Inhibitory Transmission in Locus Coeruleus Neurons Expressing GABA<sub>A</sub> Receptor Epsilon Subunit Has a Number of Unique Properties

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INTRODUCTION

Ionotropic GABA<sub>A</sub> receptors are responsible for fast, flexible synaptic transmission and have a wide range of specific properties (Barker and Ransom 1978; Chavas and Marty 2003; Gulleld and Stuart 2003; Gulleld et al. 2005; Kawaguchi and Hirano 2007; Macdonald et al. 1989; Staley et al. 1995). GABA binding results in the opening of anion-selective intrinsic channels through which primarily chloride ions flow. This, in turn, changes neuron excitability.

A wide variety of GABAergic synapses have been identified in the past decade. Diversity is due to several pre- and postsynaptic factors as well as GABA handling in and around the synaptic cleft (for review, see Mody and Pearce 2004). Receptor structure is one key factor. Ionotropic GABA<sub>A</sub> receptors are oligomeric, probably pentameric, proteins made up of 18 subunits in the mammal brain (Bonnert et al. 1999). There is good evidence that neurons in various parts of the brain express specific repertoires of subunits, leading to heterogeneous subcellular distribution of subunits and targeting mechanisms (Everitt et al. 2004; Fritschy and Mohler 1995; Klausberger et al. 2002; Somogyi et al. 1989). Numerous studies of recombinant receptors have revealed that subunit composition confers distinctive pharmacological properties on ionotropic receptors (e.g., Kaur et al. 2009; Korpi et al. 2002; Nusser et al. 1998; Puia et al. 1990; Verdoorn et al. 1990). However, the specific properties of fast synaptic transmission have been attributed to the subunit composition of native receptors in a very small number of brain areas.

Among the seven subunit families, classified on the basis of their structural homology as α, β, γ, δ, ε, θ, and ρ, the δ, ε, and θ subunits stand out as each has only a single member. The substitution of a δ subunit for a γ subunit has now been shown to have marked consequences for neural excitability (Bright et al. 2007; Glykys et al. 2007; Jia et al. 2005; Stell et al. 2003).

Much less is known regarding ε and θ subunits. These two subunits are homologous to subunits in the γ and β families, respectively (Bailey et al. 1999; Simon et al. 2004). They assemble with other GABA<sub>A</sub> subunits to form receptors with distinctive properties in various models of heterologous expression (Bonnert et al. 1999; Davies et al. 1997; Ranna et al. 2006; Thompson et al. 1998; Whiting et al. 1997). Both of these subunits exhibit restricted expression in the brain, and they are predominantly coexpressed in the modulatory nuclei (Bonnert et al. 1999; Moragues et al. 2000; Sinkkonen et al. 2000; Thompson et al. 1998). Among these, the locus coeruleus (LC), the largest source of norepinephrine in the CNS, is responsible for major brain functions, such as arousal, learning, attention, and anxiety (Aston-Jones et al. 2004; Berridge and Waterhouse 2003).

The properties of native receptors containing ε and/or θ subunits are so far unknown. We therefore appraised the expression profile of GABA<sub>A</sub> subunits in the LC, and investigated the pharmacological properties of GABA<sub>A</sub> receptors within the LC in vivo, as well as in vitro. Finally, we examined the kinetics of GABA<sub>A</sub>-mediated currents to define their functional profile.

METHODS

In vivo single-unit extracellular recording and microinjections

Adult, male Sprague-Dawley rats weighing 300–350 g were anesthetized with chloral hydrate (400 mg/kg ip). A tracheal cannula was then inserted into the right jugular vein for additional administration of anesthetic. Body temperature was maintained at 37°C throughout the experiment by a heating pad connected to a rectal probe. Animals were placed in a stereotaxic frame with the head oriented at 15° (nose down) for LC recordings or at 0° to the horizontal plane for reference experiments in the subthalamic nucleus (STN). The skull was exposed, and a 3-mm burr hole was drilled over the nucleus. Extracellular recording was performed using an Omegadot glass electrode filled with a 2% solution of Pontamine Sky Blue in 0.5% sodium acetate, broken back to a tip diameter of 1–2 μm. Pressure microinjections were made using a thick-walled pipette with a calibrated narrow inner diameter, broken 2 μm from the tip and glued adjacent to the recording micropipette. The calibrated pipette was filled with flunitrazepam (2 mM) dissolved in 25% DMSO/H2O as vehicle or vehicle only. The signal from the recording electrode was passed through a high-input impedance, amplified, and discriminated. It was continuously monitored with an audio monitor and oscilloscope and fed into a personal computer. Firing activity before and after drug microinjection was processed off-line (CED micro 1401 interface and Spike2 software, Cambridge Electronic Design). LC neurons were encountered 1.1 mm lateral to the midline, 3.7 mm caudal to the lambdoid fontanel, and 6.0–6.9 mm ventral to the dura, just ventral to a zone of relative silence (corresponding to the 4th ventricle) and medial to neurons in the mesencephalic nucleus of the 5th cranial nerve (activated by depression of the mandible). They showed long duration action potential (>2 ms), spontaneous firing at a regular rhythm (mean: 3.1 ± 0.3 Hz, n = 10), and spikes with a long-lasting positive-negative waveform. STN neurons were encountered 3.2 mm posterior to the begma, and 2.2 mm lateral and 7.3–8.0 mm ventral to the dura. All neurons recorded exhibited a biphasic waveform 1.0 to 1.5 ms in duration, firing at a more variable frequency than LC neurons (mean: 3.8 ± 1.2 Hz; n = 9). The basal firing rate was recorded ≥2 min prior to any drug administration.

The drug was ejected by applying pressure pulses (50–150 ms) to the calibrated pipette, using a solenoid-controlled pneumatic pressure device (Picospritzer II, General Valve) driven by synthetic air. Flunitrazepam was first tested in the STN as this structure expresses flunitrazepam-sensitive GABA<sub>A</sub> receptors (Baufreton et al. 2001). Injection of vehicle alone had no effect because firing rates measured after application matched basal rates (P = 0.5, n = 8). The injected volume was measured by monitoring the movement of the meniscus in the calibrated pipette, i.e., 4 nl for the 8 pmol dose.

At the end of each experiment, a 5-μA cathodic current was passed through the recording electrode to discharge Pontamine Sky Blue at the recording site. The brain was fixed by transcardial perfusion with an ice-cold solution containing (in mM) 250 sucrose, 26 NaHCO<sub>3</sub>, 7 MgCl<sub>2</sub>, 2 KCl, 1.15 NaH<sub>2</sub>PO<sub>4</sub>, 0.5 CaCl<sub>2</sub>, and 11 glucose (pH 7.35). Two coronal slices containing LC were obtained from one brain in the same solution using a vibroslicer (VT 1000S, Leica, France). Slices were incubated at 25°C in a Krebs solution containing (in mM) 124 NaCl, 26 NaHCO<sub>3</sub>, 3.6 KCl, 1.3 MgCl<sub>2</sub>, 2.4 CaCl<sub>2</sub>, 1.25 HEPES, and 10 glucose (pH 7.35) for ≥1.5 h before recording. One slice was continuously superfused (3.5 ml/min) with the oxygenated Krebs’ solution at 25°C in an immersion-type recording chamber. The slice was examined under a dissecting microscope; the LC was identified as a translucent area lying on the lateral part of the periventricular gray matter, next to the 4th ventricle.

Recordings were made using the blind patch-clamp technique in cell-attached, whole cell, and excised outside-out patch configurations. All data were analyzed using pClamp V. 9.02 software (Axon Instruments).

CELL-ATTACHED RECORDINGS. In the cell-attached mode, the intrapipette perfusion medium was Krebs’ solution and the patch was held at 0 mV (Baufreton et al. 2001). Action currents, corresponding to extracellular action potentials (Fenwick et al. 1982), were detected in cell-attached recordings by the threshold method. The cumulative number of action currents was registered every 30 s. It was transformed off-line into mean firing frequency over the 30-s bins. The mean firing frequency in control was then calculated and used to normalize all values in a recording. The mean discharge values given in RESULTS were calculated over the whole control or test periods.

WHOLE CELL RECORDINGS. Two pipette solutions were used in the whole cell configuration. Solution 1, a low-chloride pipette medium containing (in mM) 140 K-glucuronate, 11 EGTA, 10 HEPES, 1 CaCl<sub>2</sub>, 2 ATP-Mg<sub>2</sub>, and 0.4 Na-GTP, was used for current clamp recordings (Supplemental Fig. S1). In solution 2, a Ca<sup>2+</sup>-based, high-chloride pipette solution, K-glucuronate was replaced by CsCl, but it was otherwise similar to solution 1. This was used for voltage clamp in whole cell and excised outside-out patch configurations.

In all cases, the osmolality of intra-pipette solutions was between 280 and 300 mosM, and pH was adjusted to 7.25 using KOH or CsOH. Electrodes, pulled from thin glass capillaries (GC150F-10, Harvard Apparatus, Edenbridge, UK) on a vertical puller (PP-830, Narishige), had resistances of ~10 and ~6 MΩ when filled with solutions 1 and 2, respectively. Signals were recorded using an Axopatch-1D amplifier (Axon Instruments, Foster City, CA) with the filter set at 2–5 kHz, monitored on an oscilloscope and chart recorder, stored on video tape, and/or digitized at 0.5–10 kHz, using a Digidata 1200B. Access resistance (Ra) was regularly monitored. Junction potentials were measured as described by Neher (1992). Voltage values were corrected off-line (~13 mV for solution 1 and ~5 mV for solution 2).

PHARMACOLOGY OF GABA-ACTIVATED CURRENTS. All modulators were diluted in oxygenated Krebs’ solution and delivered by means of a multi-barrel, gravity-feed system, consisting of two capillaries positioned just above the recording pipette. They were tested using a three-step protocol, with GABA applied first, then an incubation of the modulator, and third, co-application of GABA and the modulator (see an example in Supplemental Fig. S2B). We compared the charge of whole cell currents (Papke and Porter Papke 2002) to minimize the possible impact of the kinetic features of drug delivery in the depth of slice on the amplitude and timing of peak responses. Whole cell currents were evoked by a submaximal concentration (10 μM) of GABA in all our whole cell experiments, due to poor space clamp with maximal doses (as exemplified by Supplemental Fig. S2A).

mIPSCs. Recordings of miniature inhibitory postsynaptic currents (mIPSCs) were only made in cells with an Ra <10 MΩ. They were interrupted every 3 min to check Ra and stopped if Ra increased by >20%. mIPSCs were recorded at ~65 mV in the presence of a cocktail of five antagonists so as to inhibit the receptors of all the neurotransmitters described in the LC other than GABA (Williams et al. 1991): yohimbine (1 μM), 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, 10 μM), t-2-amino-5-phosphonovaleric acid (APV, 50 μM),...
strycnmine (1 μM), 2-hydroxysaclofen (5 μM), and tetrodotoxin (TTX, 1 μM). Flunitrazepam and diazepam were applied via bath perfusion. Current traces were filtered at a cut-off frequency of 2.5 or 5 kHz by the amplifier and acquired at 5 or 10 kHz. mIPSCs were detected using a programmable software (Acquis 1, Biologic France) and the method reported by Perrais and Ropert (1999) or the threshold detection function of Clampfit. They were visually inspected to remove electrical artifacts and superimposed mIPSCs. The decay time constant was obtained in two ways: 1) without assuming the number of decay components: f(t)/dI/A, where I is the current and A the peak amplitude of the mIPSC; the integral being taken between the mIPSC peak and the return to baseline and 2) by fitting a single exponential on the decay phase. Mean mIPSC was obtained from averaging all fitted mIPSCs. The peak amplitude, charge, 10–90% rise time, and decay time constant were calculated for each mIPSC or from the averaging of all mIPSCs.

EXCISED PATCH EXPERIMENTS. In the outside-out excised patch experiments, GABA was repeatedly applied to membrane patches pulled out of a slice using a rapid application system (HSSE-2, ALA Scientific Instruments, Sega Electronique). The fluid interface generated between the control recording solution and the GABA-containing solution was driven rapidly across the patch. Several steps were required. First, the whole cell configuration was obtained from an LC neuron in a slice. Second, an outside-out patch was excised from the membrane. Third, the pipette was pulled out of the slice and brought in front of the fast solution exchanger. Fourth, GABA was applied repeatedly at a rate of 0.033 Hz, to avoid desensitization, and GABA-elicited responses were low-pass filtered at 2 kHz and digitized at 10 kHz. Fifth, the solution exchange time was monitored at the end of each recording by blwoing out the patch and stepping a dilute external solution across the open electrode tip to measure a liquid junction current. The success rate of these experiments was very low and, further, GABA-activated currents in excised patches run down quickly. Only eight experiments (from 7 slices) yielded currents that were maintained over three to seven applications of GABA and showed a 10–90% time exchange between solutions <1.1 ms; they were further considered for analysis. Values of exchange times in this data set ranged from 0.6 to 1.1 ms. Ensemble currents were obtained by averaging the three to seven GABA-elicited responses acquired for each patch. They were characterized using the method devised by Bianchi et al. (2001). Peak amplitude, integral and 10–90% rise time of ensemble currents were calculated using Clampfit. The desensitization or deactivation time courses of ensemble GABA currents were fitted using the Levenberg-Marquardt least-squares method with one- or two-component exponential functions of the form \( f(t) = a e^{-t/r} + C \), where \( n \) is the best number of exponential components, \( a \) is the relative amplitude of the component, \( r \) is the time constant, and \( C \) is a constant term to account for residual current. A second component was never accepted because it did not significantly improved the fit compared with a single-exponential function as determined by a statistical Fisher test on the sum of squared residuals. The contribution of desensitization was evaluated by the percentage of desensitization, the ratio of current at the end of GABA application to that at peak.

**Drugs**

GABA (Fluka Biochem) and GABA<sub>A</sub> receptor modulators [diazepam, flunitrazepam, pentobarbital, tetrahydroxycorticosterone (THDOC), allopregnanolone (3α-hydroxy-5α-pregnan-20-one), SB 205384, GABAzine (SR 95531), and bicuculline methiodide] were purchased from Sigma. Transmitter receptor inhibitors (yohimbine, CNXQ, APV, strychnine, 2-hydroxysaclofen), were also purchased from Sigma. TTX was obtained from Latoxan. All drugs were prepared as concentrated frozen stock solution aliquots. Diazepam, flunitrazepam, allopregnanolone, pentobarbital, CNXQ, and THDOC were diluted in DMSO, whereas all other drugs were prepared in distilled water. Care was taken to keep the final concentration of DMSO <0.1%.

MEMBRANE TRANSPLANTATION IN XENOPUS OOCYTES AND ELECTROPHYSIOLOGICAL RECORDINGS. The transplantation method was adapted from previous work (Miledi et al. 2002). For each experiment, we used either five locus coeruleus dissected from brain slices, obtained in the same way as those used for patch-clamp experiments, or the equivalent weight of cerebellum. Tissues were kept frozen at −80°C until used. They were homogenized in 4–μl ice-cold solution containing 5 mM glycine by passing through a 10-μl pipette and a microinjection glass pipette. Stage V and VI oocytes, prepared as previously described (Boue-Grabot et al. 2004), were microinjected (Nanoject II, Drummond) with 80 nl membrane preparations. The oocytes were then incubated in Barth’s solution containing 1.8 mM CaCl<sub>2</sub> and gentamycin (10 μg/ml, Sigma) at 19°C for 1 day prior to recording. Two-electrode voltage-clamp was performed using glass pipettes (1–2 MΩ) filled with 3 M KCl solution. Oocytes were perfused at a flow rate of 10–12 ml/min with Ringer solution (pH 7.4) containing (in mM) 115 NaCl, 5 NaOH, 2.5 KCl, 1.8 CaCl<sub>2</sub>, and 10 HEPES. Membrane currents were recorded through an OC-725B amplifier (Walter Instruments) and digitized at 500 Hz. All drugs (the same ones used in the patch experiments) were dissolved in the perfusion solution and applied using a computer-driven valve system (BPS8, Ala Scientific). All recordings were made at room temperature. The peak current was measured as usual for currents obtained by expressing recombinant channels in oocytes.

**Molecular analysis**

In situ hybridization was carried out on fresh Wistar rat brain tissues processed into 14-μm-thick cryostat serial sections as previously described (Moragues et al. 2000). The ribonucleotide probes used corresponded to sequences with no significant identity with other known rat GABA<sub>A</sub> receptor subunit nucleotide sequences or other known nucleotide sequences available in databases. The GABA<sub>A</sub> receptor θ and ε probes were as described earlier (Moragues et al. 2002). The GABA<sub>B</sub> receptor γ1-subunit probe was taken from the γ1-nucleotide sequence (nucleotides 1182–1309, Accession No. NM080856); γ2-subunit probe was taken from Accession No. NM183327, nucleotides 1074–1206; γ3 from NM024370, nucleotides 1137–1323; β1 from NM012956, nucleotides 1187–1391; and β3 from NM017065, nucleotides 1178–1381. The cDNA probes were subcloned in pBluescript (Stratagene, Amsterdam) and sequenced. Radiolabeled antisense and sense cRNA probes were prepared as described (Moragues et al. 2000) and used at 20.10<sup>5</sup> cpm/ml.

The oligonucleotides for PCR analysis of alternative splicing were selected from the AF 255612 sequence. The sense primer: AAACCCCTGGCCCAAGATAAG and reverse primer: CACGGTAGGCTTCTTCTCCA were used as previously described (Moragues et al. 2000). Binding was performed at 4°C, as previously described (Lopez-Meraz et al. 2004). Briefly, cryostat sections were preincubated in 100 mM TrHCl, pH 7.5, for 15 min, then in 5 nM [3H] flunitrazepam (PerkinElmer) in the presence or absence of 1 μM clonazepam, 5 μM flunitrazepam, or 5 μM diazepam, for 60 min. Sections were washed three times in buffer for 10 s and once in distilled water for 1 s, dried, and exposed to Hyperfilm-H<sup>3</sup>H (Amersham), together with calibrated [3H]micro-scales (Amersham), for 18 days.

IMMUNOHISTOCHEMISTRY. The ε subunit was detected using an affinity-purified rabbit antisera previously characterized for its specificity (1: 500) (Moragues et al. 2000) together with a mouse monoclonal antisera to tyrosine hydroxylase (TH) (1:300; Bio-Rad, Ivy-sur-Seine). The sections were then incubated in a mixture of goat anti-rabbit IgGs coupled with Alexa-568 (1:1000; Jackson ImmunoResearch) and goat anti-mouse IgGs coupled with fluorescein isothiocyanate (FITC; 1:200, Jackson ImmunoResearch). After washing, the sections were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and examined under a fluorescence microscope (Zeiss). The ε subunit was further analyzed at...
Electro-microscopic level using the preembedding immunogold technique. Sections, prepared as described for in vitro patch recordings, were fixed in 4% paraformaldehyde for 15 min, cryoprotected in 30% sucrose, frozen quickly at −50°C for 1 min, and then thawed. Sections were incubated in 4% NGS for 30 min and then α antibodies (1:500) supplemented with 1% NGS at 4°C for 15 h. They were then incubated in goat anti-rabbit IgGs conjugated to gold particles (0.8 nm diam; Aurion; 1:100 in PBS/BSA-C) for 2 h. The sections were then washed and postfixed in 1% glutaraldehyde for 10 min. After washing in acetate buffer (0.1 M, pH 7), the signal of the gold immunoparticles was increased using a silver enhancement kit (HQ silver; Nanoprobes) at room temperature in the dark for 2 min. Sections were finally processed for electron microscopy as previously described (Decossas et al. 2003).

Statistics

In the text, values are given as means ± SE when mean and median are close. If this is not the case, both median and mean are given. Box plots are used for graphic presentation of electrophysiological data due to the small sample size. The box plot presents the distribution with the median as a central line. The hinges and edges of the box display the 25th and 75th percentiles, whereas the “whiskers” display the 5th and 95th percentiles. The square shows the mean. Except where otherwise stated, statistical comparisons were made using nonparametric tests. The Mann and Whitney test, as well as the Wilcoxon test, were used for data obtained from independent and matched paired samples, respectively. Values of $P < 0.05$ were considered significant.

RESULTS

Flunitrazepam application has minimal action, if any, on the firing rate in the Locus Coeruleus in vivo and in vitro

Because insensitivity to benzodiazepines, such as flunitrazepam, is a major feature of recombinant receptors expressing α, we investigated the effect of local applications of flunitrazepam on the firing rate of LC neurons of anesthetized rats (Fig. 1). We first tested flunitrazepam in another structure, the subthalamic nucleus (STN) as we knew that STN neurons expressed a single class of flunitrazepam-sensitive GABA$_A$ receptors (Baufreton et al. 2001). We thus determined that injecting 8 pmol of flunitrazepam into the STN induced a marked, short-lived decrease in firing frequency as expected from a short-lived application of a GABAergic agent (Fig. 1A, top). On average, firing frequency diminished significantly by $45 \pm 10\%$ ($n = 9$, $P < 0.01$) with an apparently symmetrical 1-min onset and offset. Mean basal firing rate was $2.9 \pm 0.7$ Hz, which transiently decreased to $1.9 \pm 0.8$ Hz ($n = 9$) 1 min after the injection. Injection of the same 8 pmol dose of flunitrazepam into the LC (Fig. 1A, bottom) barely, yet significantly, decreased the discharge frequency (on average $8.0 \pm 3.8\%$ in the 1st min with mean discharge rates of $3.1 \pm 0.3$ Hz in control and $2.8 \pm 0.3$ Hz 1 min after flunitrazepam injection, $n = 10$, $P < 0.05$). Most importantly, the changes induced by flunitrazepam in the LC were significantly different from those measured in the STN (Fig. 1B).

To investigate the sensitivity of GABA$_A$ receptors in the LC to flunitrazepam on the cellular level, we recorded neurons in rat brain slices using the patch-clamp technique. We first verified that neurons in the LC exhibited electrophysiological properties in agreement with those described in the literature (Alvarez et al. 2002; Alvarez-Maubein et al. 2000; Arima et al. 1998; Hagan et al. 1999; Ishimatsu and Williams 1996; Williams et al. 1984) (see supplemental Fig. S1). We then assessed the ability of flunitrazepam to change the spontaneous firing rate of LC neurons (Fig. 2). The firing rate was calculated from action currents in the cell-attached configuration (Fenwick et al. 1982), which preserved the physiological anion gradient. In the cell-attached mode, LC neurons fired regularly. We used a two-step protocol to ascertain that a neuron experienced a GABAergic tone: 1 μM flunitrazepam was perfused, then flunitrazepam and the competitive antagonist bicuculline were co-applied (Fig. 2A). We found that co-perfusion of 1 μM flunitrazepam and of 10 μM bicuculline had no effect in 5 of 11 neurons, showing that all neurons in our slices did not receive a GABAergic tone able to alter their firing rate. It increased the firing rate in the remaining six neurons. In the same six-neuron sample, however, flunitrazepam perfusion...
Expression of GABAA subunits within the LC

Expression of ε subunit in LC from adult rats has been documented (Bonnert et al. 1999; Moragues 2000, 2002; Sinkkonen 2000). Complementary studies were performed on juvenile rat brains, i.e., those used for slice recordings. Using specific antibodies, a virtually complete co-localization of tyrosine hydroxylase- and ε-immunoreactivity was observed in the LC of young rats (Fig. 3A). RT-PCR analysis of LC mRNA, using a pair of oligonucleotide primers designed to amplify the ε N-terminal domain (Moragues et al. 2000), produced a major 207-bp band (Fig. 3B), corresponding to a short variant able to assemble with α and β subunits to form functional GABA_ε receptors (Davies et al. 2002). We also examined the subcellular distribution of the ε subunit in the LC. Preembedding immunogold localization on an electron microscopic level revealed particles representing ε subunit immunoreactivity in the cytoplasm and along the membrane (Fig. 3C). Many particles were found within the perisynaptic areas of the cell membrane (Fig. 3, C and D). The lack of labeling within the active synaptic zone was not surprising with the preembedding method. Unfortunately, the antibody used in this study did not detect the ε subunit using postembedding methods, which are more appropriate for demonstrating synaptic receptor labeling (Nusser et al. 1998).

It has been previously established by several teams using various techniques that α3 subunit (and to a lower level α2) are expressed in LC neurons and that β2 and δ subunits are not (Fritschy and Mohler 1995; Luque et al. 1994; Tohyama and Oyamada 1994). But data for other β and γ subunits are conflicting (Chen et al. 1999; Fritschy and Mohler 1995; Luque et al. 1994; Tohyama and Oyamada 1994). Thus we analyzed subunit expression in LC neurons by in situ hybridization experiments on serial adjacent sections of 21-day-old rat brains, using specific probes for θ, β1, β3, γ1, γ2, γ3, and ε subunits. The specificity of θ- and ε-probes was previously reported (Moragues et al. 2000, 2002). The specificity of β1, β3, and γ1-3-probes was verified using radiolabeled sense probes (not shown). The specificity and expression level of each probe were also assessed by reference to previous reports (Laurie et al. 1992; Wisden et al. 1992; Zhang et al. 1991). Labeling in the LC was expressed only for β1, β3, ε-, and θ-subunit probes (Fig. 4). Mean gray levels were measured for β1 and β3 probes. The signal for the β3 subunit was higher in the LC than in the cortex, reported to express this subunit moderately at P12 and very weakly in adults (Laurie et al. 1992). The β3-subunit signal was weaker in the LC than in the cortex, which expresses this subunit strongly at P12 and weakly in adults (Laurie et al. 1992). No signal above background was found in the LC using γ1, γ2, or γ3 probes, although labeling was found in the cortex (γ1-γ3), inferior colliculus (γ2), or cerebellum (γ1, γ2). Both θ and ε subunits were highly expressed in 21-day-old LC and raphe magnus nuclei, as reported in adults (Moragues et al. 2000; Sinkkonen et al. 2000). Taken together, our experiments showed an unusual subunit expression profile, with no γ subunits but ε and θ, suggesting unique functional properties.

Pharmacology of GABA-activated currents in the LC in brain slices

As expected, GABA applications in the LC induced dose-dependent currents, which were inhibited by co-application of GABA_ε antagonists (Supplemental Fig. S2) (Aston-Jones et al. 2004; Ennis and Aston-Jones 1989; Williams et al. 1991). We used GABAergic tools known to act differently on GABA_ε...
receptor subtypes (Bonnett et al. 1999; Davies et al. 1997; Ranna et al. 2006; Sergeeva et al. 2005; Whiting et al. 1997) (Fig. 5). The anesthetics, propofol and etomidate, the typical barbiturate, pentobarbital, as well as the neurosteroids, allopregnanolone and THDOC, were all active and significantly potentiated the GABA-induced current. The mean charge of the current activated by co-application of GABA (10 μM) plus propofol (5 μM) was 3,237 ± 2,04 pC, significantly different from the mean charge of the current activated by GABA alone 1,196 ± 484 pC (n = 7, P < 0.05). For etomidate (5 μM), the mean charge values were 2,548 ± 563 versus 1,458 ± 273 pC (n = 7, P < 0.05). Only eight neurons of nine were affected by pentobarbital, which had a mean lower potency. However, the mean charge of the current activated by co-application of GABA (10 μM) and pentobarbital (25 μM) was 1,110 ± 187 pC, significantly different from that of the current activated by GABA (10 μM) alone, 787 ± 149 pC (n = 9, P < 0.01). Allopregnanolone (1 μM) and THDOC (0.5 μM) also significantly potentiated the GABA-induced currents (P < 0.05). Current charge was augmented by 135 and 98%, respectively, with mean values of: 2,786 ± 579 versus 1,563 ± 345 pC (n = 7) for allopregnanolone and 2,189 ± 603 versus 1,036 ± 195 pC (n = 7) for THDOC. Median values in the same data set were 1,558 versus 3,024 pC (n = 7) for allopregnanolone and 1,030 versus 1,316 pC (n = 7) for THDOC. This first set of results is in agreement with our in situ hybridization findings and data obtained from recombinant receptors, indicating that receptors in the LC may contain αβε subunits. However, this did not exclude the possibility that the receptors consisted only of α and β subunits as such recombinant receptors are also modulated by anesthetics and steroids (Belelli et al. 2002). We therefore used zinc ions to test this hypothesis experimentally. Zinc ions are potent inhibitors of receptors consisting of α and β subunits: Zn^{2+} concentrations <1 μM fully inhibit GABA-induced currents on αβ receptors (Barberis et al. 2002; Hosie et al. 2003). On the contrary, Zn^{2+} ions at concentrations <1 μM are virtually ineffective on αβγ recombinant receptors, while αβδ and αβζ display intermediate sensitivity (Barberis et al. 2002; Nagaya and Macdonald 2001; Thompson et al. 1998, 2002; Whiting et al. 1997). Among the three doses of Zn^{2+} tested (Fig. 5C), 500 μM Zn^{2+} significantly inhibited GABA currents by 72 ± 6% (n = 7, P < 0.05), whereas the lower doses (0.5 and 50 μM Zn^{2+}) had no significant effect. The mean current charges were 2,027 ± 314 versus 2,195 ± 294 pC (n = 6, P = 0.2) and 1,904 ± 389 versus 2,472 ± 456 pC in control (n = 5, P = 0.06), 0.5, and 50 μM Zn^{2+}, respectively, suggesting that GABA did not activate receptors with only α and β subunits.

We also tested whether the channel blocker picrotoxin (100 μM) and the allosteric antagonist GABAzine (20 μM) were able to significantly change the holding current. Neither was effective (Fig. 5D), indicating that native receptors in the LC had no agonist-independent openings. Holding current in the continued presence of picrotoxin was −160 ± 46 versus 161 ± 45 pA in the prepicrotoxin period (n = 5, P = 0.8). In the same way, the current was unaltered by perfusion of GABAzine, (−132 ± 48 pA, not significantly different from the pre-GABAzine value, −133 ± 49 pA, n = 6, P = 0.3). Bicuculline did not affect the holding current either (−178 ± 52 vs. −180 ± 55 pA, n = 2; see also Supplemental Fig. S2).

There were two possible explanations why the benzodiazepine, flunitrazepam, was not effective in altering the firing discharge of LC neurons in vitro: it was unable to bind to receptors or it was capable of binding to the receptors but did not functionally interfere with receptor properties (Boileau and Czajkowski 1999). Thus we initially explored whether the LC expressed binding sites for flunitrazepam by performing [^3H]flunitrazepam binding experiments on sections of 21-day-old rat brains (Fig. 6A). These experiments showed strong labeling in central gray areas and moderate labeling in the molecular and granular layers of the cerebellum in agreement with data in adult rat brains (Walsh et al. 1999). Quantification

FIG. 3. The ε protein is expressed in the LC of 21-day-old rats. A: fluorescent double-labeling for the ε subunit (A1) and tyrosine hydroxylase (TH) (A2) in a frontal section through the LC shows virtually full co-localization, indicating that most if not all TH neurons in the LC express ε. Note that the diffuse TH labeling was due to the weak paraformaldehyde fixation protocol used in this experiment. B: RT-PCR amplification product (→) using ε 5′-terminus specific oligonucleotide primers on mRNA extracted from the LC. Ethidium bromide-stained agarose gel electrophoresis run with a DNA ladder (M), negative water control (Ct). C and D: ultrastuctural localization of the ε subunit. Immunogold labeling (→ in D) revealed sites at the plasma membrane, close to terminals (t) at synaptic junctions. Scale bars = 100 μm (A); 1 μm (C), 0.5 μm (D).
with other subunits in the α family (Meadows et al. 1998), was also active in the micromolar range. Current charge increased from 1.026 ± 280 to 1.449 ± 362 pC (P < 0.01, n = 8) following perfusion of SB 205384. We finally tested whether mIPSCs showed the same differential sensitivity to flunitrazepam and diazepam as whole cell currents. Flunitrazepam and diazepam were both applied at 1 μM. The mean charge of mIPSCs was not significantly altered by perfusion of flunitrazepam (P = 0.078), whereas it was significantly augmented by diazepam. The mean charge values measured in the sample of 7 neurons that were challenged with flunitrazepam were 1.37 ± 0.22 pC (control) and 1.91 ± 0.18 pC (n = 7). They were 0.92 ± 0.09 pC in the control and 1.98 ± 0.13 pC (n = 6) in the neuron sample challenged with diazepam. The apparent difference in the control charge transfer of mIPSCs between the two groups was not statistically significant (Mann and Whitney test, P = 0.1807). Amplitude and decay time constant were accordingly significantly augmented by diazepam (mean values were −46.9 ± 5.4 vs. −29.5 ± 1.1 pA and 32.8 ± 4.0 vs. 22.7 ± 2.6 ms, respectively), whereas flunitrazepam had no significant effect on the mIPSCs amplitude but slightly increased their decay time constant (−37.7 ± 2.9 vs. −32.3 ± 3.7 pA and 39.7 ± 3.7 vs. 31.4 ± 2.6 ms, respectively, P = 0.031). In agreement with our other results, flunitrazepam was thus unable to significantly increase the potency of the inhibitory tone on neurons in LC.

To rule out the involvement of presynaptic mechanisms in the effect of benzodiazepines and to compare the pharmacology of receptors in the LC with that of native receptors with well-defined pharmacologies, we transplanted membrane preparations (Miledi et al. 2002) from the LC and cerebellum into Xenopus oocytes (Fig. 7A, top), and recorded GABA-induced currents. Application of GABA induced measurable inward currents for concentration ranging from 10 μM to 1 mM. Sub-maximal concentration of GABA (100 μM) evoked currents with mean amplitudes of 68.7 ± 12.4 nA (n = 8) and 71.4 ± 19 nA (n = 7), respectively, in oocytes transplanted with LC and cerebellum membranes (Fig. 7). As shown in Fig. 7, A and B, flunitrazepam (1 μM) had no effect on sub-maximal GABA-induced currents in LC-transplanted oocytes (101.4 ± 2.4% of the GABA-induced control response), whereas it increased GABA-activated current by 50% (161.8 ± 23.2%) in cerebellum-transplanted oocytes. The action of diazepam was similar in both LC- and cerebellum-transplanted oocytes (Fig. 7B). A similar average increase of 25% (129.2 ± 5.2 and 119.7 ± 6.5%) and 50% (154.7 ± 8.6 and 141.4 ± 6.2%) were measured in both cases with 1 and 10 μM diazepam, respectively. Pentobarbital also potentiated currents by 100% (206.7 ± 23.3%) and 75% (181.3 ± 8.5%) in oocytes transplanted with cerebellum and LC membranes, respectively. Finally, co-application of 50 μM Zn2+ on LC-transplanted oocytes inhibited GABA currents by 25%; whereas no modulation was recorded in cerebellum-transplanted oocytes. Thus the transplantation method produced results that confirmed the properties of the GABA_A receptors described in the cerebellum (Ouardouz and Sastry 2006; Santhakumar et al. 2006; Wall 2003, 2005) and the effect of benzodiazepines on LC neurons revealed by our patch-clamp experiments on brain slices.

FIG. 4. In situ hybridization in 21-day-old rat brain indicated the expression of β1, β2, e-, and θ-subunits with no evidence supporting the expression of γ1-, γ2- or γ3-subunits. Film autoradiograms of immediately adjacent frontal sections, hybridized with 35S-labeled antisense probes specific for β1, γ1-3, and e-subunits, show labeling in a number of discrete structures, whereas the probe for the β3-subunit gave a more massive, widespread signal. Labeling in the LC was observed using β1-, β2-, e-, and θ-subunit probes. No signal above background was found in the LC when γ1-, γ2-, or γ3-probes were used, although clear labeling was found in other parts of the same brain sections. Cb, cerebellum; Ent, enthorhinal cortex; IC, inferior colliculus Rmg, raphe magnus. Scale bar = 4 mm.
Kinetic properties of inhibitory currents in the LC

Figure 8A shows four adjacent segments from a typical recording of mIPSCs, together with the average mIPSC and the distribution of peak intensities, rise times (measured between 10 and 90% of peak), and decay time constants obtained from the 285 mIPSCs detected during the 20-min recording. Mean peak intensity and charge measured in 21 neurons were $-29.3 \pm 4.0$ pA and $0.95 \pm 0.14$ pC, respectively. The average decay time constant was 25.8 $\pm 1.7$ ms ($n = 21$). Surprisingly, the mean 10–90% rise time obtained in the same neuron sample was 5.4 $\pm 0.6$ ms ($n = 21$; range: 2.0–11.3 ms). The strikingly long mean rise time evoked RC filtering. Therefore, to avoid possible interference from the passive properties of the neurons, we turned to the excised outside-out patch configuration (Fig. 8B). This configuration, together with concentration jumps of GABA mimicking that of GABA in the synaptic cleft, is used to characterize the kinetic properties of recombinant receptors expressed in nonexcitable host cells, such as HEK cells, or native receptors in dissociated or cultured neurons (Bianchi et al. 2001; Hutcheon et al. 2000; Lagrange et al. 2007; Lavoie et al. 1997). We used rapid applications of 1 mM GABA. Simultaneous activation of the GABA_A receptors in the patch when GABA was applied produced a macroscopic current called “ensemble current” (Fig. 8B). GABA-induced ensemble currents showed a variety of peak intensities (range: $-55.5$ to $-437.4$ pA; mean: $-147 \pm 42$ pA). Kinetic parameters had mean and median values that were close, suggesting homogeneity of the sample receptors (Fig. 8B, bottom). Again, rise time was high (7.2 $\pm$ 0.7 ms; range: 4.9–10.3 ms; $n = 8$). It was not significantly different from that of mIPSCs ($P > 0.05$). Desensitization and deactivation were well fitted by a single exponential, with a time constant of 108 $\pm$ 13 and 310 $\pm$ 20 ms, respectively (ranges: 80–193 and 211–376 ms; $n = 8$). The kinetic parameters of mIPSCs and that of GABA-activated ensemble currents are shown in Table 1. Both ensemble currents from outside-out excised patches and mIPSCs thus had rise times in the 5- to 7-ms range, much larger than usual rise times values.

**DISCUSSION**

Our results support the hypothesis that GABA_A transmission in the LC relies on receptors with unique properties: 1) mRNA coding for $\epsilon$, but not for any subunit in the $\gamma$ family, is expressed in the LC, and the $\epsilon$ subunit is found at the plasma membrane; 2) LC neurons in vivo display minimal sensitivity to the wide-spectrum benzodiazepine, flunitrazepam, a property conferred by the $\epsilon$ subunit on recombinant receptors; 3) sensitivity to benzodiazepine is differential because diaze-

![Figure 5](https://example.com/figure5.png)

**FIG. 5.** GABA-activated currents were modulated by the anesthetics, neurosteroids, and zinc ions but antagonists do not reveal tonic inhibition. A and B: action of anesthetics, barbiturate, and neurosteroids. All the 5 drugs tested significantly potentiated GABA-activated currents. Pentobarbital showed the lowest apparent potency, whereas allopregnanolone displayed action over a wide range. C: effect of increasing concentrations of Zn$^{2+}$. Note that application of Zn$^{2+}$ at 0.5 and 50 $\mu$M had no significant effect. Small intensity GABA-activated currents were induced in midbrain slices by GABA perfusion at a concentration close to threshold (10 or 20 $\mu$M, to avoid the limited space clamp often encountered with half-maximal or maximal concentrations of GABA). Each panel illustrates the action of one modulator. D: neither the competitive antagonist GABAzine (20 $\mu$M) nor the channel blocker picrotoxin (100 $\mu$M) were able to significantly change the holding current required to maintain neuron potential at $-60$ mV as illustrated by the histogram of the values of the current intensity during the 5 s preceding the application of the blockers, the last 5 s of the application, and the 5 s following the application. The data distribution is presented in box plots. *, **: significantly different from currents induced by GABA alone at $P < 0.05$ and 0.01, respectively.
pam, another wide-spectrum benzodiazepine, is effective; and 4) the kinetic parameters of GABAergic transmission in the LC are unusually slow.

Composition of GABA<sub>A</sub> receptors in the LC

GABA<sub>A</sub> receptors are formed by combinations of homologous subunits, 18 of which have been cloned from mammals. The number of active receptors is restricted and subunits in the 18-subunit repertoire assemble as pentamers with precise rules for subunit assembly and specific subunit partnerships (McKernan and Whiting 1996). Thus for example, two α and two β subunit assemble with one γ or δ. Monoaminergic neurons, such as those in the LC, are characterized by a high α3 expression level (Fritschy and Mohler 1995; Luque et al. 1994; Tohyama and Oyamada 1994). In contrast, there are conflicting data on subunits in the β and γ families, and the LC has been described as “lacking” a normal repertoire of GABA<sub>A</sub> receptor subunits (Moragues et al. 2000; Sinkkonen et al. 2000). Accordingly, our mRNA probes for subunits in the γ family failed to detect any expression in the LC. Sequence analysis and chromosomal organization of genes suggested that subunits θ and ε were orthologous to nonmammalian β<sub>2</sub> and γ<sub>2</sub> subunits (Bailey et al. 1999; Garret et al. 1997; Simon et al. 2004). We therefore deduce that ε is present on its own (Bonnett et al. 1999; Davies et al. 2001; Oyamada 1994). In contrast, there are conflicting data on expression level of a normal repertoire of GABA<sub>A</sub> receptor subunits (Fritschy and Mohler 1995; Luque et al. 1994; Tohyama and Oyamada 1994). In contrast, there are conflicting data on subunits in the LC. Sequence analysis and chromosomal organization of genes suggested that subunits θ and ε were orthologous to nonmammalian β<sub>2</sub> and γ<sub>2</sub> subunits (Bailey et al. 1999; Garret et al. 1997; Simon et al. 2004). We therefore deduce that ε replaces subunits from the γ family. We also suggest that β1/3 are present, together with θ, because this subunit cannot fully substitute for any subunit in recombinant receptors (Bonnett et al. 1999; Ranna et al. 2006). Moreover, θ and ε display strikingly overlapping expression patterns throughout the brain (Moragues et al. 2000, 2002; Sinkkonen et al. 2000). In addition, our experiments with zinc ions clearly eliminated the possibility that only α and β subunits were assembled in the LC. Our experiments with anesthetics and neurosteroids brought results in agreement with data obtained using various combinations of α, β, γ, θ, and ε recombinant subunits (Bonnett et al. 1999; Davies et al. 2001; Ranna et al. 2006; Thompson et al. 1998), further supporting our suggestion that α<sub>3</sub>, β<sub>1/3</sub>, θ and ε subunits compose GABA<sub>A</sub> receptors in LC.

Pharmacological signature of native ε-containing GABA<sub>A</sub> receptors

Our data using compounds in the benzodiazepine family gave results fully supporting the hypothesis that LC neurons express novel GABA<sub>A</sub> receptors. Benzodiazepine binding sites are located at the interface between the α and γ subunits. Receptors that contain both an α (with the exception of α4/6) and a γ subunit are benzodiazepine-sensitive, and the affinity and/or efficacy of the drugs is greater on receptors containing a γ2 subunit. Thus the fact that our molecular and functional data did not indicate any γ subunit expression is consistent with the ineffectiveness of flunitrazepam. We show, for the
first time, that whereas current charge through native receptors in the LC is insensitive to flunitrazepam, while the effect of diazepam was maintained (Buhr et al. 1996, 1997; Wingrove et al. 1997), providing a possible explanation for this differential sensitivity to diazepam and flunitrazepam.

All studies on recombinant ε-containing receptors have failed to establish any change in the amplitude of GABA-activated currents as well as in current charge with flunitrazepam, in accordance with our results. There is only one data set on the kinetics of GABA-induced currents in recombinant receptors with an ε subunit, but it did not explore sensitivity to benzodiazepines (Wagner et al. 2005). Incorporating θ into α1β1γ1/2-containing receptors did not affect the action of flunitrazepam (Bonnert et al. 1999). It is therefore unknown if current decay in recombinant receptors with ε and θ subunits shows the same differential sensitivity to diazepam and fluni-
The functional consequences of receptor multiplicity and the contribution of diversity to adjusting neural excitability in the brain remain poorly understood, and the role of receptors with an ε subunit in regulating neural activity is still unknown.

Background tonic inhibition has been proposed as a functional hallmark of receptors with an ε subunit. Incorporation of cDNA coding for the ε subunit in cell lines or *Xenopus* oocytes causes a significant leak current, recorded in the absence of GABA but typically blocked by the noncompetitive GABA A inhibitor, picrotoxin (Davies et al. 2001; Maksay et al. 2003; Neelands et al. 1999; Wagner et al. 2005). Native receptors containing an ε subunit are unlikely to show spontaneous agonist-independent openings. The fraction of leak current blocked by picrotoxin in single hypothalamic neurons was not correlated to ε expression as it remained the same in ε-positive and -negative neurons (Sergeeva et al. 2005). Neurons in the nucleus tractus solitarii also lacked constitutive openings of GABA channels, although they expressed the ε subunit and were barely responsive to the benzodiazepine, midazolam (Kasparov et al. 2001). We found no evidence of leak current. Picrotoxin had no effect under the same conditions where GABA readily activated currents. Our outside-out patches did not show any significant currents in the absence of GABA. Thus all the evidence obtained from native receptors in neurons expressing the ε protein eliminates the possibility of constitutive activity. This may be attributed to neuron-specific processes or to partnership between θ and ε. Alternatively, constitutive activity and the resulting leak current may be due to heterologous expression itself, as changing the ε expression level has been shown to change the level of leak current together with sensitivity to anesthetics (Thompson et al. 2002).

The kinetic properties conferred by subunit composition determine the functional role of receptors activated by GABA release. Rise times, amplitudes, and decay times of synaptic currents are considerably affected by voltage and time errors. Indeed as expected from the electrically distributed nature of the neurons (Allgaier et al. 2001; Jedema and Grace 2003; Lena et al. 1999), synaptic currents in the LC are likely to be distorted by both dendritic and somatic filtering. mIPSCs recorded in LC neurons have a 10-fold slower rising phase than those recorded in electronically compact cells (Brickley et al. 1999; Camp et al. 2006; Nusser et al. 2001). However, a similarly slow rising phase was also measured in excised-patch currents, without any filtering due to cell parameters. As α3-containing GABA A receptors present in the reticular thalamic nucleus have relatively faster rise time kinetics (<2 ms) (Zhang et al. 1997), slow activation is therefore likely a true characteristic of native receptors containing ε/θ subunits.

LC activity has been assigned a role in many important functions, including wake-sleep cycles, attention, sensory processing, synaptic plasticity, network resetting, memory formation and retrieval, decision making, performance facilitation (for review, Sara 2009). It is well established that LC shows distinctive changes in firing rate during the sleep-wake cycle and that during one sleep state, the rapid eye movement (REM) sleep, the noradrenergic neurons in LC cease firing (Aston-Jones and Bloom 1981; Chu and Bloom 1973). It has been proposed that REM sleep maintenance requires cessation of firing in LC via GABAergic inhibition (Gervasoni et al. 1998; Mallick et al. 2001), and indeed the great majority of studies highlight the need for silence in the LC noradrenergic neurons as a condition for the occurrence and maintenance of REM sleep (Gottesmann 2008). The slow activation of GABA A receptors with an ε subunit is therefore well-suited for efficiently inhibiting the huge somas of neurons in LC, thereby ensuring a persistent arrest of firing.

**Epsilon-containing receptors in subpopulations of brain neurons?**

The ε subunit is concentrated in discrete parts of the brain. The ε-immunoreactivity is thus found in cholinergic, dopaminergic, serotonergic, and noradrenergic neuron groups with wide-ranging neuromodulatory actions (Moragues et al. 2002). This raises the possibility that ε plays a unique role, specific to these regions, perhaps connected with their influence on basic brain functions via their long-range projections throughout the neuraxis. It will thus be interesting to examine whether ε has specific targeting and plasticity mechanisms and whether it shares them with α3. In this context, the anxiety-modulating properties of α3-specific compounds (Atack et al. 2005; Dias et al. 2005) require further appraisal, and ε may be instrumental in the clinical effects of some allosteric GABA A-receptor modulators.

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Epsilon-containing GABA<sub>A</sub> receptors in LC

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