, spontaneous currents accounted for $\sim 1\%$ (0.03 vs 3 nA) of maximal GABA-mediated currents (see above).

In cultured neurons, which, in contrast to acutely isolated neurons, present an intact soma with processes, effects of picrotoxin were smaller ($p = 0.028$; Wilcoxon test): 3.5 ± 2.2 pA ($n = 4$) and 3.9 ± 1.5 pA ($n = 6$) in ϵ -negative and ϵ -positive neurons, respectively. Bicuculline (50 μ M) was more effective than picrotoxin in cultured neurons. It shifted the baseline by 31.7 ± 8.3 pA ($n = 7$) and by 36.3 ± 6.4 pA ($n = 4$) in ϵ -negative and ϵ -positive neurons, respectively.

Propofol-evoked currents in hypothalamic neurons

Propofol reliably activated GABA_ARs at concentrations higher than 6 μ M (Fig. 4*A*). At a concentration of 48 μ M, propofol activated $19.7 \pm 12.8\%$ ($n = 4$) and $12.1 \pm 2.3\%$ ($n = 7$) of maximal GABA (500 μ M)-mediated current in ϵ -positive and ϵ -negative neurons, respectively. The difference between these two neuronal groups was not significant ($p = 0.715$; Wilcoxon test). Propofol-evoked currents were blocked by gabazine (20 μ M; $n = 3$) or bicuculline (50 μ M; $n = 4$), indicating involvement of GABA_ARs (Fig. 4*B*).

Propofol-modulation of GABA responses in hypothalamic neurons

Propofol ($>1 \mu$ M) potentiated submaximal (not maximal) GABA-mediated responses (Fig. 4*C,D*). Dose–response curves

Figure 4. Propofol modulates and activates hypothalamic GABA_ARs. *A*, Example of propofol-mediated currents ($>6 \mu\text{M}$) in acutely isolated TM neurons. At $<6 \mu\text{M}$, propofol (ppf) potentiates GABA responses. This includes potentiation of the amplitude of submaximal (not maximal) GABA-mediated responses and prolongation of the washout kinetic of maximal responses. *B*, The GABA_AR antagonist bicuculline (bic) abolishes propofol-mediated currents. The bottom-most trace shows a superimposition of the top traces. *C*, Example of GABA-response potentiation by different concentrations of propofol. *D*, Averaged dose–response curves for propofol potentiation of control GABA responses (at $EC_{17.3} \pm 6.1$ vs $EC_{16.6} \pm 4.2$ in ϵ -negative and ϵ -positive neurons, respectively). All amplitudes are plotted as percentage of maximal GABA-mediated response. Data are obtained from four ϵ -negative and five ϵ -positive neurons.

revealed no different potencies of propofol in modulating GABA-evoked responses ($p = 0.465$; Wilcoxon test) between ϵ -negative ($5.3 \pm 0.4 \mu\text{M}$; $n = 4$) and ϵ -positive ($8.1 \pm 0.5 \mu\text{M}$; $n = 5$) cells (Fig. 4*D*). The corresponding slope functions (0.89 ± 0.06 and 0.78 ± 0.04) were also not different ($p = 0.16$; Wilcoxon test). At $6 \mu\text{M}$, propofol potentiated GABA responses to $190 \pm 16\%$ in ϵ -negative neurons ($n = 12$; $EC_{25} \pm 2.8$) and to $180 \pm 23\%$ in ϵ -positive neurons ($n = 6$; $EC_{26} \pm 4.2$). At $12 \mu\text{M}$, propofol potentiated GABA responses to $267 \pm 17\%$ in ϵ -negative neurons ($n = 15$; $EC_{27} \pm 2.6$) and to $276 \pm 33\%$ in ϵ -positive neurons ($n = 7$; $EC_{27} \pm 3.1$). The difference in amplitude potentiation between these two neuronal groups was not significant for both concentrations of propofol (Fig. 5). We noticed, however, that potentiated GABA responses in ϵ -negative neurons were essentially devoid of noise, similar to maximal GABA responses, whereas ϵ -positive neurons displayed no reduction of steady-state current fluctuations (noise). We quantified current fluctuations with stationary noise analysis and normalized the obtained variances (σ^2) on variances in control response (Fig. 5). For $6 \mu\text{M}$ propofol, the ratio $\sigma^2/\sigma^2_{\text{control}}$ was significantly different ($p = 0.028$; Wilcoxon test) between ϵ -negative and ϵ -positive neurons [0.44 ± 0.07 ($n = 12$) vs 1.08 ± 0.15 ($n = 6$), respectively]. The same was true for $12 \mu\text{M}$ propofol (Fig 5): 0.185 ± 0.04 ($n = 12$) and 1.14 ± 0.26 ($n = 6$) for ϵ -negative and ϵ -positive neurons, respectively. GABA concentrations were taken at $EC_{24} \pm 2.7$ and $EC_{19} \pm 5.4$ for ϵ -negative ($n = 12$) and ϵ -positive ($n = 6$) neurons, respectively.

Diazepam modulation of GABA-evoked currents was similar in the amplitude of potentiation (by $106 \pm 28\%$, $n = 12$ vs $112 \pm$

Figure 5. Noise variance analysis of GABA currents in control and in the presence of propofol. Representative GABA-mediated currents and their modulation by propofol (top left corner) in two acutely isolated TM neurons (HDC-positive) differing in ϵ -subunit expression are shown. To the right, enhanced fragments of steady-state or baseline currents (indicated on the corresponding pictures to the left by arrows) are shown. At the bottom are histograms illustrating averaged relative noise variances and relative amplitude values ($\pm$ SEM) in control and in the presence of propofol (ppf), obtained from the 12 ϵ -negative and six ϵ -positive neurons.

21%, $n = 7$) in ϵ -negative and ϵ -positive neurons, respectively ($p = 0.4$; Wilcoxon test). GABA-evoked currents at the concentrations tested represented 30 ± 3 and $26 \pm 6\%$ of maximal currents in ϵ -negative ($n = 12$) and ϵ -positive ($n = 7$) neurons, respectively. The current fluctuation variances (σ^2) in the presence of diazepam ($1 \mu\text{M}$) represented $90 \pm 15\%$ (GABA taken at $EC_{32} \pm 5$; $n = 9$) versus $106 \pm 13\%$ (GABA taken at $EC_{27} \pm 7$; $n = 6$) of control values in ϵ -negative versus ϵ -positive neurons ($p = 0.75$; Wilcoxon test), respectively.

Propofol modulation of GABAergic sIPSCs in hypothalamic neurons

sIPSCs were studied in cultured hypothalamic neurons in the presence of TTX ($0.3 \mu\text{M}$). All of them were completely blocked by either gabazine ($20 \mu\text{M}$) or bicuculline ($50 \mu\text{M}$), selective GABA_AR antagonists, indicating that all spontaneous synaptic potentials in cultured hypothalamic neurons are mediated by GABA. In 11 neurons in which propofol ($3 \mu\text{M}$) was applied for 30–60 sec and sIPSCs were analyzed, single-cell RT-PCR was successfully performed. In these cells, mRNAs encoding for more than two GABA_AR subunits were detected. Six neurons were ϵ -subunit positive; among them, three were OX and three were NI cells. Five neurons (one HDC-positive and four NI cells) were ϵ -subunit negative; however, all of them expressed at least $\alpha 2$ and $\beta 3$ GABA_AR subunits. Mean amplitudes of control sIPSCs were not different ($p = 0.22$; Wilcoxon test) between ϵ -positive (45.1 ± 10.3 pA; $n = 6$) and ϵ -negative (57.6 ± 10.4 ; $n = 5$) neurons. Propofol did not change sIPSC amplitudes (Kolmogorov–Smirnov test applied separately to each neuron) in both neuronal groups [mean

Figure 6. Spontaneous IPSCs in cultured hypothalamic neurons. Photographs of two patched hypothalamic neurons placed near the application tube (at the right) and examples of corresponding recordings (middle) and plots for the decay time constants and cumulative amplitude fractions (below), which were analyzed with the Kolmogorov–Smirnov test, are shown. This analysis demonstrated no difference in amplitudes in control and under propofol, although decay time constants were increased significantly in both neurons ($p < 0.05$). Note that short events are not potentiated by propofol in the ϵ -positive neuron.

amplitudes were 41.55 ± 8.3 ($n = 6$) and 55.78 ± 12.3 ($n = 5$) in ϵ -positive and ϵ -negative neurons, respectively]. Mean sIPSC frequencies were not different ($p = 0.66$; Wilcoxon test) in control and in the presence of propofol (0.49 ± 0.11 vs 0.52 ± 0.12 Hz, respectively; $n = 11$, both neuronal groups are considered together). Propofol significantly prolonged ($p < 0.05$; Kolmogorov–Smirnov test) the duration of synaptic events in all five ϵ -subunit-negative neurons (mean values of τ_{dec} were 37.8 ± 4.6 and 62.2 ± 5.8 msec in control and in the presence of propofol, respectively) and in two of six ϵ -subunit-positive neurons (from 56 ± 8.8 to 94 ± 18 msec). In the remaining four positive neurons, decay time constants of sIPSCs were not changed (51.7 ± 8.8 vs 63.8 ± 11.8 msec in control and in the presence of propofol, respectively). The difference in cumulative decay time distribution curves in control and in the presence of propofol in two former ϵ -positive neurons (Fig. 6, right) was significant, but shorter events were obviously resistant to the propofol modulation. Thus, in 33% of the neurons, propofol-resistant receptor population properties were found to be masked by the other GABA_AR populations. Propofol sensitivity of sIPSCs in ϵ -negative (five of five neurons) and ϵ -positive (two of six cells) groups was different ($p = 0.0015$; Fisher's exact probability test).

Discussion

We correlated single-cell expression of the GABA_AR ϵ -subunit in native neurons with the pharmacological properties of GABA-mediated responses, as well as with spontaneous inhibitory synaptic currents. We demonstrated a somatic localization of the ϵ -subunit protein in two neuronal groups: histaminergic neurons from the TM nucleus and both orexinergic and histaminergic

neurons from the perifornical area. Non-identified hypothalamic neurons were investigated as well. Several properties of the human or rat ϵ -subunit determined in artificially expressed systems are not readily found in native cells, e.g., constitutive activity of ϵ -containing GABA_ARs. In accordance, cardiac parasympathetic preganglionic neurons in brainstem slices with transfected ϵ -subunits did not display significant constitutive GABA_AR activity (Irnatzen et al., 2002). The shift of baseline current by GABA_AR antagonists in our study represented no more than 1% of the maximal GABA-mediated currents and was not observed in all ϵ -positive cells; in contrast, some ϵ -negative cells demonstrated spontaneous activity sensitive to GABA_AR antagonists. For comparison, in HEK 293 cells transfected with the rat ϵ -subunit, such currents represented 83% of the maximal GABA-mediated currents (Davies et al., 2002) and 38% in oocytes transfected with the human ϵ -subunit (Maksay et al., 2003). It is therefore likely that the high constitutive activity of heterologously expressed ϵ -subunits occurs only in artificially constructed receptors. The background constitutive current of $\sim 1\%$ of the maximal GABA currents detected in the ϵ -subunit-lacking and -expressing hypothalamic neurons might be of physiological importance especially because it was recorded during perfusion of the cells in the absence of any of spillover GABA from synaptic activity. Relevant constitutive or “tonic” inhibition was studied previously in rat slice preparations of postnatal and adult hippocampus (Otis et al., 1991), cortex (Salin and Prince, 1996), and cerebellum (Brickley et al., 1996; Wall and Usowicz, 1997). The ϵ -subunit, although not detected in the aforementioned structures, was suggested as a candidate responsible for tonic inhibition (Bai et al., 2001). Our study does not support such a role for the ϵ -subunit in hypothalamic neurons. More recent studies have identified $\alpha 6\delta$ (Brickley et al., 2001) and $\alpha 4\delta$ (Stell et al., 2003) GABA_AR types to be responsible for the tonic inhibition in cerebellar granule and dentate granule cells, respectively. In previous studies, expression of $\alpha 4$ (Sergeeva et al., 2002), $\alpha 6$ and δ (Wisden et al., 1992) GABA_AR subunits was not detected in the posterior hypothalamus. Therefore, the receptor type responsible for tonic inhibition in this brain region remains to be elucidated.

We found two features of ϵ -positive hypothalamic neurons, which can be used for their identification. First, the membrane noise of propofol-potentiated submaximal GABA currents is not different from the noise of the control responses. In contrast, noise is dramatically reduced in ϵ -negative cells, like in maximal GABA-evoked responses. Second, decay time constants (τ_{dec}) of spontaneous IPSCs in ϵ -positive cells are partially or completely propofol resistant, in contrast to ϵ -negative cells. Both of these findings can be explained by the presence of a propofol-resistant GABA_AR population (which is only found in ϵ -positive neurons). In line with previous studies (Davies et al., 1997, 2001) on heterologously expressed ϵ -containing GABA_ARs, we did not find differences in the direct activation of GABA_ARs by propofol between ϵ -positive and ϵ -negative neurons.

We show that diazepam similarly modulates GABA-mediated responses in all hypothalamic neurons, and noise variance analysis did not reveal any difference in the current fluctuations between ϵ -positive and ϵ -negative neurons, indicating that diazepam-resistance is not specific for the ϵ -containing receptors. Association of the ϵ -subunit with different subunits could occur in different brain regions, giving rise to receptors with different properties. For example, together with the θ - and $\alpha 3$ -subunits in locus ceruleus (Sinkkonen et al., 2000), in which the $\gamma 2$ -subunit is not expressed, diazepam-insensitive receptors may be formed, whereas, during association with the $\gamma 2$ -subunit (Da-

vies et al., 2001) in other brain regions, receptors may be modulated by diazepam. Kasparov et al. (2001) reported previously that the caudal aspect of the nucleus tractus solitarius of the rat contains neurons, whose GABA_ARs are not modulated by diazepam, and this coincides with the presence of the ϵ -subunit in this region. Diazepam may fail to enhance GABA currents in several GABA_AR types, including δ -containing and γ -subunit-lacking receptors. Previously, we showed that TM neurons with intermediate GABA sensitivity exhibit spontaneous IPSCs, which are not prolonged by zolpidem, a diazepam-site agonist (Sergeeva et al., 2002). Therefore, absence of a significant correlation between the noise of diazepam-potentiated responses and presence of the ϵ -subunit indicates the occurrence of diazepam-insensitive receptor populations in ϵ -negative neurons. Thus, diazepam-site agonists are not useful for the pharmacological identification of ϵ -positive neurons.

Expression of splice variants of the ϵ -subunit was not analyzed in the present study because PCR primers amplified a cDNA fragment outside of the splice region. The ϵ antibody was directed toward the N-terminal part of the mature protein, which is present in both the short and long isoforms described in rodent brain (Sinkkonen et al., 2000). However, Western blots performed in a previous study (Moragues et al., 2000) with protein extracted from hypothalamus showed that the size of the protein labeled by the anti- ϵ antibody correspond to a short isoform.

Our data are in keeping with the recently suggested role of the TM nucleus as a key to the sedative component of anesthetics (Nelson et al., 2002). Only TM and ventrolateral preoptic area neuronal activities were changed after anesthesia in this study. Injection of a GABA_AR antagonist into the TM nucleus abolished the sedative response to GABAergic anesthetics. Injection of propofol, on the other hand, induced a sedative response. In addition to the histaminergic TM (Haas and Panula, 2003), other aminergic, as well as cholinergic and orexinergic, nuclei are involved in sleep–wake regulation (Saper et al., 2001). Interestingly, in all the latter structures, the ϵ -subunit of GABA_AR is highly expressed (Moragues et al., 2000, 2002, 2003). Thus, the TM nucleus is unique among those with its infrequent expression of the ϵ -subunit and the ensuing vulnerability to anesthesia. Moreover, we demonstrated previously that ϵ -positive TM neurons are always GAD65 (GABA-producing enzyme) positive (Sergeeva et al., 2002); therefore, if they release GABA and are not inhibited by GABAergic anesthetics, they would provide a positive loop in the propofol action.

In conclusion, we characterized native ϵ -containing GABA_ARs in two types of hypothalamic neurons involved in sleep–waking regulation and present protocols for their pharmacological identification. Resistance to the anesthetic propofol is typical for GABA_A receptors containing this subunit and provides a molecular correlate for the proposed role of the TM nucleus in the sedative response to anesthesia.

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