0026-895X/96/030458-09$3.00/0

MOLECULAR PHARMACOLOGY, 50:458–466 (1996).

Pharmacological Properties of γ-Aminobutyric Acid$_A$ Receptors from Acutely Dissociated Rat Dentate Granule Cells

JAIDEEP KAPUR and ROBERT L. MACDONALD

Departments of Neurology (J.K., R.L.M.) and Physiology (R.L.M.), University of Michigan Medical Center, Ann Arbor, Michigan 48104-1687

Received February 15, 1996; Accepted May 8, 1996

SUMMARY

The pharmacological properties of γ-aminobutyric acid (GABA) type A receptor (GABAR) currents recorded from hippocampal dentate granule cells acutely dissociated from 28–35-day-old rats were characterized using the whole-cell patch-clamp technique. Granule cells were voltage-clamped to 0 mV, and GABA was applied using a modified U-tube rapid-application technique. All granule cells were moderately sensitive to GABA (EC_{50} = 47 μM). All granule cell GABAR currents were uniformly sensitive to Zn^{2+} (IC_{50} = 29 μM), diazepam (EC_{50} = 158 nM), zolpidem (EC_{50} = 75 nM), and dimethoxyl-4-ethyl-β-carboline-3-carboxylate (IC_{50} = 60 nM). GABAR currents from only 50% of granule cells were sensitive to loreclezole (EC_{50} = 9 μM). These data suggest that hippocampal dentate granule cells expressed GABARs with distinctive pharmacological properties of two types: loreclezole-sensitive and -insensitive receptors. It is likely that these distinctive properties were due to the specific GABAR subtypes that assembled to produce distinct granule cell GABAR isoforms.

Fast inhibitory synaptic transmission in the CNS is primarily mediated by the neurotransmitter GABA interacting with postsynaptic GABARs. The classic properties of GABARs include formation of a chloride channel; GABAR currents are enhanced by barbiturates, benzodiazepines, and neurosteroids and antagonized by penicillin, picrotoxin, bicuculline, and Zn^{2+}. The concept of a single type of GABAR was challenged by early pharmacological studies demonstrating that GABARs had varying sensitivity to benzodiazepines. Recent molecular cloning of the receptor indicated that GABAR isoforms are likely composed of varying combinations of five subunits structurally homologous to the nicotinic acetylcholine receptor (1). Five families of GABAR subunits (α, β, γ, δ, and ρ) and multiple subunit subtypes (α1-6, β1-4, γ1-4, δ1, and ρ1-2) have been cloned, suggesting the that multiple GABAR isoforms may be expressed in the CNS.

There is growing evidence for the structural diversity of native CNS GABARs. *In situ* hybridization studies have demonstrated an heterogeneous distribution of GABAR subtype mRNAs in the brain. Immunoprecipitation studies have shown that different specific combinations of GABAR subtypes occur in the different regions of the brain. McKernan *et al.* (2) found that a majority of native GABARs contain only a single α subtype, and that α1, α2, α3, or α5 subtypes coassemble with a β and the γ2 subtype. Subunit-specific serum for the δ subunit precipitated a specific set of GABARs that contained α1, α3, β2/3, and γ2 subtypes but not the α5 subtype (3). Expression of specific GABAR isoforms in different neurons may result in distinct forms of GABAergic inhibition because GABARs composed of different subunit subtype combinations expressed in heterologous systems have been shown to have different pharmacological and biophysical properties.

Hippocampal dentate granule cells can potentially express GABARs with distinct properties because they express 10 different GABAR subtype mRNAs. *In situ* hybridization studies have demonstrated high levels of expression of mRNAs for α1, α2, α4, α5, β1, β2, β3, γ1, γ2, and δ1 subtypes in the dentate gyrus (4). It is not known which of the subtypes assemble to form the native GABAR(s) on the granule cell. α4, α5, β1, γ1, and δ1 subtype mRNAs have a restricted distribution in the brain, and each subtype, if expressed in granule cell GABARs, would confer distinct pharmacological properties. The immunoprecipitation experiments predicted that granule cells expressed either δ1 or α5 subtypes but not both in combination with α1, α3, β2/3, and γ2 subtypes. These studies made no prediction regarding the expression of α2, α4, β1, or γ1 subtypes by granule cells. Immunoprecipi-

This work was supported by grants from American Epilepsy Society and the National Epifellows Foundation, by United States Public Health Service Grant KO8-NS01748 (J.K.), grants from the Lucille P. Markey Charitable Trust Fund, and by United States Public Health Service Grant RO1-NS33300 (R.L.M.).

ABBREVIATIONS: GABA, γ-aminobutyric acid; GABAR, γ-aminobutyric acid$_A$ receptor; CNS, central nervous system; DMCM, methyl-6,7-dimethoxyl-4-ethyl-β-carboline-3-carboxylate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

tation studies did not reveal whether subtype combinations precipitated from brains were functionally expressed by granule cells. Finally, these studies also did not determine whether more than one GABAR isoform was expressed by single granule cells.

The pharmacological properties of individual hippocampal dentate granule cell GABAR isoforms were characterized to seek evidence for functional expression of the subtypes whose mRNA was detected in the dentate gyrus by *in situ* hybridization study of Wisden *et al.* (4). The pharmacological properties of individual neurons were characterized to determine the presence or absence of hippocampal dentate granule cell GABAR isoform heterogeneity.

Materials and Methods

Isolation of dentate granule cells. All experiments were performed on dentate granule cells isolated according to the method described originally by Kay and Wong (5) and later modified by Coulter *et al.* (6). Male or female Sprague-Dawley rats (28–35 days old) (Harlan Bioproducts for Science, Indianapolis, IN) were killed and decapitated. The brain was dissected free, and the region containing the hippocampus was blocked and chilled in an oxygenated bicarbonate buffer (4°) for 1 min. The bicarbonate buffer solution contained 120 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM D-glucose, and 20 mM $NaHCO_3$, pH 7.4. After blot drying, the brain was mounted on a vibratome stage, and 500-μm coronal sections containing the hippocampus were cut. The sections were allowed to recover in oxygenated (95% O_2/5% CO_2) bicarbonate buffer for 30–60 min. Hippocampal sections were then incubated in oxygenated Sigma type XXIII (Sigma Chemical, St. Louis, MO) protease enzyme in a PIPES buffer at 32° for 30–45 min. The PIPES buffer solution had the same composition as the bicarbonate buffer solution except that the PIPES replaced bicarbonate as the buffer, and pH of the solution was titrated to 7.0 with NaOH. The dentate gyrus was dissected and cut into 0.5-mm cubes, which were triturated in a cold (4°) PIPES-buffered medium with fire-polished glass pipettes to isolate neurons. The isolated neurons were plated onto 35-mm polystyrene petri dishes (Corning Glass Works, Corning, NY), and the recordings were made within 1 hr of isolation. Only the soma and the proximal dendrites of the dentate granule cells were preserved during the isolation procedure (Fig. 1).

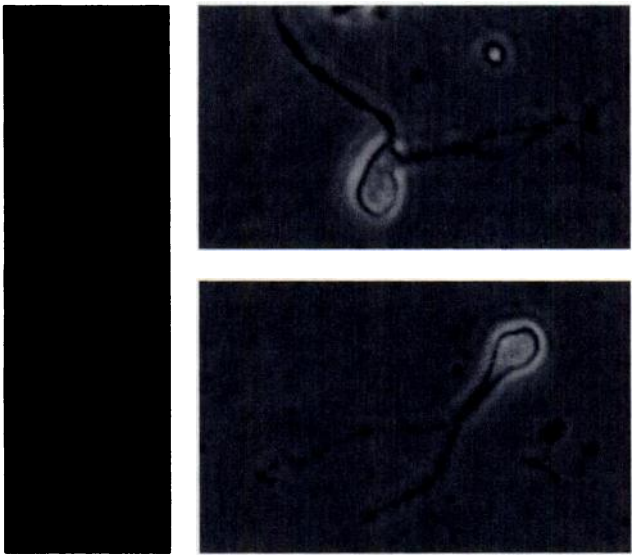

Fig. 1. Acutely isolated hippocampal dentate granule cells. Phase-contrast micrographs of granule cells isolated from the dentate gyrus of a hippocampal slice of a 30-day-old rat.

Recording. Whole-cell voltage-clamp recordings were made using the technique described by Hamill *et al.* (7). The extracellular recording solution consisted of 142 mM NaCl, 1.0 mM $CaCl_2$, 8.09 mM KCl, 6 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH adjusted to 7.4 and osmolarity of 310–320 mOsM (all reagents were from Sigma). Glass recording patch pipettes were filled with a solution consisting of the following 115 mM Trizma phosphate (dibasic), 30 mM Trizma base, 11 mM EGTA, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 4 mM Mg-ATP, pH 7.35. Recording pipettes also contained an ATP regeneration system consisting of 50 units/ml creatinine phosphokinase, 22 mM phosphocreatine, and 10 mM ATP. The ATP regeneration system maintained intracellular energy stores and reduced run down of GABAR currents as previously described by Stelzer and Wong (8). This maintenance solution was used to fill the shank of the recording pipette but was omitted from the solution used to fill the tip of the recording pipette because it interfered with seal formation. All recordings were obtained at room temperature (24°). Patch pipettes (resistance of 6–8 MΩ) were pulled on P-87 Flaming Brown puller by a four-stage pull. Currents were recorded with an Axopatch 1-D amplifier (Axon Instruments, Burlingame, CA), and low-pass filtered at 2 kHz with an eight-pole Bessel filter before digitization, storage, and display. Currents were displayed on a Gould 2400S chart recorder (Gould, Cleveland, OH), and peak whole-cell currents were measured manually from the chart paper. Currents were also recorded on a hard disk (Acer-286turbo PC) using the Axotape program (Axon Instruments) (digitized at 208 Hz) and on a video cassette tape recorder (Sony SL-HF360) via a digital audio processor (Sony PCM-501 ES, 14-bit, 44 kHz).

Drug application. GABA, zolpidem, and $ZnCl_2$ dissolved in extracellular solution were applied to neurons using a modified U-tube "multipuffer" rapid-application system (9, 10) with the tip of application pipette placed 100–200 μm from the cell. The rate of solution exchange was rapid with a mean 10–90 rise time of 54.4 ± 4.96 msec (five cells). Loreclezole and diazepam were first dissolved in dimethylsulfoxide and then diluted in extracellular buffer with the final dimethylsulfoxide dilution being ≥1:50,000. GABA, picrotoxin. pentobarbital, and $ZnCl_2$ were obtained from Sigma; zolpidem was obtained from Research Biochemicals (Natick, MA); and loreclezole was a gift from Janssen Pharmaceuticals (Beerse, Belgium).

Study of allosteric regulators. Allosteric regulators known to enhance GABAR currents were coapplied with 10 μM GABA. This concentration of GABA was selected for several reasons. First, it was on the rising phase of the GABA concentration-response curve, allowing accurate assessment of enhancement without saturation of the maximum current (11). Second, with 10 μM GABA, desensitization was minimal and GABAR current run-down was slow, thus reducing trial-to-trial variability of responses. Finally, past quantitative studies of enhancement of GABAR currents by allosteric regulators have been made at GABA concentrations less than the EC_{50} for the GABA concentration-response relationship, thus allowing comparison of the current study with the previous studies. For similar reasons, allosteric regulators known to inhibit GABAR currents were coapplied with 30 μM GABA.

Data analysis. The magnitude of the enhancement or inhibition of GABAR current by a drug was measured by dividing the peak amplitude of GABAR current elicited in the presence of a given concentration of the drug and GABA by the peak amplitude of control current elicited by GABA alone and multiplying the fraction by 100 to express it as percentage of control. Thus, the control response was 100%. Peak GABAR currents at various drug concentrations were fitted to a sigmoidal function using a four-parameter logistic equation (sigmoidal concentration-response) with a variable slope. The equation used to fit the concentration-response relationship was:

$$I = \frac{I_{max}}{1 + 10^{(Log_{EC_{50}} - Log_{drug}) \cdot n_H}}$$

where I is the GABAR current at a given GABA concentration, and I_{max} is the maximal GABAR current. Maximal current and concentration-response curves were obtained after pooling data from all neurons tested for GABA and for all drugs except loreclezole. Concentration-response curves were also obtained from individual neurons for GABA, Zn^{2+}, diazepam, and zolpidem. The curve-fitting algorithm minimized the sum of the squares of the actual distance of points from the curve. Convergence was reached when two consecutive iterations changed the sum of squares by <0.01%. The curve fit was performed on an IBM-compatible personal computer using Prism (GraphPAD, San Diego, CA). All data are presented as mean ± standard error.

Results

Dentate granule cell GABA concentration-response relationship. Granule cells are the principal output neurons of the dentate gyrus and are abundantly present in this region, and thus a large number of granule cells could be isolated for every experiment. They were identified by their characteristic granular shape, small size, and a single large principal dendrite (Fig. 1). Neurons that had >30-μm soma diameters, were polygonal or pyramidal, or had multiple dendrites were not studied.

GABA was applied to the granule cells at concentrations ranging from 1 to 1000 μM with recovery intervals of ≥2 min (Fig. 2A). The rate of activation of GABAR currents was concentration dependent, increasing with increasing GABA concentrations (Fig. 2A).

Desensitization of GABAR currents was GABA concentration dependent (Fig. 2A). Desensitization was virtually absent at GABA concentrations of <10 μM and became more prominent and increased in rate as GABA concentration was increased (Fig. 2A). GABA concentration-response curves were obtained from individual granule cells for GABA concentrations ranging from 3 to 1000 μM. EC_{50} values for individual cells (five cells) ranged from 30 to 113 μM (median, 34 μM). The data from individual cells were pooled and fitted to a sigmoidal logistic function (Fig. 2B). Mean GABA EC_{50} was 46 ± 10 μM, maximal GABAR current was 842 ± 54 pA, and the Hill slope was 1.2. It was possible that the peak currents elicited by high concentrations of GABA did not represent the true GABAR maximal current for at least two reasons: if the rate of desensitization was sufficiently fast or if there was significant redistribution of chloride ions by application of high concentration of GABA, peak GABAR currents would not have been achieved. The mean GABAR current elicited by 1 mM GABA from these five neurons was 838 pA compared with 842 pA maximal current derived by the best fit to Hill equation. This suggested that at high GABA concentrations, peak currents were not compromised by rapid desensitization or by chloride ion redistribution.

Diazepam enhancement of dentate granule cell GABAR currents. Diazepam (1–1000 nM) was coapplied with 10 μM GABA. There was no enhancement of GABAR current by 1 or 10 nM diazepam (11 cells). However, higher concentrations of diazepam (30 nM to 1 μM) uniformly enhanced GABAR currents in a concentration-dependent fashion (Fig. 3A). Detailed diazepam concentration-response relationships were obtained in six neurons. The data from these neurons could be fitted to a sigmoidal function (Fig. 3B) with EC_{50} values ranging from 96 to 317 nM (median, 122 nM). Because the GABAR currents in all the dentate granule cells were uniformly enhanced by diazepam, the concentration-response data from these cells were pooled (Fig. 3C). The enhancement of GABAR current by diazepam was a sigmoidal function of diazepam concentration with a Hill slope of 1.2 ± 0.3, maximal enhancement of 210 ± 10%, and an EC_{50} of 158 ± 13 nM (Fig. 3C).

Zolpidem enhancement of dentate granule cell GABAR currents. Varying concentrations of zolpidem (10–1000 nM) were coapplied with 10 μM GABA. Zolpidem enhanced hippocampal dentate granule cell GABAR currents in all nine neurons studied. Complete concentration-response data were obtained in seven cells (Fig. 4A) and could be fitted to a sigmoidal function in each case (Fig. 4B). EC_{50} values for zolpidem enhancement of GABAR currents for individual cells varied from 40 to 126 nM (median, 64 nM). The data from individual neurons were pooled (Fig. 4C). Zolpidem enhanced granule cell GABAR currents with an EC_{50} of 75 ± 13 nM, maximal enhancement of 165 ± 6%, and a Hill slope of 1.1 ± 0.4 (Fig. 4C).

DMCM inhibition of hippocampal dentate granule cell GABAR currents. From 10 to 300 nM DMCM was

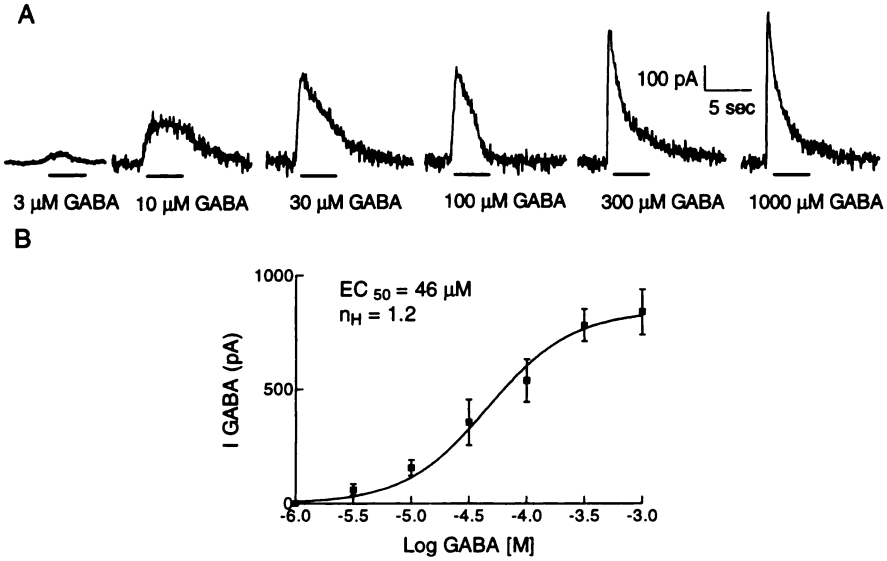

Fig. 2. A, GABA concentration-response relationship for a dentate granule cell isolated from a 30-day-old rat. Traces were from a single neuron and show responses to six concentrations of GABA. The concentration of GABA eliciting the current appears below the trace. *Bar*, duration of GABA application. B, Pooled data from six neurons. Each point represents the mean of five observations. *Error bar*, standard error. *Line*, best fit of the data to a sigmoidal function. The EC_{50} was derived from the equation for the sigmoid function that best fit the data.

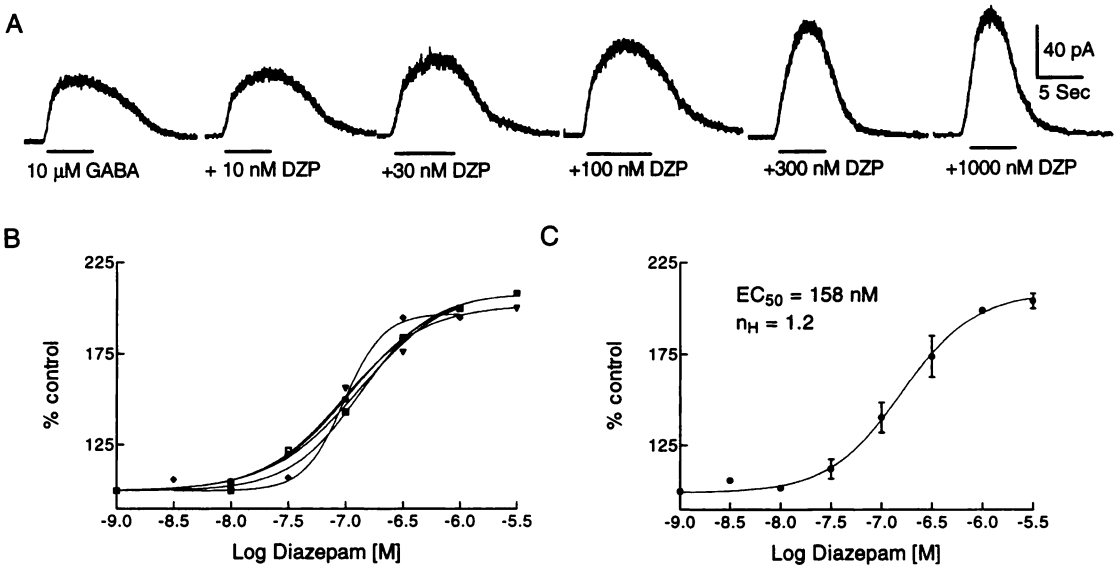

Fig. 3. A, Diazepam (*DZP*)-enhanced dentate granule cell GABAR currents. The traces were from a single neuron. Concentrations of drug applied with 10 μM GABA are shown below the traces. *Horizontal bars*, duration of application of the drug. Recovery between drug application was not shown. B, Diazepam concentration/dentate granule cell GABAR current enhancement relationship for seven neurons were plotted individually. C, *Points*, mean of seven observations in B; *error bars*, standard errors; *lines*, best fit of the data to a sigmoidal function. The EC_{50} was derived from the equation for the sigmoid function that best fit the data.

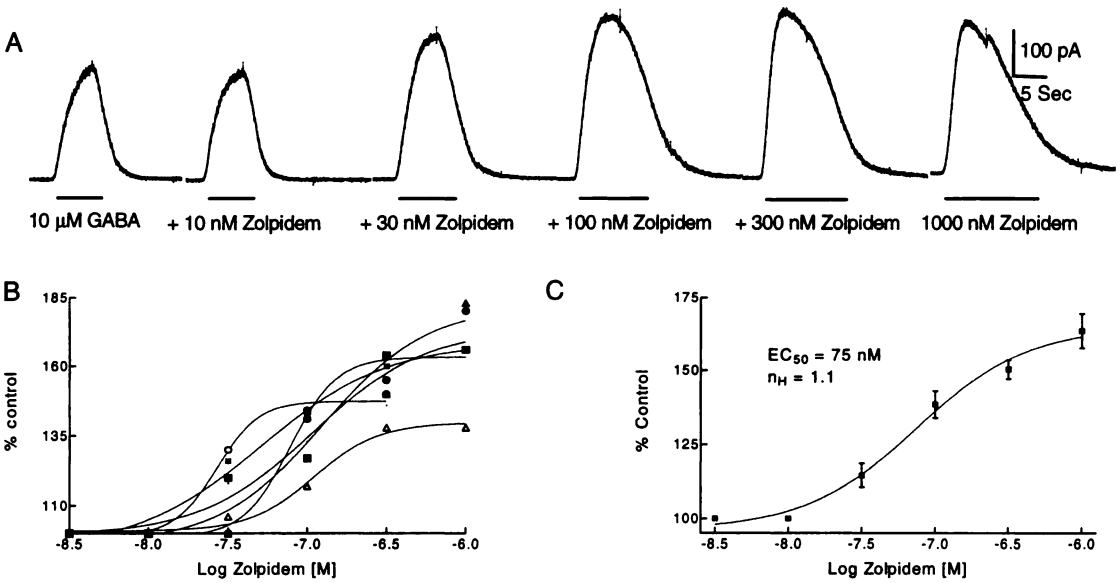

Fig. 4. A, Zolpidem-enhanced dentate granule cell GABAR currents. The traces were from a single neuron. Concentrations of drug applied with 10 μM GABA are shown below the traces. *Horizontal bars*, duration of application of the drug. Recovery between drug application was not shown. B, Zolpidem concentration-response relationship for GABAR current enhancement. Data for seven neurons were plotted individually. C, *Points*, mean of seven observations in B; *error bars*, standard errors; *lines*, best fit of the data to a sigmoidal curve. The EC_{50} was derived from the equation for the sigmoid function that best fit the data.

coapplied with 30 μM GABA to four hippocampal dentate granule cells (Fig. 5A). In all four neurons tested, DMCM inhibited GABAR currents in a concentration-dependent fashion (Fig. 5B). The IC_{50} for DMCM inhibition of GABAR currents was 60 ± 7 nM, and the maximal inhibition of GABAR currents was 61 ± 5%.

Zn^{2+} inhibition of dentate granule cell GABAR currents. The action of Zn^{2+} on 30 μM GABAR currents in hippocampal dentate granule cells was studied. Zn^{2+}, ranging in concentration from 1 to 1000 μM, was coapplied with GABA after stable GABAR currents were obtained. Zn^{2+} has been reported to be a noncompetitive blocker of GABAR currents (12, 13); thus, the magnitude of Zn^{2+} block was expected to be independent of GABA concentration. In eight hippocampal dentate granule cells, GABAR currents were reduced by Zn^{2+} in a concentration-dependent fashion (Fig. 6A). Zn^{2+} inhibition of GABAR currents was similar among all granule cells tested: currents in all cells were inhibited by 100 μM, and none were inhibited by 1 μM Zn^{2+}. The Zn^{2+} IC_{50} values for individual neurons also were distributed over a narrow range of 13–51 μM (median, 29 μM) (Fig. 6B). Because the data obtained from individual granule cells suggested a homogeneous population of cells, the data were pooled and fitted to a single sigmoidal concentration-response curve. The

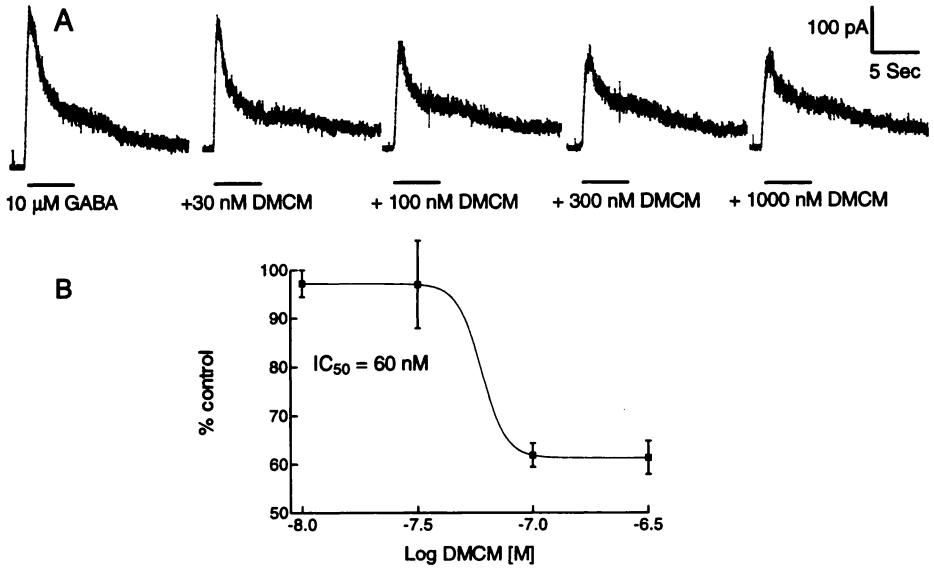

Fig. 5. DMCM inhibited dentate granule cell GABAR currents. The traces were from a single neuron. Drug concentrations applied with 30 μM GABA are shown below the trace. *Horizontal bars*, duration of drug application. B, *Points*, mean of four observations; *error bars*, standard errors; *lines*, best fit of the data to a sigmoidal function. The IC_{50} was derived from the equation for the sigmoid function that best fit the data.

IC_{50} of Zn^{2+} inhibition of GABAR currents was 28.5 ± 11 μM, maximal inhibition of GABAR currents was 77 ± 3%, and the Hill slope was 2.0 ± 0.4 (eight cells) (Fig. 6C). The IC_{50} of Zn^{2+} inhibition of the entire group of dentate granule cell GABAR currents was similar to the median of the individual IC_{50} values, also suggesting a single population of cells.

The incomplete inhibition of hippocampal dentate granule cell GABAR currents by Zn^{2+} may result from the presence of two subpopulations of GABARs, one Zn^{2+} sensitive and the other Zn^{2+} insensitive, or by incomplete Zn^{2+} block of a single GABAR population. Based on the studies of recombinant GABARs, Zn^{2+}-insensitive GABARs were likely to be diazepam sensitive. The possibility was tested that the residual granule cell GABAR current following Zn^{2+} block was contributed by a diazepam-sensitive GABAR subpopulation. In four granule cells, 30 μM GABA-elicited currents were inhibited by 100 μM Zn^{2+}, and the residual current was 34 ± 3.2%. When 10 nM diazepam was applied in addition to 100 μM Zn^{2+}, the residual current was 35 ± 3.9%. The hippocampal dentate granule cell GABAR currents were enhanced by 100 (30%) and 1000 nM diazepam (50%) in the presence of 100 μM Zn^{2+} compared with Zn^{2+} alone applied with 30 μM GABA. Thus, Zn^{2+}-insensitive residual current was no more diazepam sensitive than the Zn^{2+}-sensitive GABAR currents in the granule cells.

Effect of loreclezole on dentate granule cell GABAR currents. Ten granule cells were tested for enhancement of GABAR by 1 or 10 μM loreclezole. In five of these cells, loreclezole did not enhance the GABAR currents (Fig. 7A). In the remainder of cells, GABAR currents were enhanced in a concentration-dependent fashion (Fig. 7B). In both of the cells in which GABAR currents were enhanced and those in which these currents were not enhanced, increasing concentrations of loreclezole enhanced the rate of apparent desen-

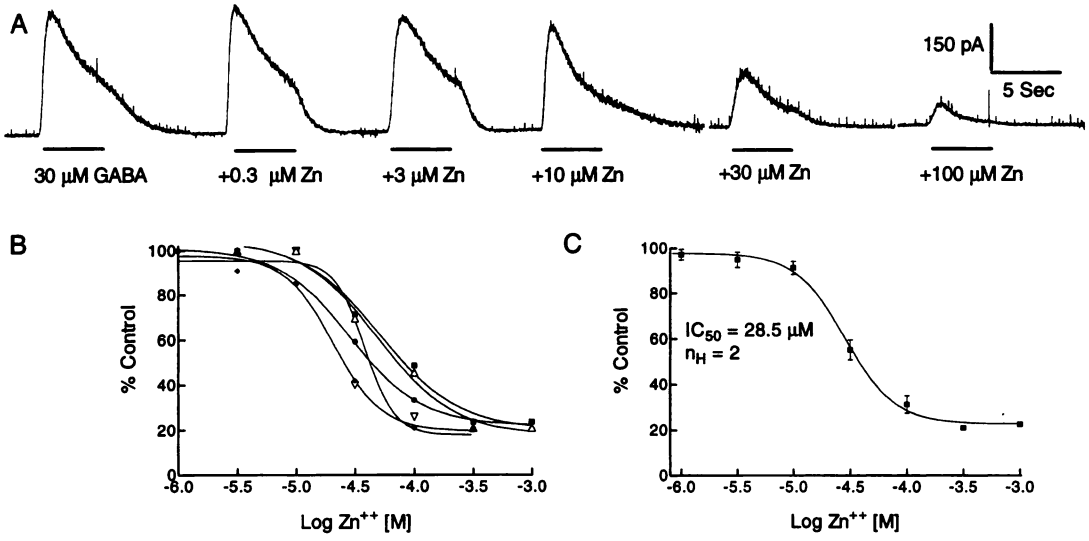

Fig. 6. A, Zn^{2+} inhibited dentate granule cell GABAR currents. The traces were from a single neuron. Drug concentrations applied with 30 μM GABA are shown below the trace. *Horizontal bars*, duration of drug application. Recovery between drug applications was not shown. Note the Zn^{2+} inhibition of GABAR currents was incomplete. B, Zn^{2+} concentration-response relationship for GABAR current inhibition. Data were from eight neurons plotted individually. C, *Points*, mean of the data from eight neurons in B; *error bars*, standard errors; *lines*, best fit of the data to a sigmoidal function. The IC_{50} was derived from the equation for the sigmoid function that fit the data.

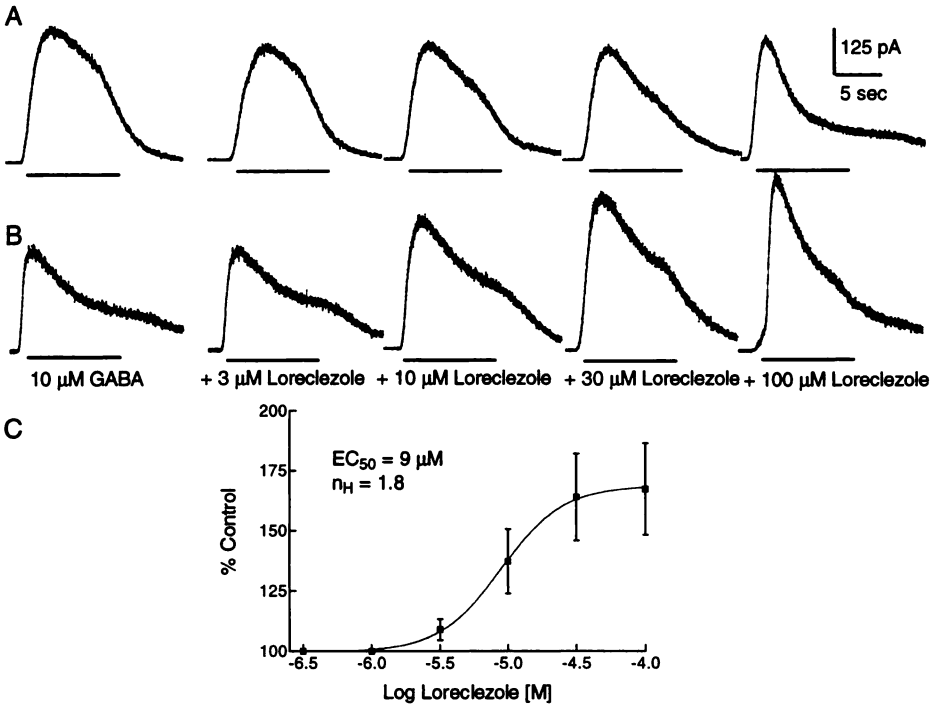

Fig. 7. A, Loreclezole did not enhance dentate granule cell GABAR currents in 50% of the neurons tested. The traces were from a single neuron. B, Loreclezole enhanced dentate granule cell GABAR currents in 50% of the neurons tested. The traces were from a single neuron. In both sets of neurons, loreclezole in high concentration enhanced the rate of apparent desensitization. The concentrations of drug applied with 10 μM GABA are shown below the traces. *Horizontal bars*, duration of drug application. Recovery between drug applications was not shown. C, Loreclezole concentration-response curves obtained from dentate granule cells sensitive to loreclezole. *Points*, mean of three observations; *error bars*, standard errors; *lines*, best fit of the data to a sigmoidal function. The EC_{50} was derived from the equation for the sigmoid function that best fit the data.

sitization of GABAR currents (Fig. 7, A and B). For loreclezole-enhanced neurons, concentration-response curves were obtained. The EC_{50} for loreclezole was 9 ± 1.8 μM, maximal enhancement of GABAR currents was 168 ± 11%, and the Hill slope was 1.9 (four cells).

Discussion

Dentate granule cell GABARs had a distinct set of pharmacological properties, which were likely subunit and subunit subtype composition dependent. These properties included the presence of both benzodiazepine sensitivity and moderate Zn^{2+} sensitivity on individual cells, intermediate diazepam sensitivity, and high/moderate zolpidem sensitivity and existence of granule cells with and without loreclezole sensitivity.

GABAR pharmacology of single neurons. We characterized the pharmacology of GABARs present on a single identified neuron type: granule cells of the dentate gyrus. Complete drug concentration-response curves, with drug concentration ranging from 2 to 3 $\log_{10}$ units performed on single acutely isolated neurons, have not been previously reported. The modification of the U-tube system enabled application of multiple drug concentrations in a relatively short time. Inclusion of an ATP regeneration system in the recording pipette allowed prolonged recordings of GABAR currents from acutely isolated neurons without significant rundown. A potential confounding factor present in this study was that it was unknown whether enzymatic dissociation of neurons altered GABAR pharmacology. However, multiple studies of GABAR currents recorded from acutely enzymatically dissociated neurons have been reported (8, 14, 15), several with the enzyme used in the current study, reporting pharmacological properties of GABARs that were similar to those reported for cultured neurons (6, 16).

This method offered multiple advantages over others for characterizing the properties of native GABARs. Receptor binding assays have been commonly used to characterize native receptor pharmacology, but these studies have been unable to characterize receptors on single neurons. Membrane preparations used for radiolabeled ligand binding assays are derived from entire regions such as hippocampus, spinal cord, or cerebral cortex. These regions contain multiple neuronal types that may express different GABAR isoforms. Quantitative receptor autoradiography with tritium-labeled ligands provides better resolution, but it still averages several neurons in the tissue volume. Other factors interfering with quantitative receptor autoradiography include variable tissue quenching of tritium and the presence of endogenous GABA in the sections (17). Finally, radiolabeled ligand binding studies are performed at a cold temperature (0–4°), which alters the binding affinities of the ligands for GABARs (18).

GABA sensitivity of dentate granule cell GABARs. The GABA EC_{50} of acutely dissociated hippocampal dentate granule cell GABARs was 46.5 μM, which is similar to that observed in acutely dissociated pyramidal neurons (25.4 μM) (14) and adult cortical (40.3 μM) and thalamic neurons (23 μM) (16). The total range of EC_{50} values for individual granule cells was ~0.5 $\log_{10}$ unit of GABA concentration, and the individual EC_{50} values were evenly distributed around the mean. These findings suggested that the limited number of granule cells sampled were uniform with regard to GABA EC_{50}.

Benzodiazepine pharmacology of dentate granule cell GABARs. We studied the pharmacology of a well-established benzodiazepine agonist, diazepam; a BZ 1-preferring agonist, zolpidem; and a benzodiazepine inverse agonist, DMCM, on the hippocampal dentate granule cell GABARs. Benzodiazepines like diazepam act on an allosteric site on the GABAR. Benzodiazepine receptor sites have been designated BZ 1, BZ 2, or BZ 3 based on high, low, or no affinity for certain benzodiazepine agonists such as CL 218,872 or zolpi-

dem (1, 19). DMCM acts at the benzodiazepine site but inhibits GABAR function. All dentate granule cell GABARs were sensitive to the allosteric regulators diazepam, zolpidem, and DMCM, which bind to the benzodiazepine site.

The hippocampal dentate granule cells expressed GABARs with a high EC_{50} (100–200 nM) for diazepam. Native GABAR currents were previously found to be either highly diazepam sensitive, enhanced by 1–10 nM diazepam (20, 21), or insensitive to micromolar concentrations of diazepam, in the case of GABAR currents in cultured cerebellar granule cells (22). The diazepam EC_{50} value of granule cell GABAR currents was intermediate and unlike any previously characterized native receptors. Benzodiazepine agonists like diazepam and flunitrazepam bind with high affinity (low nanomolar value for K_d) to the dentate gyrus region of the hippocampus (17, 23, 24), but the binding affinity often does not correlate with the EC_{50} for GABAR agonists and allosteric modulators (1).

All hippocampal dentate granule cells tested in this study had an EC_{50} for zolpidem intermediate between BZ 1 and BZ 2 receptors. Maximal enhancement of GABAR currents by zolpidem was 165% compared with 210% by diazepam. These findings suggested that hippocampal dentate granule cell GABARs had properties intermediate between BZ 1 and BZ 2 that were not characteristic of either. This finding was in contrast to the conclusion reached in radioligand binding studies addressing this question. Niddam *et al.* (24) reported that relative binding density of [^{3}H]zolpidem compared with that of [^{3}H]flunitrazepam in the dentate gyrus region was only 20–50%. Olsen *et al.* (17) found that the [^{3}H]-2-oxoquazepam (a BZ 1-preferring ligand) binding in the dentate gyrus region was significantly lower than that of [^{3}H]flunitrazepam binding. Both studies concluded that this brain region predominantly contained BZ 2 receptors. These studies used ligand binding at one standard concentration, assuming no regional variability of K_d in the brain. This assumption was challenged by a recent study (25) that reported three different K_d values for [^{3}H]zolpidem within the hippocampus: one high affinity (~20 nM), one intermediate affinity (~200 nM), and one low affinity (~6 μM). Thus, the benzodiazepine pharmacology of hippocampal dentate granule cell GABARs was unusual with regard to diazepam EC_{50} and the result of intermediate EC_{50} for zolpidem enhancement of currents. Inhibition by DMCM was a property shared with other native GABARs.

Expression of various combinations of recombinant subunit subtypes in oocytes or mammalian cell lines suggested an explanation for the benzodiazepine sensitivity of hippocampal dentate granule cell GABARs. Early studies indicated that benzodiazepine sensitivity was conferred by the presence of a γ2 subtype (1). Because all hippocampal dentate granule cell GABAR were diazepam, zolpidem, or DMCM sensitive, a likely explanation would be that each contained a γ2 subtype. There was no obvious explanation, however, for moderate diazepam sensitivity (EC_{50} = >100 nM). Recombinant GABARs containing α4 (26) or a6 subtypes with a β and γ2 subtype are diazepam insensitive. The mRNA for the α4 subtype, which renders GABARs diazepam insensitive (26), was present in dentate granule cells. One possible explanation for moderate diazepam sensitivity of granule cell GABAR was that hippocampal dentate granule cells expressed a GABAR with two different α subtypes, one of which was the α4 in addition to the γ2 subtype. Although several studies have demonstrated expression of multiple α subtypes in native and recombinant GABARs, none have used α4 as one of the subtypes. Immunohistochemical studies using antibodies directed against α4 subtype are needed to confirm this possibility. Alternatively, factors other than subunit composition, such as phosphorylation of GABARs, participate in determining the EC_{50} of benzodiazepines for GABARs, and the phosphorylation state of granule cell GABARs rendered them less sensitive to diazepam.

There also was no explanation for intermediate zolpidem sensitivity of hippocampal dentate granule cell GABARs from the study of recombinant GABARs. Zolpidem had a relatively higher affinity for recombinant GABARs containing the α1 subtype but did not bind to GABARs containing the α5 subtype (27, 28). The data in this study suggested the absence of α5 subtype and could not directly suggest the presence of the α1 or α2 subtype in addition to γ2 subtype in the granule cell GABARs. Because all of the GABAR currents recorded from dentate granule cells were enhanced by zolpidem, it was likely that none of the cells expressed GABAR isoforms containing the α5 subtype as the only α subtype. The immunohistochemical studies report the presence of both the α1 and α2 subtypes on synaptic junctions between basket cell and granule cell somata (29, 30).

GABAR currents from all cells tested were inhibited by DMCM with a low IC_{50}. The finding of high affinity inhibition of dentate granule cell GABAR currents suggested the absence of functional expression of the α6 subtype in these cells because GABAR currents in recombinant GABARs containing the α6 subtype were enhanced by DMCM (31).

Zn^{2+} sensitivity of dentate granule cell GABARs. GABAR currents from all dentate granule cells studied were moderately sensitive to Zn^{2+}. The GABAR currents were inhibited to 23% of control by Zn^{2+} with an IC_{50} of 30 μM. Zn^{2+} inhibition of GABAR currents from cultured hippocampal neurons has been studied extensively, but these hippocampal cell cultures were made from embryonic or newborn rat hippocampi before the development of the dentate gyrus. Although the Zn^{2+} sensitivity of dentate granule cell GABARs has not been reported, the Zn^{2+} pharmacology of recombinant GABARs has been recently characterized (32–34). Recombinant GABAR isoforms expressed in human embryonic kidney cells composed of α1 or β1 subtypes (32) or both α1 and β1 subtypes (33) were highly sensitive to Zn^{2+}, with IC_{50} values of <2 μM. On the addition of the γ2 subunit to these GABAR subtypes, 100 μM Zn^{2+} failed to significantly inhibit GABAR currents. Zn^{2+} sensitivity of recombinant GABARs was also modified by α subtype. Recombinant GABARs containing the α6 subtype were more sensitive to Zn^{2+} than were GABARs containing the α1 subtype (35). The δ subunit also modified Zn^{2+} sensitivity of recombinant GABAR isoforms containing α1, β1, and γ2 subtypes expressed in mouse L929 fibroblast cells. Coexpression of the δ subunit with α1, β1, and γ2 subtypes partially restored Zn^{2+} sensitivity (34). The α1, β1, γ2, and δ1 subtype-containing GABAR isoform was sensitive to 100 μM Zn^{2+} but not to 10 μM Zn^{2+}.

The finding of moderate sensitivity of dentate granule cell GABAR currents to Zn^{2+} in combination with diazepam sensitivity in this study suggests that either both γ2 and δ1 subtypes are present or the expressed α subtype(s) modified the Zn^{2+} sensitivity. The incomplete inhibition of hippocam-

pal dentate granule cell GABAR currents by Zn^{2+} may result from the presence of two subpopulations of GABARs with regard to Zn^{2+} sensitivity or from Zn^{2+} block of a single GABAR population that was incomplete. Based on the studies of recombinant GABARs, Zn^{2+}-insensitive GABARs were likely to be diazepam sensitive. Experimentally, the Zn^{2+}-insensitive residual GABAR currents in granule cells were no more sensitive to diazepam than were Zn^{2+}-sensitive GABAR currents. These data suggested a single population of moderately Zn^{2+}-sensitive GABARs with incomplete Zn^{2+} block rather than two subpopulations of GABARs. Although the Zn^{2+} sensitivity of dentate granule cell GABARs has not been described previously, cerebellar granule cells in culture also express relatively Zn^{2+}-sensitive GABARs (IC_{50} = 57.2 μM), and cerebellar granule cells also contain mRNA for δ1 and γ2 subtypes (36).

Homogeneity of the properties of hippocampal dentate granule cell GABARs with regard to GABA, Zn^{2+}, diazepam, zolpidem, and DMCM. Dentate granule cell GABARs seemed to belong to a single population of GABARs with regard to GABA sensitivity, Zn^{2+} and DMCM inhibition, and diazepam and zolpidem enhancement. This assessment was based on the findings that there was a narrow range of EC_{50} or IC_{50} values for these ligands on individual cells and that Hill analysis of pooled population data showed characteristics (EC_{50} or IC_{50} and Hill slope) similar to those obtained from analysis of individual neurons.

However, because of the limited number of neurons tested, this description of the hippocampal dentate granule cell population is qualitative. These features do not rule out the existence of subpopulations of receptors because the cell sample size for each drug tested was small, limited to 5–10 neurons, which could result in subpopulations of 10–20% of the hippocampal dentate granule cell GABAR isoforms being missed.

Two subpopulations of hippocampal dentate granule cell GABARs. We found that dentate granule cell GABAR currents were enhanced in only 50% of cells by the novel antiepileptic drug loreclezole. Loreclezole has been shown to be an antiepileptic drug with a wide spectrum of action (37) and has been shown to enhance GABAR currents (38). In experimental models of epilepsy, the pharmacological profile of loreclezole was similar to that of barbiturates and different from that of benzodiazepines (39). Loreclezole enhanced recombinant GABAR currents via a specific modulatory site on GABAR β subunits. The action of loreclezole depended on the β subtype expressed; isoforms containing β2 or β3 subtypes had a 300-fold lower EC_{50} for loreclezole enhancement of GABAR current than isoforms containing the β1 subtype (40). The recombinant GABAR studies suggested that two subpopulations of hippocampal dentate granule cell GABARs may exist: those containing the β1 subtype and those containing the β2 or β3 subtype. Because mRNA for β1, β2, and β3 subtypes has been demonstrated in dentate granule cells, it was likely that the β1 subtype was expressed in dentate granule cell GABARs that were not enhanced by loreclezole and that the β2 and/or β3 subtype was expressed in the loreclezole-sensitive cells.

Synaptic terminals of multiple distinct types of interneurons act through GABARs on the granule cells (41, 42). The synaptic inputs from these GABAergic interneurons are spatially segregated (42). These findings have raised the possibility that diverse and spatially segregated GABAergic input into granule cells coincides with selective expression of different subtypes of the GABAR on the surface of granule cells (29). Results of the current study with loreclezole suggested that at least two different GABAR isoforms can be presented by the postsynaptic element to match the diversity of GABAergic inputs.

Distinct properties of hippocampal dentate granule cell GABARs. Fast inhibitory neurotransmission in the forebrain is achieved by a single neurotransmitter, GABA, which gates one class of chloride channels, the GABARs. GABAR-mediated fast inhibitory transmission is used in diverse neuronal circuits in the brain to achieve widely differing consequences. For example, GABAergic inhibition is involved in generation of rhythmic oscillations (θ rhythm) the hippocampus, lateral inhibition in the retina, and switching between relay mode and bursting mode of the principal neurons in the thalamus. Much of the diversity of GABAergic inhibition has been attributed to the neuronal connections and the properties of the neuronal elements of the circuit (43). The properties of the postsynaptic element, the GABARs, were not believed to vary among the hippocampus, the retina, and the thalamus. Before cloning of the GABAR subunits, limited varieties of native GABARs were recognized based on radioligand binding studies. The molecular cloning of five families of GABAR subunits (α, β, γ, δ, and ρ) and multiple subunit subtypes (α1-6, β1-4, γ1-4, δ1, and ρ1-2) has lead to the formulation of a hypothesis of GABAR heterogeneity, which states that multiple GABAR isoforms are assembled in the CNS (44) and that individual GABAR isoform properties are brain region or even neuron specific (45).

The presence of both benzodiazepine sensitivity and moderate Zn^{2+} sensitivity on single cells, intermediate pharmacology, and the existence of two GABAR subpopulations (those enhanced by loreclezole and those insensitive to loreclezole) defines the distinct properties of the GABARs present on soma and proximal dendrites of hippocampal dentate granule cells. These findings are consistent with the GABAR heterogeneity hypothesis and suggest that a specific neuronal population, hippocampal dentate granule cells, expresses GABARs with distinct properties that may distinguish them from GABAR isoforms expressed in other neurons. These distinct properties may also play a role in determining the properties of fast synaptic inhibition in the context of hippocampal neural network.

Acknowledgments

We thank Nadia Esmaiel for her assistance with the experiments and Dr. Elizabeth I Tietz for comments on the manuscript.

References

1. Macdonald, R. L., and R. W. Olsen. $GABA_A$ receptor channels. *Annu. Rev. Neurosci.* **17**:569–602 (1994).
2. McKernan, R. M., K. Quirk, R. Prince, P. A. Cox, N. P. Gillard, C. I. Ragan, and P. Whiting. $GABA_A$ receptor subtypes immunopurified from rat brain with α subunit-specific antibodies have unique pharmacological properties. *Neuron* **7**:667–676 (1991).
3. Mertens, S., D. Benke, and H. Mohler. $GABA_A$ receptor populations with novel subunit combinations and drug binding profiles identified in brain by α 5- and δ-subunit-specific immunopurification. *J. Biol. Chem.* **268**:5965–5973 (1993).
4. Laurie, D. J., W. Wisden, and P. H. Seeburg. The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J. Neurosci.* **12**:4151–4172 (1992).

5. Kay, A. R., and R. K. Wong. Isolation of neurons suitable for patch-clamping from adult mammalian central nervous systems. *J. Neurosci. Methods* **16:**227–238 (1986).
6. Coulter, D. A., J. R. Huguenard, and D. A. Prince. Differential effects of petit mal anticonvulsants and convulsants on thalamic neurones: GABA current blockade. *Br. J. Pharmacol.* **100:**807–813 (1990).
7. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100 (1981).
8. Stelzer, A., A. R. Kay, and R. K. Wong. $GABA_A$-receptor function in hippocampal cells is maintained by phosphorylation factors. *Science (Washington D. C.)* **241:**339–341 (1988).
9. Bormann, J. U-tube drug application, in *Practical Electrophysiological Methods* (H. Kettenmann and R. Grantyn, eds.). Wiley-Liss, New York, 136–140 (1992).
10. Greenfield, L. J., M. D. Uhler, and R.L. Macdonald Cyclic GMP-dependent protein kinase II regulates recombinant $GABA_A$ receptor currents in L929 cells. *Soc. Neurosci. Abstr.* **20:**503(1994).
11. Akaike, N., K. Hattori, N. Inomata, and Y. Oomura. *gamma*-Aminobutyric-acid- and pentobarbitone-gated chloride currents in internally perfused frog sensory neurones. *J. Physiol. (Lond.)* **360:**367–386 (1985).
12. Legendre, P., and G. L. Westbrook. Noncompetitive inhibition of γ-aminobutyric $acid_A$ channels by Zn^{2+}. *Mol. Pharmacol.* **39:**267–274 (1991).
13. Celentano, J. J., M. Gyenes, T. T. Gibbs, and D. H. Farb. Negative modulation of the γ-aminobutyric acid response by extracellular zinc. *Mol. Pharmacol.* **40:**766–773 (1991).
14. Celentano, J. J., and R. K. S. Wong. Multiphasic desensitization of the GABA(A) receptor in outside-out patches. *Biophys. J.* **66:**1039–1050 (1994).
15. Yakushiji, T., Y. Oyama, and N. Akaike. Comparative study on barbiturates using isolated single neurons: GABA-mimetic action and augmentatory action on GABA response. *Brain Res.* **488:**357–360 (1989).
16. Oh, K.-S., C.-J. Lee, J. W. Gibbs, and D. A. Coulter. Postnatal development of $GABA_A$ receptor function in somatosensory thalamus and cortex: whole-cell voltage clamp recordings in acutely isolated rat neurons. *J. Neurosci.* **15:**1341–1351 (1995).
17. 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).
18. Yang, J. S., and R. W. Olsen. γ-Aminobutyric acid receptor binding in fresh mouse brain membranes at 22°: ligand-induced changes in affinity. *Mol. Pharmacol.* **32:**266–277 (1987).
19. Sieghart, W., and G. Drexler. Irreversible binding of [^{3}H]flunitrazepam to different proteins in various brain regions. *J. Neurochem.* **41:**47–55 (1983).
20. De Deyn, P.P., and R. L. Macdonald. Effects of non-sedative anxiolytic drugs on responses to GABA and on diazepam-induced enhancement of these responses on mouse neurones in cell culture. *Br. J. Pharmacol.* **95:**109–120 (1988).
21. Skerritt, J. H., and R. L. Macdonald. Benzodiazepine receptor ligand actions on GABA responses: β-carbolines, purines. *Eur. J. Pharmacol.* **101:**135–141 (1984).
22. Mathews, G. C., A. M. Bolos-Sy, K. D. Holland, K. E. Isenberg, D. F. Covey, J. A. Ferrendelli, and S. M. Rothman. Developmental alteration in $GABA_A$ receptor structure and physiological properties in cultured cerebellar granule neurons. *Neuron* **13:**149–158 (1994).
23. Young, W. S., 3d, and M. J. Kuhar. Autoradiographic localisation of benzodiazepine receptors in the brains of humans and animals. *Nature (Lond.)* **280:**391–394 (1979).
24. Niddam, R., A. Dubois, B. Scatton, S. Arbilla, and S. Z. Langer. Autoradiographic localization of [^{3}H]zolpidem binding sites in the rat CNS: comparison with the distribution of [^{3}H]flunitrazepam binding sites. *J. Neurochem.* **49:**890–899 (1987).
25. Ruano, D., M. Vizuete, J. Cano, A. Machado, and J. Vitorica. Heterogeneity in the allosteric interaction between the *gamma*-aminobutyric acid (GABA) binding site and three different benzodiazepine binding sites of the $GABA_A$/benzodiazepine receptor complex in the rat nervous system. *J. Neurochem.* **58:**485–493 (1992).
26. Wisden, W., A. Herb, H. Wieland, K. Keinanen, H. Luddens, and P. H. Seeburg. Cloning, pharmacological characteristics and expression pattern of the rat $GABA_A$ receptor *alpha* 4 subunit. *FEBS Lett.* **289:**227–230 (1991).
27. Pritchett, D. B., H. Sontheimer, B. D. Shivers, S. Ymer, H. Kettenmann, P. R. Schofield, and P. H. Seeburg. Importance of a novel $GABA_A$ subunit for benzodiazepine pharmacology. *Nature (Lond.)* **338:**582–585 (1989).
28. Puia, G., S. Vicini, P. H. Seeburg, and E. Costa. Influence of recombinant γ-aminobutyric $acid_A$ receptor subunit composition on the action of allosteric modulators of γ-aminobutyric acid-gated Cl^- currents. *Mol. Pharmacol.* **39:**691–696 (1991).
29. Nusser, Z., J. D. Roberts, A. Baude, J. G. Richards, W. Sieghart, and P. Somogyi. Immunocytochemical localization of the α1 and β2/3 subunits of the $GABA_A$ receptor in relation to specific GABAergic synapses in the dentate gyrus. *Eur. J. Neurosci.* **7:**630–646 (1995).
30. Fritschy, J. M., and H. Mohler. $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J. Comp. Neurol.* **359:**154–194 (1995).
31. von Blankenfeld, G., S. Ymer, D. B. Pritchett, H. Sontheimer, M. Ewert, P. H. Seeburg, and H. Kettenmann. Differential benzodiazepine pharmacology of mammalian recombinant $GABA_A$ receptors. *Neurosci. Lett.* **115:**269–273 (1990).
32. Smart, T. G., S. J. Moss, X. Xie, and R.L. Huganir. $GABA_A$ receptors are differentially sensitive to zinc: dependence on subunit composition. *Br. J. Pharmacol.* **103:**1837–1839 (1991).
33. 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).
34. Saxena, N. C., and R. L. Macdonald. Assembly of $GABA_A$ receptor subunits: role of the δ subunit. *J. Neurosci.* **14:**7077–7086 (1994).
35. Saxena, N. C., and R. L. Macdonald. Properties of putative cerebellar γ-aminobutyric $acid_A$ receptor isoforms. *Mol. Pharmacol.* **49:**567–579 (1996).
36. Kilic, G., O. Moran, and E. Cherubini. Currents activated by GABA and their modulation by Zn^{2+} in cerebellar granule cells in culture. *Eur. J. Neurosci.* **5:**65–72 (1993).
37. de Beukelaar, F., and L. Tritsmans. Loreclezole. *Epilepsy Res. Suppl.* **3:**125–128 (1991).
38. Ashton, D., J. Fransen, J. Heeres, G. H. Clincke, and P. A. Janssen. *In vivo* studies on the mechanism of action of the broad spectrum anticonvulsant loreclezole. *Epilepsy Res.* **11:**27–36 (1992).
39. Dawson, G. R., R. Curnow, P. Bayley, A. Rambridge, and M. D. Tricklebank. Lack of effect of flumazenil and CGS 8216 on the anxiolytic-like properties of loreclezole. *Eur. J. Pharmacol.* **252:**325–328 (1994).
40. Wafford, K. A., C. J. Bain, K. Quirk, R. M. McKernan, P. B. Wingrove, P. J. Whiting, and J. A. Kemp. A novel allosteric modulatory site on the GABA(A) receptor β subunit. *Neuron* **12:**775–782 (1994).
41. Han, Z. S., E. H. Buhl, Z. Lorinczi, and P. Somogyi. A high degree of spatial selectivity in the axonal and dendritic domains of physiologically identified local-circuit neurons in the dentate gyrus of the rat hippocampus. *Eur. J. Neurosci.* **5:**395–410 (1993).
42. Buhl, E. H., K. Halasy, and P. Somogyi. Diverse sources of hippocampal unitary inhibitory postsynaptic potentials and the number of synaptic release sites [see comments]. *Nature (Lond.)* **368:**823–828 (1994).
43. Shepherd, G. M., and C. Koch. Introduction to synaptic circuits, in *The Synaptic Organization of the Brain* (G.M. Shepherd, ed.). Oxford University Press, New York, 3–31 (1990).
44. Burt, D. R., and G. L. Kamatchi. $GABA_A$ receptor subtypes: from pharmacology to molecular biology. *FASEB J.* **5:**2916–2923 (1991).
45. Olsen, R. W., and A. J. Tobin. Molecular biology of $GABA_A$ receptors. *FASEB J.* **4:**1469–1480 (1990).

Send reprint requests to: Jaideep Kapur, M.D., Ph.D., Department of Neurology, Neuroscience Laboratory Building, 1103 East Huron St., Ann Arbor, Michigan 48104-1687. E-mail: jkapur@umich.edu