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ACCELERATED COMMUNICATION

Immortalized Hypothalamic GT1-7 Neurons Express Functional γ-Aminobutyric Acid Type A Receptors

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SUMMARY

Neuronal cell lines provide a source of pure populations of neurons and allow the properties of many neurotransmitter receptors to be studied. However, none of these cells have been reported to express functional γ-aminobutyric acid $(GABA)_A$ receptors. Indeed, there have been no reports of cell lines expressing functional amino acid receptors. Using biochemical and electrophysiological techniques, we have identified a neuronal cell line expressing functional $GABA_A$ receptors. Membranes from immortalized hypothalamic (GT1-7) neurons bound [^{3}H]muscimol but not [^{3}H]flunitrazepam. GABA-activated chloride currents, recorded from GT1-7 cells, were blocked by bicuculline and Zn^{2+} but were insensitive to diazepam. These results suggest that $GABA_A$ receptors on GT1-7 cells lack γ subunits. The neurosteroid 5α-pregnan-3α-ol-20-one and pentobarbital both modulated $GABA_A$ receptors in these cells. Polymerase chain reaction analysis of the cells revealed the presence of mRNAs encoding α1, β1, and β3 polypeptides. GT1-7 cells provide a useful model system for studying the regulation of $GABA_A$ receptor polypeptide expression.

Hetero-oligomeric $GABA_A$ receptors are present in neurons throughout the mammalian nervous system. Like nicotinic acetylcholine receptors (1), these inhibitory receptors are believed to consist of five subunits. So far, 14 different varieties of subunit have been cloned, including α1-6, β1-3, γ1, γ2S, γ2L, γ3, and δ (2). This subunit diversity provides the potential for numerous $GABA_A$ receptor subtypes (2). Indeed, multiple subtypes appear to exist in the nervous system, based on binding, protein chemistry, and immunoblotting studies (3-5). Attempts to characterize $GABA_A$ receptor polypeptides in specific neurons have been hindered by the difficulties of obtaining pure cell populations with which to carry out biochemical analyses. Not only is it unclear which $GABA_A$ receptor polypeptides are present in specific types of neurons, but also the receptor subunit stoichiometry remains unresolved.

Neuronal cell lines provide a source of pure populations of neurons. Cell lines with neuronal characteristics can be exploited to study the properties of various neurotransmitter receptors (6). None of these cell lines, however, have been reported to express functional $GABA_A$ receptors. Several types of immortalized neurons, derived from tumors in transgenic mice, are now available (7). For example, Mellon *et al.* (8) obtained GT1-7 cells from transgenic mice by targeting oncogene expression to the promotor region of the GnRH gene.

In this paper we report that [^{3}H]muscimol binds to GT1-7 membranes. In addition, GABA activates bicuculline-sensitive chloride currents recorded from voltage-clamped GT1-7 cells. Using a PCR-based method, we determined that these cells contain mRNAs for α1, β1, and β3 polypeptides. In contrast, we detected no amplification products corresponding to α2, α4, α6, β2, γ2, and δ mRNAs. GT1-7 membranes did not bind [^{3}H] flunitrazepam, and GABA-activated currents recorded from the cells were insensitive to diazepam but blocked by Zn^{2+}; both properties are associated with $GABA_A$ receptors lacking γ subunits (9-11). The identification of a neuronal cell line expressing functional $GABA_A$ receptors, consisting of α1, β1, and β3 subunits, should enable us to study the regulation of gene expression and the properties of a defined $GABA_A$ receptor subtype within a neuronal environment.

Materials and Methods

Cell cultures. GT1-7 cells were cultured in 75-cm^2 tissue culture flasks containing 20 ml of culture medium comprising Dulbecco's

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ABBREVIATIONS: GABA, γ-aminobutyric acid; GT1-7 cells, immortalized gonadotropin-releasing hormone-secreting neurons; PCR, polymerase chain reaction; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GnRH, gonadotropin-releasing hormone; $[Cl^-]_i$, intracellular chloride concentration.

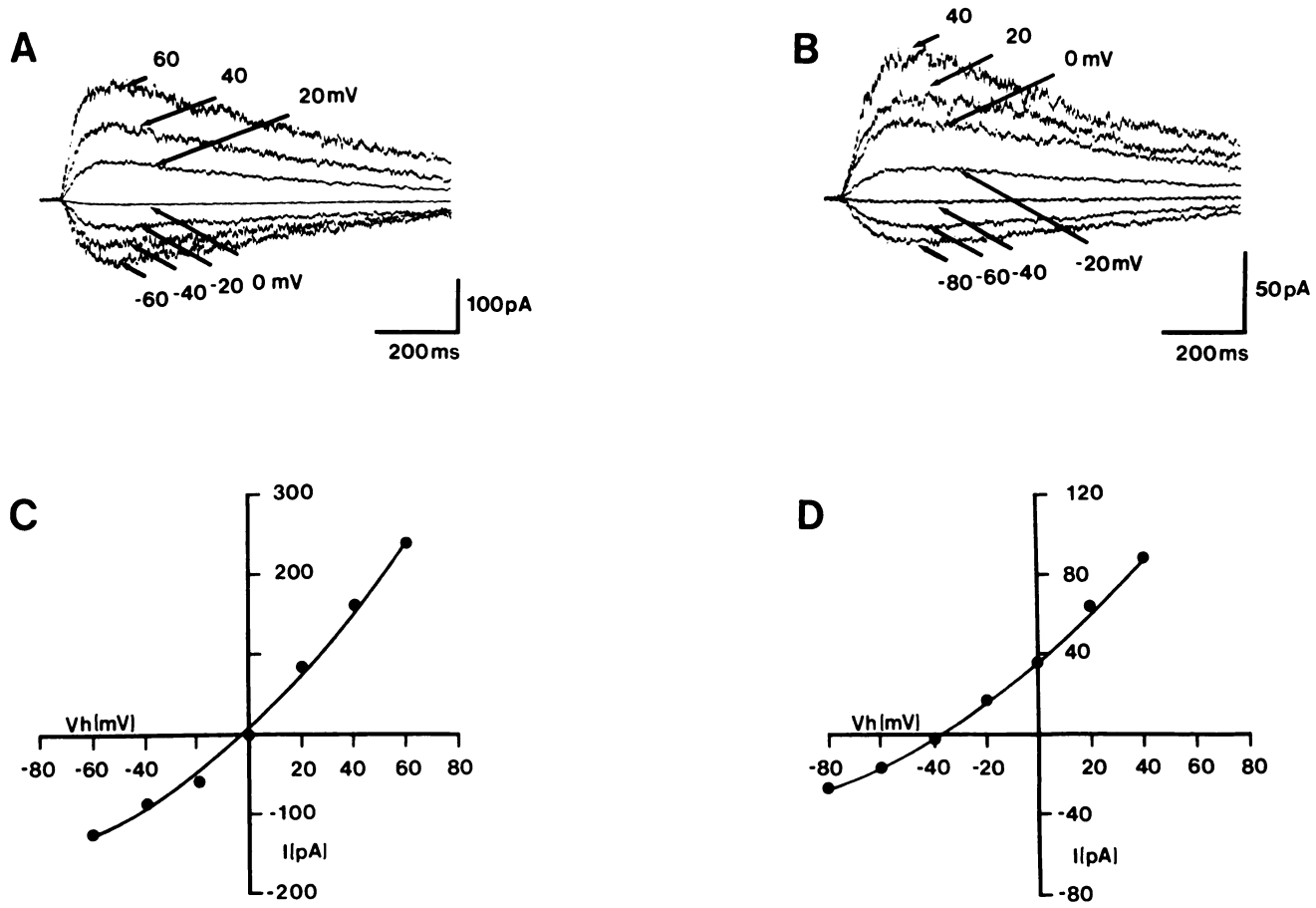

Fig. 1. GABA activates chloride currents in GT1–7 cells. A, GABA (100 μM)-activated currents, recorded from a cell with 148 mM Cl^- on both sides of the cell membrane. Currents were recorded from GT1–7 cells at holding potentials between −60 and 60 mV. B, Currents recorded from a cell with 30 mM $[Cl^-]_i$ and 148 mM extracellular Cl^-. Currents were recorded from cells at holding potentials between −80 and 40 mV. Currents in A and B were recorded from separate cells and low pass filtered at 1 kHz. C, Relationship between the holding potential and the response amplitude, for the currents in A, depicted graphically. D, Graph of the relationship between the response amplitude and voltage for the GABA-evoked currents illustrated in B. *Curves* were fitted to data points by eye, and the I_{GABA} equilibrium potentials were estimated to be approximately 0 mV for the cell in A and −40 mV for that in B.

modified Eagles medium supplemented with 5% (v/v) calf serum, 5% (v/v) horse serum, 5 × 10^4 IU/liter penicillin, and 50 mg/liter streptomycin. Having reached confluency, cells were harvested by replacing the culture medium with 2.5 ml of Ca^{2+}-, Mg^{2+}-, and bicarbonate-free Hanks', containing 500 mg/liter trypsin, 200 mg/liter EDTA, and 10 mg/liter phenol red. After 10 min in the trypsin solution, 10 ml of culture medium were added, to prevent further digestion. Cells were washed by centrifugation (100 × *g* for 5 min) and resuspension in 10 ml of culture medium. For binding and PCR assays, cells were centrifuged (100 × *g* for 5 min) and washed with phosphate-buffered saline. After recentrifugation, the supernatant was removed and pellets were stored at −70° until the day of the experiment. For electrophysiological experiments, 0.15 ml of the cell suspension was added to 35-mm-diameter dishes (Falcon) containing 1.5 ml of culture medium and glass coverslips. Cells were used 2–7 days after subculturing.

Membrane preparation. Cells were homogenized (Tissuemizer-Ultre Turrax) for 15 sec in 10 volumes of ice-cold buffer (10 mM K_2HPO_4/KH_2PO_4, pH 7.4) and were centrifuged for 10 min at 48,000 × *g* at 4°. The pellet was washed once by resuspension and centrifugation in 10 volumes of buffer containing 50 mM KCl. The membranes were stored at −20° until they were used 1–10 days later.

[^{3}H]Flunitrazepam binding assay. On the day of the assay, membranes were thawed and centrifuged. The pellet was washed once by resuspension and recentrifugation in 10 volumes of 50 mM Tris·HCl buffer (pH 7.5). Protein concentration was adjusted to approximately 1 mg/ml, as measured using the method in Ref. 12. The incubation mixture (500 μl) contained 200–300 μg of protein and 2 nM [^{3}H]flunitrazepam (76.0 Ci/mmol; DuPont New England Nuclear). After a 60-min incubation at 4°, samples were filtered through Whatman GF/B glass fiber filters, using a filter manifold (Hoefer Scientific Instruments). Filters were washed three times with 4 ml of ice-cold buffer and counted with 1 ml of Ecolume scintillation fluid (ICN Biomedicals), in a Beckman LS5801 scintillation counter. Single-point binding assays were performed on triplicate samples. Specific binding was defined as the amount of bound [^{3}H]flunitrazepam that was displaced by 1 μM clonazepam.

[^{3}H]Muscimol binding assay. On the day of the assay, membranes were thawed and centrifuged. The pellet was washed three additional times by resuspension and recentrifugation in 10 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.4) containing 50 mM KCl. Aliquots (200–300 μg of protein) of tissue were incubated in a final volume of 500 μl. The incubation (30 min at 4°) was started with the addition of 10 mM [^{3}H]muscimol (17.1 Ci/mmol; DuPont New England Nuclear) and was stopped by centrifugation at 48,000 × *g* for 20 min. The supernatant was discarded and the pellet was gently washed twice with 4 ml of ice-cold water and then solubilized overnight with 1:1 Soluene 350 (Packard)/toluene (Baker). The next day, samples were counted as described for the [^{3}H]flunitrazepam binding assay. Specific [^{3}H]muscimol binding was de-

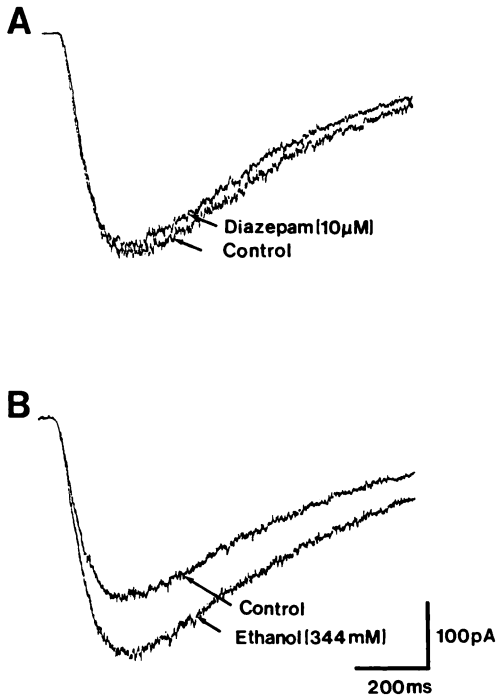

Fig. 2. GT1-7 cells express $GABA_A$ receptors sensitive to high doses of ethanol but not diazepam. A, Diazepam (10 μM) had no discernible effect on currents activated by locally applied GABA (100 μM). B, In the same cell as in A, ethanol (344 mM) increased the amplitude of GABA responses. Currents, recorded at −60 mV, were low pass filtered at 1 kHz, and superimposed *traces* are averages of three currents.

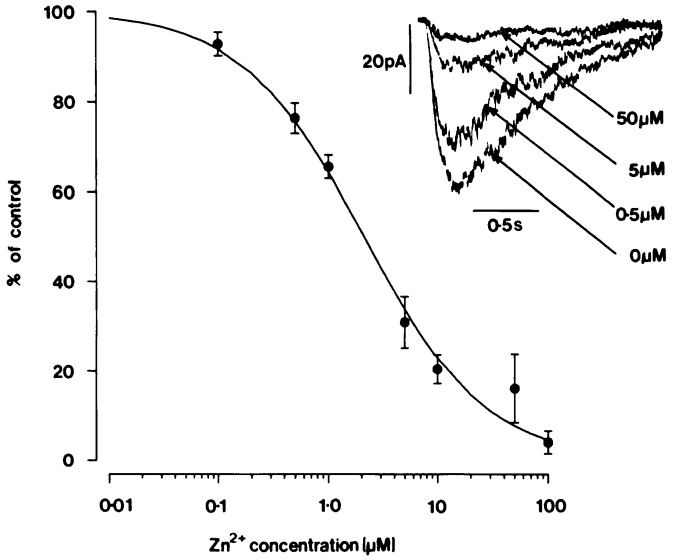

Fig. 3. Zinc blocks GABA-evoked currents recorded from GT1-7 cells. *Inset traces*, GABA-evoked currents recorded from a GT1-7 cell in the absence and presence of Zn^{2+} (0.5, 5, and 50 μM). GABA (100 μM) was applied locally by pressure ejection (at a pressure of 1.4×10^5 Pa, a frequency of 0.04 Hz, and a duration of 20 msec), and Zn^{2+} was applied to the bath. Traces are computer-generated averages of four currents recorded from the same cell, voltage-clamped at −60 mV. The dose-response curve for the block of GABA-evoked currents by Zn^{2+} is illustrated graphically. Each data point represents the mean amplitude of currents, recorded from at least four separate cells, expressed as a percentage of the amplitude of control responses. *Error bars*, ±standard error. All experiments were carried out at −60 mV. The *curve* represents the least squares fit to the data points for the relation $I/[Zn^{2+}] = I_0 \cdot K_i^n/([Zn^{2+}]^n + K_i^n)$, where $I_0 = 100$ and $K_i = IC_{50}$ (11). The IC_{50} for Zn^{2+} is 2.1 μM; n is 0.79.

fined as the amount of radioactivity of bound [^{3}H]muscimol that was displaced by 0.1 mM GABA.

Electrophysiology. For electrophysiological experiments, 0.1 ml of the inoculum was added to 2 ml of culture medium in 35-mm-diameter culture dishes containing glass coverslips. GABA-evoked whole-cell currents were recorded using a List Electronics L/M EPC7 patch-clamp amplifier. Cells were continuously superfused (1.5 ml/min) with extracellular solution consisting of (in mM) NaCl, 140; KCl, 2.8; $MgCl_2$, 2.0; $CaCl_2$, 1.0; and HEPES, 10 (pH 7.2). The electrode solution contained (in mM) CsCl, 140; $MgCl_2$, 2.0; $CaCl_2$, 0.1; EGTA, 1.1; ATP (Mg^{2+} salt), 0.1; and HEPES, 10 (pH 7.2). In some experiments, the electrode solution was supplemented with 114 mM sodium gluconate and CsCl was reduced to 26 mM, to achieve $[Cl^-]_i$ of 30 mM. Equilibrium potentials were compensated for liquid junction potentials. GABA (100 μM) was applied by pressure ejection (1.4×10^5 Pa for 10–30 msec, at 0.04 Hz) from modified patch pipettes. Other compounds were bath applied. Experiments were carried out at 20–24°. Currents were filtered (1 kHz low-pass, eight-pole Bessel, Frequency Devices D02LPF), recorded on chart paper (Gould Brush 2200), and simultaneously acquired (Labmaster DMA) and digitized (2 kHz) for storage on the hard disk of an IBM PC. Currents were averaged, superimposed, and measured using the computer. Drugs used were GABA, (+)-bicuculline, sodium pentobarbital, diazepam, 5α-pregnan-3α-ol-20-one, and 5α-pregnan-3β-ol-20-one (all from Sigma). Stock solutions of pregnane steroids and diazepam, in ethanol, were diluted to achieve an ethanol concentration of <0.1% (17.2 mM).

RNA preparation and PCR amplification. Total cellular RNA was extracted from GT1-7 cells by the acid phenol method (13). First-strand cDNA synthesis was performed using random hexamers (Pharmacia) as primers and murine leukemia virus reverse transcriptase (BRL). The 500-μl reaction contained reaction buffer (BRL), 10 μg of total cellular RNA, 400 ng of the random hexamers, 10 units of RNasin (Promega), 2000 units of murine leukemia virus reverse transcriptase, and 0.25 mM levels of each nucleotide triphosphate. The reaction was incubated at 42° for 2 hr. For PCR amplifications, the samples were brought to final concentrations of 10 mM Tris (pH 8.3), 50 mM KCl, 4% dimethylsulfoxide, 0.01% gelatin, 2.5 mM $MgCl_2$, 200 μM each nucleotide triphosphate, 50 pmol of both the reverse and forward primers (see Table 1 for sequences and locations), 50 ng of template DNA, and 2.5 units of Taq I polymerase (BRL), in a total volume of 100 μl. The mixtures were overlaid with mineral oil and amplified with a Coy Temp Cycler. Each amplification cycle consisted of denaturing at 94° for 30 sec, annealing at 55° for 30 sec, and extending at 72° for 1 min. PCR was carried out for 30 cycles. The PCR products were analyzed by electrophoresis in 1.2% agarose gels containing 0.5 μg/ml ethidium bromide.

Results

[^{3}H]Muscimol binding to GT1-7 membranes. Membranes of GT1-7 cells bound the radiolabeled $GABA_A$ receptor agonist [^{3}H]muscimol (213 ± 16 fmol of [^{3}H]muscimol bound/mg of protein, at 10 nM). In contrast, no significant clonazepam-displaceable binding of the radiolabeled benzodiazepine [^{3}H]flunitrazepam was detected. In addition, the benzodiazepine diazepam (1–100 μM) was unable to enhance specific [^{3}H]muscimol binding. These data provide evidence for the existence of $GABA_A$ receptors in GT1-7 cells. The absence of clonazepam-displaceable [^{3}H]flunitrazepam binding and the lack of modulation of [^{3}H]muscimol binding by diazepam suggest that these receptors are not associated with a central benzodiazepine recognition site.

Properties of GABA-activated currents in GT1-7 cells. We used the patch-clamp technique to study the properties of GABA receptors in single voltage-clamped GT1-7 cells. GABA (100 μM), applied locally by pressure ejection,

TABLE 1

Primer sequences for PCR

Published sequences were obtained as follows: $\alpha 1$ and $\beta 1$, Ref. 27; $\alpha 2$, Ref. 28; $\alpha 4$, Ref. 16; $\alpha 6$, Ref. 29; $\beta 2$ and $\beta 3$, Ref. 30; $\gamma 2$ and δ, Ref. 24; and NF68, Ref. 31.

Subunit	Direction	Primer sequence	Beginning at base[a]
$\alpha 1$	Forward	5′-GACTATCTTTGGGCCTGGACCCTCA-3′	25
	Reverse	5′-CGGGCTGGCTCCCTTGTCCACTC-3′	608
$\alpha 2$	Forward	5′-GAGGACAAAATTGAGCACTTGCA-3′	3
	Reverse	5′-GAGTTGTTAAGTCGAAGGATATT-3′	347
$\alpha 4$[b]	Forward	5′-ACTTTGGCTTTTCACAAATGCCAA-3′	80
	Reverse	5′-AAAAGGTTGAGAGGGAGACGTT-3′	371
$\alpha 6$	Forward	5′-GCTCTTCAGTTTACTATGGATA-3′	11
	Reverse	5′-TCATGGTGTACAGGATCGTTCCA-3′	445
$\beta 1$	Forward	5′-GGAGCGAGCAAACAAGACCAGATGCAAT-3′	1018
	Reverse	5′-GATGCGACCCTTTCCGGGCACCCGTGCCT-3′	1287
$\beta 2$	Forward	5′-ATAAACTCATCACCAAGAAAGTTG-3′	644
	Reverse	5′-AAGTCCCATTACTGCTTCTGATGT-3′	1158
$\beta 3$	Forward	5′-TGGAGCACCGTCTGGTCTCCAGGA-3′	643
	Reverse	5′-TCGATCATTCTTGGCCTTGGCTGT-3′	1061
$\gamma 2$	Forward	5′-GTGGAGTATGGTACCCTGCACTATTTTGTG-3′	1048
	Reverse	5′-CAGAAGGCGGTAGGGAAGAAGATCCGAGCA-3′	1358
δ	Forward	5′-GACTACGTGGGCTCCAACCTGGA-3′	88
	Reverse	5′-ACTGTGGAGGTGATGCGGATGCT-3′	485
NF68	Forward	5′-AGCGTGGGTAGCATAACCAGCGGCTAC-3′	475
	Reverse	5′-AGAGGGGGGCTCATCCTTGGCCTCCTC-3′	681

[a] Bases are numbered from the beginning of the coding region.
[b] $\alpha 4$ has also been termed $\alpha 5$ (17).

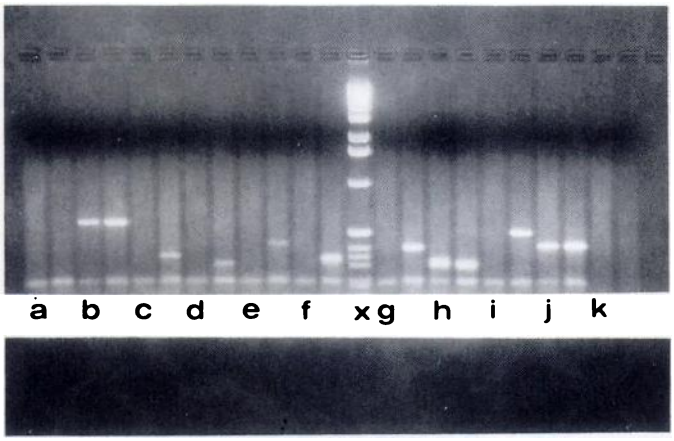

Fig. 4. GT1–7 cells express $\alpha 1$, $\beta 1$, and $\beta 3$ $GABA_A$ receptor subunit mRNAs. PCR amplification products were analyzed by electrophoresis on a 1.2% agarose gel. *Lane x*, 1-kilobase DNA ladder (BRL). All other lettered lanes represent target cDNAs (50 ng) from GT1–7 cells. Each lettered lane is paired (to the *right*) with one containing rat brain cDNA amplified with the same primer as its pair. *Lane k* and its pair contain no primers; all other lanes contain a primer for neurofilament protein (68 kDa), in addition to a $GABA_A$ receptor subunit-specific primer. *Lane a*, no $GABA_A$ receptor subunit primers; *lane b*, primers for $\alpha 1$; *lane c*, primers for $\alpha 2$; *lane d*, primers for $\alpha 4$; *lane e*, primers for $\alpha 6$; *lane f*, primers for $\gamma 2$; *lane g*, primers for δ; *lane h*, primers for $\beta 1$; *lane i*, primers for $\beta 2$; *lane j*, primers for $\beta 3$. All lanes (except *x* and its pair) display a band for neurofilament protein. *Lanes b, h,* and *j* also exhibit $\alpha 1$, $\beta 1$, and $\beta 3$ bands, respectively. Bands were evident in rat brain lanes for the other $GABA_A$ receptor subunits tested. For primer sequences and location, see Table 1.

evoked inward currents in 100% of cells voltage-clamped at −60 mV (n = 98). GABA-evoked current amplitudes ranged between 20 and 400 pA. Whole-cell GABA-activated currents had a reversal potential of 2.3 ± 1.2 mV (mean ± standard error; n = 4), with equal chloride in the electrode and extracellular solutions (Fig. 1, A and C). Reducing the $[Cl^-]_i$ to 30 mM shifted the equilibrium potential to −41.6 ± 0.3 mV (n = 3) (Fig. 1, B and D). These data are consistent with GABA activating chloride currents in GT1–7 cells. Bath application of the $GABA_A$ receptor antagonist bicuculline (1 μM) reversibly reduced the amplitude of GABA-evoked currents to 18 ± 6% (n = 3) of control amplitude (data not shown). Taken together, these observations demonstrate that functional $GABA_A$ receptors are expressed by GT1–7 cells.

Benzodiazepines and ethanol exhibit subunit-specific actions on the $GABA_A$ receptor. Receptors lacking $\gamma 1$, $\gamma 2$, or $\gamma 3$ subunits are insensitive to benzodiazepines (9, 10, 14). Modulation of the $GABA_A$ receptor by ethanol appears also to be subunit specific. The long form of the $\gamma 2$ subunit specifically confers sensitivity to ethanol (15). GABA-evoked currents recorded from GT1–7 cells were insensitive to diazepam (1 μM, n = 4; 10 μM, n = 3) (Fig. 2A). In addition, a low dose of ethanol (17.2 mM) had no effect on currents activated by GABA (n = 4) (data not shown). In contrast, high concentrations of ethanol (172 and 344 mM) potentiated the amplitude of GABA-activated currents to 119 ± 14% (n = 3) and 138 ± 12% (n = 3) (Fig. 2B) of control.

$GABA_A$ receptors incorporating $\gamma 1$ or $\gamma 2$ subunits, in transfected kidney cells, are insensitive to Zn^{2+}. In contrast, GABA-evoked currents recorded from cells lacking γ subunits are blocked by Zn^{2+} (11). Low concentrations of bath-applied zinc (0.5–100 μM) (Fig. 3) blocked GABA-activated currents recorded from GT1–7 cells. This effect of zinc was dose dependent, with an IC_{50} of 2.1 μM (Fig. 3). The lack of [^{3}H]flunitrazepam binding, the insensitivity of GABA-evoked currents to diazepam and low doses of ethanol, and the zinc block of GABA responses suggest that $GABA_A$ receptors of GT1–7 cells lack $\gamma 1$, $\gamma 2$, and $\gamma 3$ subunits.

Analysis of $GABA_A$ receptor subunit mRNAs. We used PCR analysis to examine the steady state levels of mRNAs encoding the $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 6$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 2$, and δ $GABA_A$ receptor subunits in GT1–7 cells (Table 1). Total cellular RNA was extracted from the cells, and cDNA was produced by

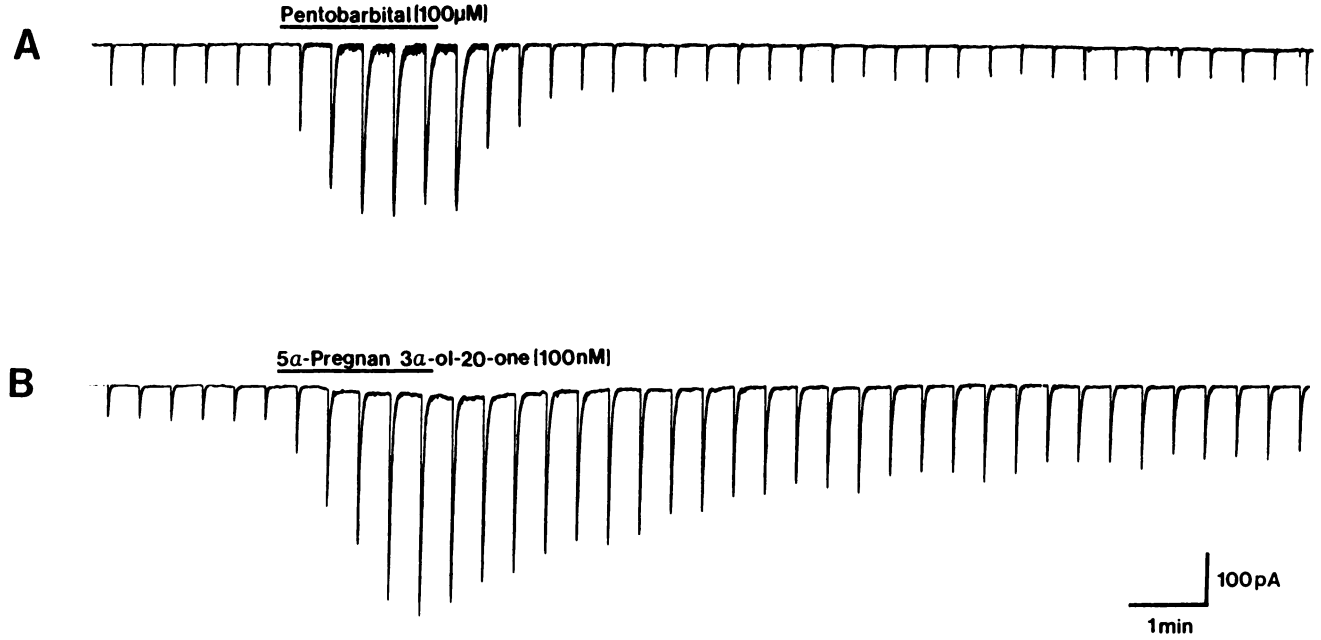

Fig. 5. Pentobarbital and 5α-pregnan-3α-ol-20-one modulate $GABA_A$ receptors in GT1–7 neurons. A, Currents activated by locally applied GABA (100 μM) were potentiated by bath-applied pentobarbital (100 μM). B, GABA-evoked currents were also increased in amplitude by the anesthetic steroid 5α-pregnan-3α-ol-20-one (100 nM). Currents were recorded at −60 mV and low pass filtered at 1 kHz.

reverse transcription and amplified by PCR. The amplification products were analyzed by electrophoresis. The efficiency of PCR was monitored in each reaction by including a set of primers specific for neurofilament (NF68) mRNA (Table 1). Rat brain cDNA served as a standard for the PCR reaction.

Visual inspection of the gel in Fig. 4 suggests that GT1–7 cells contain mRNAs for α1, β1, and β3. After 30 PCR cycles, there were no perceptible bands corresponding to α2, α4 [nomenclature of Khrestchatisky *et al.* (16)], α6, β2, γ2, or δ $GABA_A$ receptor polypeptides (Fig. 4). Whole rat brain cDNA, in contrast, contained mRNAs for all $GABA_A$ receptor subunits tested. No attempt was made to quantify the difference between the levels of $GABA_A$ receptor mRNAs in rat brain and GT1–7 cells. These data do not rule out the presence of mRNAs for α3 or α4 [nomenclature of Pritchett and Seeburg (17)] but complement the electrophysiological and ligand binding data that suggest the absence of γ subunits from GT1–7 cells.

Effects of a barbiturate and an anesthetic steroid on GABA-evoked currents. $GABA_A$ receptors of neurons and chromaffin cells are modulated by depressant barbiturates (18). When bath-applied to GT1–7 cells, pentobarbital (100 μM) increased the amplitude of GABA-evoked currents to 450 ± 61% of control, an effect that was fully reversed after washing ($n = 5$) (Fig. 5A).

$GABA_A$ receptors of neurons and chromaffin cells are also sensitive to anesthetic steroids (19, 20). GABA-activated currents recorded from GT1–7 cells were potentiated by the endogenous anesthetic steroid 5α-pregnan-3α-ol-20-one (100 nM) to 413 ± 89% of control ($n = 6$) (Fig. 5B). 5α-Pregnan-3β-ol-20-one (100 nM), a nonanesthetic steroid, had no effect on currents induced by GABA in cells responsive to its stereoisomer ($n = 3$) (data not shown).

Discussion

This study characterizes a functional $GABA_A$ receptor expressed by GT1–7 cells. GT1–7 membranes bound [^{3}H]muscimol but not [^{3}H]flunitrazepam, and cells responded to GABA. GABA, when applied to single voltage-clamped neurons, activated chloride currents blocked by the $GABA_A$ receptor antagonist bicuculline.

A number of compounds exert subunit-specific actions on $GABA_A$ receptors. For example, recombinant $GABA_A$ receptors lacking γ subunits, expressed in *Xenopus* oocytes and human embryonic kidney 293 cells, are insensitive to benzodiazepines (2). The GABA-modulatory actions of ethanol are also subunit specific, requiring the presence of the γ2L subunit (15). Diazepam and low doses of ethanol had no effect on GABA-evoked currents recorded from GT1–7 cells. These findings are consistent with the absence of the γ2 and γ3 subunits from the $GABA_A$ receptor in these cells (2, 14). High doses of ethanol, however, provoked small increases in the amplitude of GABA-evoked currents in GT1–7 cells. The degree of potentiation achieved by high concentrations of ethanol was similar to that observed in a comparable study using dorsal root ganglion neurons (21). If γ2L subunits were present in the GABA receptors of GT1–7 cells, [^{3}H]flunitrazepam binding and potentiation of GABA-evoked currents by diazepam would have been expected. This was not the case; hence, modulation of the $GABA_A$ receptor by high doses of ethanol appears to be independent of the γ2 subunit.

Zinc blocks GABA responses recorded from embryonic neurons (22). This effect of zinc exhibits subunit selectivity. Draguhn *et al.* (11), using recombinant rat $GABA_A$ receptors expressed in kidney cells, demonstrated that receptors incorporating γ subunits are insensitive to Zn^{2+}. In contrast, GABA responses mediated via receptors containing neither γ1 nor γ2 subunits were blocked noncompetitively by Zn^{2+} (11). Zn^{2+} blocks GABA-evoked currents recorded from GT1–7 cells, adding to the pharmacological evidence suggesting that $GABA_A$ receptors in GT1–7 cells lack γ subunits.

We used PCR to detect mRNAs coding $GABA_A$ receptor polypeptides. GT1–7 cell mRNA was copied into cDNAs, and

the products were amplified by PCR with primers for α1, α2, α4, α6, β1, β2, β3, γ2, and δ mRNAs. We detected amplification products only when primers for α1, β1, and β3 mRNAs were used. In contrast, parallel reactions using rat whole-brain cDNAs yielded amplification products in the presence of all subunit-specific primers used. These data, combined with the radioligand binding and electrophysiology data, suggest that $GABA_A$ receptors of GT1-7 cells contain α1, β1, and β3 subunits. Previous *in situ* hybridization studies documented the existence of α1, β1, β2, β3, γ1, and γ2 subunit mRNAs in the rat hypothalamus (10, 23–25). The lack of β2 and γ2 subunit mRNAs in the GnRH-secreting neuronal line suggests the possibility of $GABA_A$ receptor heterogeneity within the hypothalamus. Alternatively, the immortalization procedure may have disrupted the expression of these $GABA_A$ receptor mRNAs in GT1-7 cells.

GT1-7 cells may be a system in which the subunit stoichiometry of a native neuronal $GABA_A$ receptor can be determined (2). They may also prove useful for studying the regulation of α1, β1, and β3 polypeptide expression.

Anesthetic barbiturates (18) and steroids (19, 20) modulate $GABA_A$ receptors. Pentobarbital and the endogenous neurosteroid 5α-pregnan-3α-ol-20-one potentiated GABA-activated currents recorded from GT1-7 cells. In studies using recombinant $GABA_A$ receptors, modulation by anesthetic barbiturates and steroids is a feature of all functional receptor subunit combinations tested (26).

GT1-7 cells are the first neuronal cell lines to be identified as expressing functional $GABA_A$ receptors. We have used these cells to characterize $GABA_A$ receptors expressed by GnRH-secreting hypothalamic neurons. Using a similar approach to study different immortalized neurons (7), it may be possible to determine the pharmacological properties and subunit combinations of other $GABA_A$ receptor subtypes in the brain.

Acknowledgments

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References

1. Unwin, N. The structure of ion channels in the membranes of excitable cells. *Neuron* **3**:665–676 (1989).
2. Burt, D. R., and G. L. Kamatchi. $GABA_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J.* **5**:2916–2923 (1991).
3. Olsen, R. W., R. T. McCabe, and J. K. Wamsley. $GABA_A$ receptor subtypes: autoradiographic comparison of GABA, benzodiazepine, and convulsant binding sites in the rat central nervous system. *J. Chem. Neuroanat.* **3**:59–76 (1990).
4. Bureau, M., and R. W. Olsen. Multiple distinct subunits of the γ-aminobutyric acid-A receptor protein show different ligand-binding affinities. *Mol. Pharmacol.* **37**:497–502 (1990).
5. Whiting, P., R. M. McKernan, and L. L. Iversen. Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site. *Proc. Natl. Acad. Sci. USA* **87**:9966–9970 (1990).
6. Lambert, J. J., J. A. Peters, T. G. Hales, and J. Dempster. The properties of 5-HT-3 receptors in clonal cell lines studied by patch-clamp techniques. *Br. J. Pharmacol.* **97**:27–40 (1989).
7. Cepko, C. L. Immortalization of neural cells via retrovirus mediated oncogene transduction. *Annu. Rev. Neurosci.* **12**:47–65 (1989).
8. Mellon, P. L., J. J. Windle, P. C. Goldsmith, C. A. Padula, J. L. Roberts, and R. I. Weiner. Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron* **5**:1–10 (1990).
9. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. H. Seeburg. Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. *Nature (Lond.)* **338**:582–585 (1989).
10. Ymer, S., A. Draguhn, W. Wisden, P. Werner, K. Keinänen, P. R. Schofield, R. Sprengel, D. B. Pritchett, and P. H. Seeburg. Structural and functional characterization of the γ1 subunit of $GABA_A$/benzodiazepine receptors. *EMBO J.* **9**:3261–3267 (1990).
11. Draguhn, A., T. A. Verdorn, M. Ewert, P. H. Seeburg, and B. Sakmann. Functional and molecular distinction between recombinant rat $GABA_A$ receptor subtypes by Zn^{2+}. *Neuron* **5**:781–788 (1990).
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
13. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).
14. Knoflach, F., T. Rhyner, M. Villa, S. Kellenberger, U. Drescher, P. Malherbe, E. Sigel, and H. Möhler. The γ3-subunit of the $GABA_A$-receptor confers sensitivity to benzodiazepine receptor ligands. *FEBS Lett.* **293**:191–194 (1991).
15. Wafford, K. A., D. M. Burnett, N. J. Leidenheimer, D. R. Burt, J. B. Wang, P. Kofuji, T. V. Dunwiddie, R. A. Harris, and J. M. Sikela. Ethanol sensitivity of the $GABA_A$ receptor expressed in *Xenopus* oocytes requires 8 amino acids contained in the γ2L subunit. *Neuron* **7**:27–33 (1991).
16. Khrestchatisky, M., A. J. MacLennan, M.-Y. Chiang, W. T. Xu, M. B. Jackson, N. Brecha, C. Sternini, R. W. Olsen, and A. J. Tobin. A novel α subunit in rat brain $GABA_A$ receptors. *Neuron* **3**:745–753 (1989).
17. Pritchett, D. B., and P. H. Seeburg. γ-Aminobutyric $acid_A$ receptor α_5-subunit creates novel type II benzodiazepine receptor pharmacology. *J. Neurochem.* **54**:1802–1804 (1990).
18. Macdonald, R. L., J. H. Skerrit, and M. A. Werz. Barbiturates and benzodiazepine actions on mouse neurons in cell culture, in *Molecular and Cellular Mechanisms of Anesthetics* (S. H. Roth and K. W. Miller, eds.). Plenum Publishing Corp., New York, 17–25 (1986).
19. Barker, J. L., N. L. Harrison, G. D. Lange, and D. G. Owen. Potentiation of γ-aminobutyric-acid-activated chloride conductance by a steroid anaesthetic in cultured rat spinal neurones. *J. Physiol. (Lond.)* **386**:485–501 (1987).
20. Lambert, J. J., J. A. Peters, and G. A. Cottrell. Actions of synthetic and endogenous steroids on the $GABA_A$ receptor. *Trends Pharmacol. Sci.* **8**:224–227 (1987).
21. Nishio, M., and T. Narahashi. Ethanol enhancement of GABA-activated chloride current in rat dorsal root ganglion neurons. *Brain Res.* **518**:283–286 (1990).
22. Smart, T. G., and A. Constanti. Differential effect of zinc on the vertebrate $GABA_A$ receptor complex. *Br. J. Pharmacol.* **99**:643–654 (1990).
23. Lolait, S. J., A. M. O'Carroll, K. Kusano, and L. C. Mahan. Pharmacological characterization and region-specific expression in brain of the β2- and β3-subunits of the rat $GABA_A$ receptor. *FEBS Lett.* **258**:17–21 (1989).
24. Shivers, B. D., I. Killisch, R. Sprengel, H. Sontheimer, M. Kohler, P. R. Schofield, and P. H. Seeburg. Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. *Neuron* **3**:327–337 (1989).
25. Zhang, J. H., M. Sato, and M. Tohyama. Region-specific expression of the mRNAs encoding β subunits (β1, β2 and β3) of $GABA_A$ receptor in the rat brain. *J. Comp. Neurol.* **303**:637–657 (1991).
26. Puia, G., M. R. Santi, S. Vicini, D. B. Pritchett, R. H. Purdy, S. M. Paul, P. H. Seeburg, and E. Costa. Neurosteroids act on recombinant human $GABA_A$ receptors. *Neuron* **4**:759–765 (1990).
27. Schofield, P. R., M. G. Darlison, N. Fujita, D. R. Burt, F. A. Stephenson, H. Rodriguez, L. M. Rhee, J. Ramachandran, V. Reale, T. A. Glencorse, P. H. Seeburg, and E. A. Barnard. Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor super-family. *Nature (Lond.)* **328**:221–227 (1987).
28. Levitan, E. S., P. R. Schofield, D. R. Burt, L. M. Rhee, W. Wisden, M. Köhler, N. Fujita, H. F. Rodriguez, F. A. Stephenson, M. G. Darlinson, E. A. Barnard, and P. H. Seeburg. Structural and functional basis for $GABA_A$ receptor heterogeneity. *Nature (Lond.).* **335**:76–79 (1988).
29. Kato, K. Novel $GABA_A$ receptor α subunit is expressed only in cerebellar granule cells. *J. Mol. Biol.* **214**:619–624 (1990).
30. Ymer, S., P. R. Schofield, A. Draguhn, P. Werner, M. Köhler, and P. H. Seeburg. $GABA_A$ receptor β subunit heterogeneity: functional expression of cloned cDNAs. *EMBO J.* **8**:1665–1670 (1989).
31. Lewis, S. A., and N. J. Cowan. Genetics, evolution, and expression of the 68,000-mol-wt neurofilament protein: isolation of a cloned cDNA probe. *J. Cell. Biol.* **100**:843–850 (1985).

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