Human Neuronal γ-Aminobutyric Acid$_A$ Receptors: Coordinated Subunit mRNA Expression and Functional Correlates in Individual Dentate Granule Cells

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γ-Aminobutyric acid$_A$ receptors (GABARs) are heteromeric proteins composed of multiple subunits. Numerous subunit subtypes are expressed in individual neurons, which assemble in specific preferred GABAR configurations. Little is known, however, about the coordination of subunit expression within individual neurons or the impact this may have on GABAR function. To investigate this, it is necessary to profile quantitatively the expression of multiple subunit mRNAs within individual cells. In this study, single-cell antisense RNA amplification was used to examine the expression of 14 different GABAR subunit mRNAs simultaneously in individual human dentate granule cells (DGCs) harvested during hippocampectomy for intractable epilepsy. α4, β2, and δ-mRNA levels were tightly correlated within individual DGCs, indicating that these subunits are expressed coordinately. Levels of α3- and β2-mRNAs, as well as ε- and β1-mRNAs, also were strongly correlated. No other subunit correlations were identified. Coordinated expression could not be explained by the chromosomal clustering of GABAR genes and was observed in control and epileptic rats as well as in humans, suggesting that it was not species-specific or secondary to epileptogenesis. Benzodiazepine augmentation of GABA-evoked currents also was examined to determine whether levels of subunit mRNA expression correlated with receptor pharmacology. This analysis delineated two distinct cell populations that differed in clonazepam modulation and patterns of α-subunit expression. Clonazepam augmentation correlated positively with the relative expression of α1- and γ2-mRNAs and negatively with α4- and δ-mRNAs. These data demonstrate that specific GABAR subunit mRNAs exhibit coordinated control of expression in individual DGCs, which has significant impact on inhibitory function.

Key words: GABA$_A$ receptors; dentate granule cells; hippocampus; human; gene expression; mRNA; patch clamp; zinc; benzodiazepine

Fast synaptic inhibition in the brain is mediated primarily by the neutral amino acid γ-aminobutyric acid (GABA) interacting with postsynaptic GABA$_A$ receptors (GABARs). GABARs are heteromeric protein complexes composed of multiple subunits that form ligand-gated anion-selective channels (Vicini, 1991; Macdonald and Olsen, 1994). Different GABAR subunit families have been identified, and multiple subtypes exist within each family (α1–α6; β1–β4; γ1–γ3; ρ1–ρ3; δ; ε; π) (Macdonald and Olsen, 1994; Barnard et al., 1998). Studies of recombinant receptors show that varying the receptor subunit composition produces distinct functional and pharmacological properties. The α- and γ-subtypes confer differences in benzodiazepine pharmacology and inhibition by zinc (Draguhn et al., 1990; Pritchett and Seeburg, 1990; von Blankenfeld et al., 1990; Luddens and Wisden, 1991; White and Gurley, 1995; Fisher and Macdonald, 1998). The subtype of β-subunit affects channel properties (Verdoorn et al., 1990), benzodiazepine efficacy (Sigel et al., 1990; von Blankenfeld et al., 1990), affinity for GABA analogs, and the efficacy of allosteric modulation by the barbiturates, loreclezole, and steroids (Bureau and Olsen, 1990, 1993; Donnelly and Macdonald, 1996). Subunit expression varies in different brain regions and cell types and during different times in ontogeny (Laurie et al., 1992). Regional and developmental heterogeneity of native GABAR function also is well recognized, but the contribution of receptor subunit composition to this functional heterogeneity has not been defined precisely.

Our understanding of the functional consequences of different receptor subtypes is based mainly on the heterologous expression of recombinant receptors (Macdonald and Olsen, 1994), which may not predict fully the properties of the native receptor in neurons, in which multiple subunits are expressed simultaneously. Further, little is known regarding how the expression of different GABAR subunits is coordinated within individual neurons, because quantitative analysis of multiple subunit mRNAs within individual cells cannot be performed readily by using techniques such as *in situ* hybridization and RT-PCR. Even less information is specifically available about GABAR subunit expression and structure–function relationships in human neurons. Interspecies differences in GABAR composition and function, however, may not allow for direct extrapolation from rodent studies to humans.

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For example, the recently cloned GABAR ε-subunit is expressed in the human dentate gyrus, but a functional homolog has not yet been identified in rats (Davies et al., 1997; Whiting et al., 1997). In fact, because of major sequence variation, the rat and human homologs probably have distinctive structures that affect their function (E. F. Kirkness, personal communication). Thus, because aspects of GABAR structure and function may be quite distinct between species, dedicated studies of human neurons are required to delineate fully the structure–function relationships of native human GABARs.

In the current study we combine single-cell antisense RNA (aRNA) amplification and whole-cell patch-clamp techniques to profile quantitatively the expression of 14 different GABAR subunit mRNAs within individual acutely isolated human dentate granule neurons, and we correlate this expression with receptor function and pharmacology in the same cells. This approach permits an analysis of structure–function relationships of the native GABAR and provides insight into how expression of different subunit mRNAs may be coordinated within individual human neurons.

MATERIALS AND METHODS

Acute isolation of neurons. Neurons were acutely isolated according to previously published protocols (Brooks-Kayal et al., 1998b; Shumate et al., 1998). Hippocampal tissue was collected from six patients with medically intractable epilepsy (four male, two female; ages 16–54 years), using the Spencer procedure (Spencer et al., 1984). Tissue was placed immediately into a chamber containing cold (4° C), oxygenated (95%O_2/5%CO_2) artificial CSF (aCSF) solution composed of (in mM) 201 sucrose, 3 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose and was transported rapidly to the laboratory (5–10 min transit time). Hippocampal slices (450 μm) were cut on a vibratome and incubated for 1 hr in an oxygenated medium containing (in mM) 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 25 glucose, and 20 piperazine-*N,N′*-bis[2-ethanesulfonic acid] (PIPES), pH-adjusted to 7.0 with NaOH at 32°C. Slices were digested enzymatically for 30–60 min in 3 mg/ml Sigma protease XXIII (St. Louis, MO) in PIPES, thoroughly rinsed, and incubated another 30 min in PIPES medium before dissociation. The dentate gyrus was visualized with dark-field microscopy, 1 mm^2 chunks were cut, and then the cells were dissociated mechanically and plated onto 35 mm culture dishes in HEPES medium composed of (in mM) 155 NaCl, 3 KCl, 1 $MgCl_2$, 3 $CaCl_2$, 0.0005 tetrodotoxin, and 10 HEPES-Na^+, pH-adjusted to 7.4 with NaOH. A total of 36 cells was isolated successfully and examined (1–13 cells from each patient specimen).

Voltage-clamp recordings in isolated neurons. Using the whole-cell variant of the patch-clamp technique, we voltage-clamped neurons at −20 mV with electrodes containing a pipette solution composed of (in mM): 100 Trizma phosphate (dibasic), 28 Trizma base, 11 EGTA, 2 $MgCl_2$, 0.5 $CaCl_2$, and 4 Mg^{2+}-ATP plus 1 U/μl RNasin, pH 7.35. Given the intracellular and extracellular chloride concentrations, this provided a 50 mV driving force for chloride currents as assessed by the Goldman–Hodgkin–Katz equation. All voltages were corrected *post hoc* for a 4 mV junction potential. Recordings were amplified with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and filtered at 5 kHz before storage on a PCM device at 44 kHz (Neurodata Instruments, New York, NY). Electrode glass was autoclaved, and all solutions were prepared from nuclease-free chemicals and autoclaved ultrapure water. In addition, all personnel wore gloves throughout all experiments to minimize potential nuclease contamination. All drugs were applied with a 14-barrel "sewer pipe" perfusion system, with a 100–200 msec solution change time. GABA and clonazepam (CNZ) were obtained from Sigma. and zolpidem (ZOL) was obtained from Research Biochemicals (Natick, MA). CNZ and ZOL were dissolved as stock solutions in DMSO. DMSO at comparable concentrations to final dilutions (0.01%) had no effect on cell properties or GABA responses. Curves were fit by using the Marquardt–Levenberg nonlinear least-squares algorithm (ORIGIN, Microcal Software, Northampton, MA). Recording duration was limited to 10–15 min because this seemed to facilitate the success of subsequent aRNA amplification.

mRNA measurement. The relative expression of GABAR mRNAs within individual acutely isolated dentate granule cells (DGCs) was measured by using the technique of single-cell aRNA amplification (VanGelder et al., 1990; Eberwine et al., 1992), modified as recently described in detail (Brooks-Kayal et al., 1998a,b). After patch-clamp recording, the neuronal contents were aspirated into the micropipette. The contents of each microelectrode were expelled into a microcentrifuge tube, and first-strand cDNA synthesis was performed by using 1 mM deoxynucleotide triphosphates (dNTPs), 0.5 U/μl avian myeloblastosis virus reverse transcriptase (AMVRT; Seikagaku America, Rockville, MD), and 2 ng/μl oligonucleotide-T7 primer (5′-AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC T_{24}-3′) at 42°C for 60–90 min. After phenol-chloroform extraction and ethanol precipitation with 1 μg of *Escherichia coli* tRNA as a carrier, double-stranded DNA was made by incubation with dNTPs (1 mM), T4 DNA polymerase (1 U), and the Klenow fragment of DNA polymerase I (1 U; 14°C for 14–18 hr). The single-stranded hairpin loop was removed with S1 nuclease (1 U); the ends of the double-stranded template were blunted with T4 DNA polymerase (0.5 U) and the Klenow fragment of DNA polymerase I (0.5 U) at 37°C for 2 hr and then drop-dialyzed for 4 hr against RNase-free water to remove unincorporated dNTPs. Twenty-five percent of the cDNA recovered from the filter was used for the synthesis of amplified RNA (aRNA) (in mM): 40 Tris, pH 7.4, 10 NaCl, 10 $MgCl_2$, and 5 dithiothreitol, with the addition of 250 μM ATP, GTP, and UTP, 50 μM CTP, 15 pmol of α-[^{32}P]-CTP (3000 Ci/mmol, Amersham, Arlington, IL), 20 U of RNasin, and 2000 U of T7 RNA polymerase (Epicentre Technologies, Madison, WI) at 37°C for 4 hr. Then aRNA was synthesized again into a single-stranded cDNA template for a second round of amplification. The final aRNA synthesis included 25 pmol of α-[^{32}P]-CTP in an *in vitro* transcription reaction with the same composition as the first aRNA amplification reaction, except for 1 μM CTP. The aRNA amplification technique results in the linear amplification of all cellular mRNAs, permitting a quantitative analysis of the relative amounts of each RNA analyzed (VanGelder et al., 1990; Eberwine et al., 1992).

Slot blot preparation and expression profiles. Fourteen GABAR subunit cDNAs (α1–α6, β1–β3, γ1–γ3, δ, ε), cyclophyllin (internal reference), glial fibrillary acidic protein (GFAP; control for glial contamination), neurofilament-L (NF-L; marker for neuronal phenotype), and pBluescript plasmid (background) cDNAs were included on each slot blot. GABAR cDNAs were obtained from the late Dr. Dolan Pritchett, except ε cDNA, which was provided by Dr. Ewen Kirkness (The Institute for Genomic Research, Rockville, MD). The identity of all cDNAs was confirmed by sequencing. All GABAR cDNAs included the full human coding region, except α4, α6, β3, γ3, and δ, for which rat clones were used because human clones were unavailable. α6, β3, and γ3 included the full coding region, and α4 and δ were each >1 kb fragments, including the distal 3′ coding region (α4 bp 694–1725; δ bp 524–1580). Each blot was prehybridized for 12 hr at 42°C in 5 ml of prehybridization solution (50% formamide, 5× saline sodium citrate solution, pH 7.0, 5× Denhardt's solution, 0.1% SDS, 1 mM sodium pyrophosphate, and 100 μg/ml salmon sperm DNA) and then hybridized with the radiolabeled aRNA probe from an individual cell for 48 hr (42°C). The blots were washed to a final concentration of 0.2× SSC at 42° C for 30 min and then directly exposed for 2 hr to a Molecular Dynamics PhosphorImage screen (Sunnyvale, CA) with a linear dynamic range over five orders of magnitude. All hybridization signals fell well within this dynamic range.

Quantitation and statistical analysis. Intensity of the autoradiographic signal was measured by three-dimensional laser scanning densitometry, using Image-Quant software from Molecular Dynamics. The hybridization signal for a given cDNA on the blot was quantified as the integrated intensity of all pixels on the PhosphorImage screen within the area containing that cDNA (i.e., the "slot"). The presence of a subunit mRNA was defined as hybridization signal above background by ≥1% of the total hybridization signal for all GABAR subunits on the blot. This value was selected because it represents 1 SD of the estimated variability in background noise (based on differences in hybridization signal for pBluescript plasmid cDNA and GFAP cDNA). Correlation analysis with Bonferroni adjustment for multiple comparisons was performed comparing the hybridization signal of the different subunit cDNAs on each blot (minus background hybridization to plasmid cDNA). In addition, the relative abundance of each subunit mRNA (calculated as the hybridization signal for each subunit cDNA divided by the total hybridization signal of all GABAR subunit cDNAs on the blot) was correlated to the augmentation of GABA responses by clonazepam and zolpidem in the subset of cells for which these data were available. All correlation analyses were performed by using the statistical software program

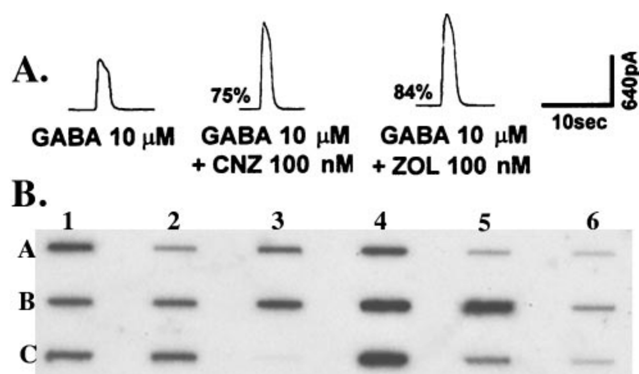

Figure 1. Whole-cell patch-clamp recordings coupled with aRNA expression profiling in a single human dentate granule neuron. *A*, Responses to concentration-clamp application of GABA (10 μM) and modulation of the 10 μM GABA response by coapplied clonazepam (*CNZ*; 100 nM) and zolpidem (*ZOL*; 100 nM). *B*, Slot blot demonstrating hybridization intensities of GABAR subunit mRNAs for the cell for which the physiological responses are illustrated in *A*. The radiolabeled amplified aRNA probe from the individual DGC recorded above was hybridized against a slot blot containing GABAR subunit cDNAs: α1–α6 (*A1–A6*), β1–β3 (*B1–B3*), γ1–γ3 (*B4–B6*), δ and ϵ (*C1, C2*), GFAP (*C3*), NF-L (*C4*), cyclophyllin (*C5*), and pBluescript (*C6*). The value for the slot containing pBluescript cDNA is considered background; NF-L expression serves as a marker for neuronal phenotype, GFAP expression as a control for glial contamination, and cyclophyllin expression as an internal reference value.

STATA. Student's *t* test or Mann–Whitney Rank sum test (for groups with unequal variances) were used for the statistical comparison of differences in the mean subunit expression and clonazepam augmentation among cell groups.

RESULTS

$GABA_A$ receptor subunit expression in individual human dentate granule cells

Whole-cell patch-clamp recording and single-cell aRNA amplification were used to examine postsynaptic GABAR function and subunit mRNA expression in individual acutely isolated DGCs from six different patients. Representative analysis of a single human DGC is shown in Figure 1. The expression of 13 different GABAR subunit mRNAs (α1–α6; β1–β3; γ1–γ3; ϵ) was examined in each cell (n = 36). The expression of δ-subunit mRNA was examined in only a subset of these cells (n = 28) because δ cDNA was not available during early portions of the study. In agreement with earlier results in other species (Brooks-Kayal et al., 1998a,b), the expression of multiple different subunit mRNAs was seen in each cell (Fig. 2). The majority of neurons expressed 10 or more different subunit mRNAs, with the most abundantly expressed subunits being α1, α4, β1, β2, β3, γ1, γ2, and δ. α2, α3, and ϵ mRNAs were expressed moderately in the majority of cells, whereas α5 mRNA was expressed at low levels (1–2% of total GABAR expression) in approximately one-half of cells. Neither α6 nor γ3 mRNA was detected in any of the cells that were examined. The pattern of subunit mRNA expression in these DGCs from epileptic humans was, in large part, similar to that previously seen in DGCs from rats with temporal lobe epilepsy after pilocarpine-induced status epilepticus (Brooks-Kayal et al., 1998b), with a few notable exceptions. Relative expression (as a fraction of total GABAR subunit expression) of α2 mRNA appeared lower, and the expression of β2 and γ1 mRNAs appeared higher in human than in either control or epileptic rat DGCs. The significance of such differences is difficult to interpret, however, because of multiple confounding factors including potential differences in hybridization efficiency caused by sequence variation between the subunit homologs in rat and human as well as the potential effects on the human cells of the antiepileptic drugs that all patients had received.

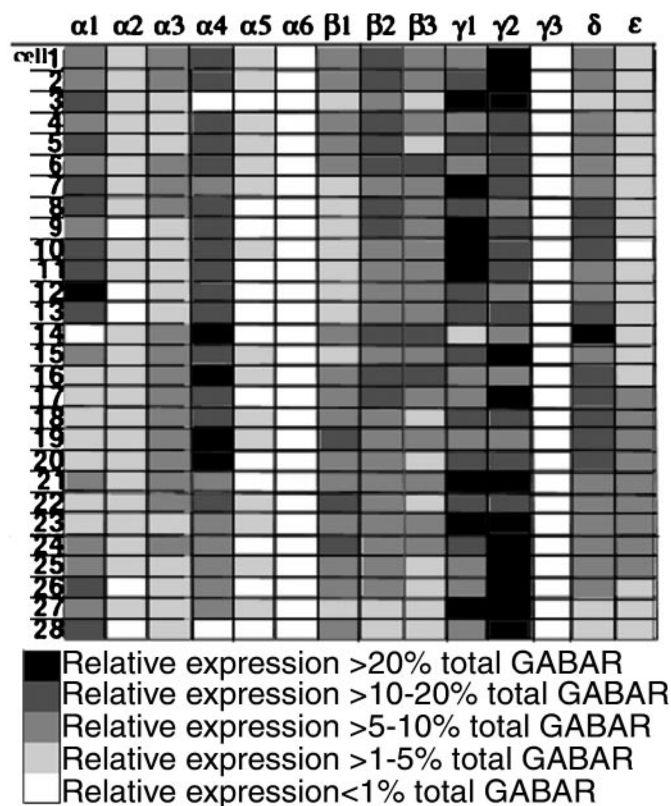

Figure 2. Relative expression of GABAR subunit mRNAs in individual human DGCs. The relative expression of 14 different GABAR subunit mRNAs was performed in 28 individual human DGCs harvested from six patients who underwent temporal lobectomy for intractable epilepsy (the relative expression of 13 subunits was examined in an additional eight cells; data not shown). The majority of neurons expressed 10 or more different subunit mRNAs, with the most abundantly expressed subunits being α1, α4, β1, β2, β3, γ1, γ2, and δ. Relative expression is defined as the hybridization signal for a single GABAR subunit divided by the sum of hybridization signals for all GABAR subunits within an individual cell.

To examine whether the expression of certain subunits might be coordinated within individual neurons, we performed a correlation analysis among the hybridization signals (normalized to background) of the different subunits in each cell. Subunits demonstrating correlation coefficients (r) with Bonferroni adjustment of >0.90 and a p value <0.001 were considered highly correlated. In the use of these criteria, several strong correlations were apparent (Fig. 3). The expression of α4, β2, and δ mRNAs was all highly correlated (r = 0.90 for α4:β2; r = 0.91 for α4:δ and β2:δ). The expression of β2 mRNA also correlated with the expression of α3 mRNA (r = 0.93). The expression of ϵ mRNA was found to correlate strongly with β1 mRNA (r = 0.97). No other strong subunit associations (r > 0.9) were identified among the 14 subunit mRNAs that were examined. Of note, despite their high levels of expression, α1, γ1, and γ2 mRNA levels were not highly correlated with levels of any other subunit, with their strongest correlations being with each other (α1:γ1, r = 0.75; α1:γ2, r = 0.67; γ1:γ2, r = 0.72). The small number of highly specific correlations (5 of 91 or 5% of potential subunit associations) makes it extremely unlikely that these result from artifacts arising from the

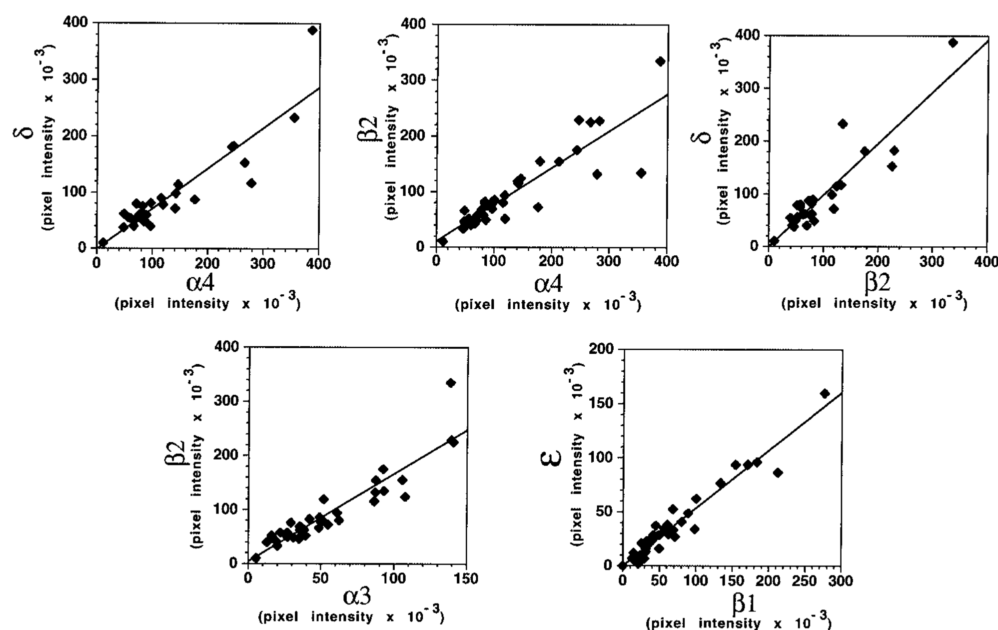

Figure 3. Coordinated expression of GABAR subunit mRNAs within individual DGCs. Correlation analysis was performed among the hybridization signals (integrated intensity of pixels normalized to background) of 14 different GABAR subunits in each cell. The hybridization of α4-, δ-, and β2-subunit mRNAs in individual DGCs correlates positively with each other (*top row*). β2 mRNA hybridization also correlates with α3-subunit mRNA (*bottom row, left*), whereas ϵ-mRNA hybridization correlates strongly with that of β1-mRNA (*bottom row, right*). Subunits demonstrating correlation coefficients (r) with a Bonferroni adjustment of >0.90 and a p value <0.001 were considered to be highly correlated. None of the other 86 potential subunit pairings was found to be highly correlated.

technique (which would be expected to result in broad associations among multiple subunits), and chance associations would be expected to occur in <1 of 1000 possible associations at the selected p value of <0.001.

To determine whether subunit associations similar to those seen in human DGCs also occurred in other species, we performed a similar correlation analysis, using the data previously obtained on GABAR expression, in acutely isolated dentate granule cells from control rats and rats with temporal lobe epilepsy after pilocarpine-induced status epilepticus (Brooks-Kayal et al., 1998b). Many of the same subunit associations seen in human dentate granule cells were also apparent in the rat cells. In the 17 neurons from control rats and 23 neurons from epileptic rats, strong correlations were seen in both groups between expression of α4 and β2 ($r = 0.85$ for controls, 0.86 for epileptic; $p < 0.001$), α4 and δ ($r = 0.89$ for controls, 0.84 for epileptic; $p < 0.001$), and β2 and δ ($r = 0.90$ for controls, 0.81 for epileptic; $p < 0.001$). The finding that these subunit associations could be identified in control and epileptic rat as well as human DGCs suggests that these correlations are not species-specific or secondary to the process of epileptogenesis. In contrast, other correlations were present in DGCs from epileptic rats, but not control rats, such as α1 and β1 ($r = 0.90$; $p < 0.001$) and β1 and γ2 ($r = 0.93$; $p < 0.001$). These correlations thus may be related, at least in part, to epilepsy-associated changes in subunit expression, such as the decreased expression of α1 and β1 mRNA in rat DGCs after pilocarpine-induced status epilepticus (Brooks-Kayal et al., 1998b). Finally, some subunit associations were clearly different between the two species. In contrast to their tight correlation in human DGCs, β1 and ϵ-mRNA expression was not correlated in the DGCs from either the control or epileptic rats. This difference may not be unexpected, considering the major sequence variation between the rat and human homologs for the ϵ-subunit (as discussed above).

Correlation of GABAR subunit mRNA levels to receptor pharmacology

Next we evaluated whether levels of specific GABAR subunit mRNAs in dentate granule cells predicted the function of the native receptors. To do this, we determined the percentage of augmentation of the 10 μM GABA response by clonazepam (100 nM) in 13 cells (from four different patients) and compared it with the relative expression of each of the different subunit mRNAs in the same cell. Augmentation by the benzodiazepine (BZ)1-specific agonist zolpidem (100 nM) also was examined in a subset of seven of these cells. Significant correlations with clonazepam augmentation were identified for only 4 of the 14 subunit mRNAs that were examined: α1, α4, γ2, and δ. As seen in Figure 4, these correlation plots clearly define two distinct populations of cells. Augmentation of the GABA response by clonazepam in individual cells correlated positively with the relative expression of α1 and γ2 mRNA ($r > 0.9$; $p < 0.01$ for each) and negatively with α4 and δ mRNA levels ($r > 0.85$; $p < 0.03$ for each) in cells within each group. As expected from the correlation analysis, the mean augmentation by clonazepam was markedly different between the two groups, with the mean augmentation by clonazepam being 13.6 ± 4.2% for cells in group I ($n = 6$) and 51.3 ± 12.9% for cells in group II ($n = 7$; Mann–Whitney, $p < 0.05$) (Fig. 5*A*). Augmentation by zolpidem was examined in only two of the cells that distributed into group I, and thus it was impossible to evaluate any correlation with subunit expression for this group. Zolpidem augmentation was examined in five cells in group II. As seen for clonazepam augmentation, there was a positive correlation between the percentage of augmentation by zolpidem (ZOL) in each cell and the expression of α1 and γ2 mRNAs in the same cells ($r = 0.68$ for α1:ZOL ratio; $r = 0.62$ for γ2:ZOL) and a negative correlation between zolpidem augmentation and the expression of α4 and δ mRNAs ($r = -0.67$ for α4:ZOL; $r = -0.64$ for δ:ZOL). In the small number of cells that were examined, however, these correlations did not meet statistical significance.

Next we attempted to identify characteristics that might discriminate these two groups of cells. Cells from each of the four patients were present in each group, arguing against the effects of particular medications or historic factors that might be unique to certain patients. We also compared the relative expression of the different subunits among the cells in each group. Although no

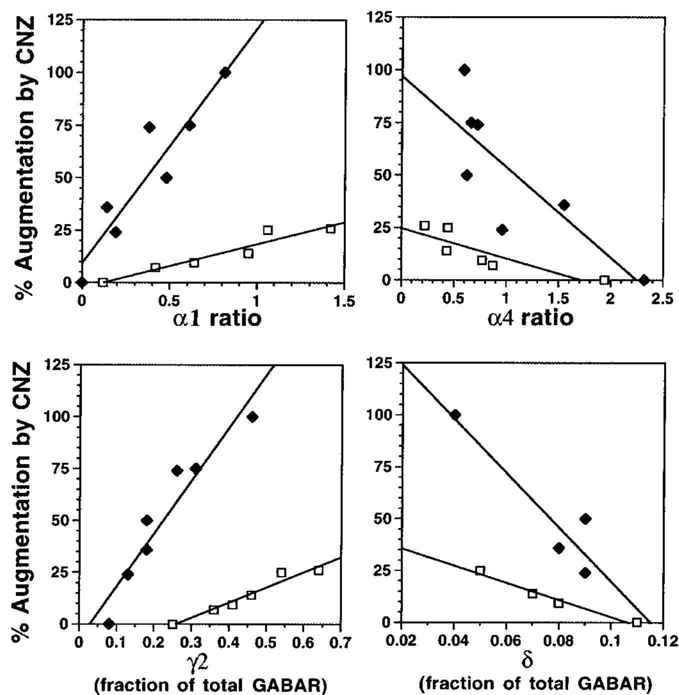

Figure 4. Augmentation of GABA response by clonazepam correlates with GABAR subunit expression in human DGCs. The percentage of augmentation of the 10 μM GABA response by 100 nM clonazepam positively correlated with the ratio of expression of α1 mRNA to other α-subunits (*top left*) and negatively correlated with the ratio of expression of α4 mRNA to other α-subunits (*top right*) in individual acutely isolated human DGCs ($n = 13$). The relative expression of γ2-subunit mRNA also correlated positively with clonazepam augmentation (*bottom left*; $n = 13$), whereas the relative expression of δ-subunit mRNA correlated negatively with clonazepam augmentation (*bottom right*; $n = 8$). Note that two populations of DGCs are identified on the basis of clonazepam augmentation, with cells in group I (*open squares*) demonstrating a lower mean augmentation by clonazepam than those in group II (*filled diamonds*). Relative expression is defined as the hybridization signal for a single GABAR subunit divided by the sum of hybridization signals for all GABAR subunits within an individual cell.

significant differences in the mean relative expression of any single subunit mRNA existed between the groups, a difference in the pattern of expression of the α-subunits was apparent (Fig. 5*B*). The mean ratio of α1 mRNA expression to the expression of all other α-subunits was twofold higher in group I cells than in group II cells (0.76 ± 0.19 vs 0.30 ± 0.09, respectively; $p = 0.05$, t test). In addition, there was a marked difference in the overall level of GABAR mRNA expression between the two groups. In group I, the total expression of GABAR subunit mRNA compared with the expression of mRNA for cyclophyllin (a cellular housekeeping protein) was 16.5 ± 2.2, compared with 30.1 ± 4.2 for group II cells (Mann–Whitney, $p < 0.03$) (Fig. 5*C*). These data suggest that two distinct populations of cells with differing GABAR pharmacology and subunit expression are present in epileptic human dentate gyrus.

DISCUSSION

The results that were obtained demonstrate strong correlations in the expression of selected GABAR subunits within individual dentate granule cells, which appear independent of species or epileptic condition. These findings suggest that the expression of certain GABAR subunit mRNAs in individual dentate granule cells, particularly the α4, β2, and δ-subunits, may be controlled

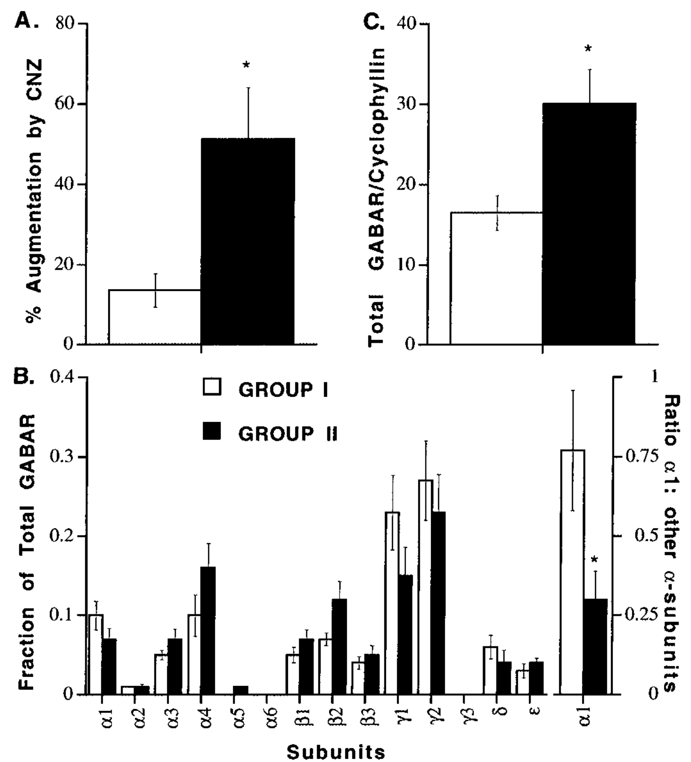

Figure 5. Two populations of DGCs in epileptic human hippocampus differ in the mean augmentation of GABA response by clonazepam and GABAR subunit expression. *A*, The mean percentage of augmentation of the 10 μM GABA response by 100 nM clonazepam is more than threefold lower in DGCs in Group I (*white bars*) as compared with the DGCs in Group II (*black bars*). *B*, Pattern of GABAR subunit expression in the different populations of DGCs. Shown are histograms demonstrating the mean ± SE relative expression of the 14 different GABAR subunit mRNAs (*left*) and the ratio of the expression of α1 to all other α-subunit mRNAs (*right*; note the change in the scale of the *vertical axis*). Note the significant difference in the expression of α1-mRNA as compared with the expression of the other α-subunits between the two groups. Relative expression is defined as a hybridization signal for a single GABAR subunit divided by the sum of hybridization signals for all GABAR subunits within an individual cell. *C*, Total GABAR subunit expression in the two populations of DGCs in epileptic human hippocampus. Shown is the mean ± SE total expression of all GABAR subunit mRNAs as a fraction of cyclophyllin mRNA. Note the significant difference in the total expression of GABAR mRNA between the two groups (*$p < 0.05$).

coordinately. Coordinated expression of this subset of subunits might be predicted on the basis of previous studies of subunit coassembly. Immunoprecipitation studies of GABARs in hippocampus have demonstrated the association of the α4-subunit with α3, β2/β3, γ2, and, to a lesser extent, α1 and α2 subunits (Kern and Sieghart, 1994; Khan et al., 1996; Benke et al., 1997). Immunoprecipitation studies that used δ-subunit-specific antibodies have shown the association of the δ-subunit with α1, α3, β2/β3, and γ2 subunits (Mertens et al., 1993). The association of the α4- and δ-subunits previously has not been examined specifically, but it might be expected on the basis of the close homology of the α4- to the α6-subunit (McKernan and Whiting, 1996). A specific association between α6- and δ-subunits has been demonstrated in cerebellar granule cells. Genetically deleted mice lacking α6 protein demonstrate a selective loss of δ-subunit protein from cerebellar granule cells (Jones et al., 1997). Interestingly, in these α6 knock-out animals δ-subunit mRNA levels were not altered, suggesting a post-translational loss of the δ-subunit. By contrast,

in the human and rat neurons studied here, the coordinated expression of the α4- and δ-subunits appears to be, at least in part, "pretranslational" at the level of transcription or mRNA stability.

Many of the GABAR subunit genes occur in clusters within the human (and rat) genome (McKernan and Whiting, 1996). In humans, genes encoding the α1, α6, β2, and γ2 subunits are clustered on chromosome 5; those encoding α2, α4, β1, and γ1 subunits are located on chromosome 4; α5, β3, and γ3 subunit genes are on chromosome 15; α3, β4, and ϵ genes are together on the X-chromosome; and the δ-gene is located on chromosome 1 (Levin et al., 1996; McKernan and Whiting, 1996; Davies et al., 1997; Whiting et al., 1997). Although coordinated transcription from a cluster of subunit genes might be expected, in fact none of the coordinated expression seen in the current study could be attributed to such an effect. The subunit genes for which the expression were coordinated most strongly, α4, β2, and δ, are on three different chromosomes. This suggests that, at least in dentate granule cells, subunit gene expression is not coordinated on the basis of shared chromosomal localization. It remains to be determined whether a stronger association between gene clusters and coordinated gene expression may occur in other cell types, such as cerebellar granule cells, in which many of the predominant subunits that are expressed (α1, α6, β2, and γ2) are located in a single chromosomal cluster.

Our results further demonstrate that subunit mRNA levels correlate closely with receptor pharmacology within individual DGCs, as would be predicted by studies of recombinant receptors, suggesting that the production of subunit mRNA may be a critical limiting step determining levels of protein expression. Studies of recombinant heterotrimeric GABARs (α_x, β_y, γ_z) have shown that the α- and γ-subunits have the strongest influence on the affinity of the expressed GABAR for BZ ligands. α1-, α2-, α3-, and α5-containing GABARs all demonstrate high affinity for the benzodiazepines flunitrazepam, diazepam, and clonazepam (Pritchett et al., 1989a; Luddens and Wisden, 1991), whereas α4-containing receptors have no affinity for BZ binding site agonists (Wisden et al., 1991). The presence of a γ-subunit also is required for modulation of the GABAR by BZs (Pritchett et al., 1989b), and the specific isoform of the γ-subunit modifies BZ affinity. Positive modulation by most BZ agonists, including clonazepam, is reduced when the γ2-subunit is replaced by a γ1-subunit (Ymer et al., 1990; Puia et al., 1991; Wisden et al., 1991). If the γ- is replaced by δ-, a BZ-insensitive receptor is created (Saxena and Macdonald, 1994, 1996). Native GABARs immunoprecipitated by using a δ-subunit-specific antibody demonstrate no high-affinity BZ binding (Quirk et al., 1995). Thus, the current results showing that augmentation of the GABA response by clonazepam in individual neurons correlated positively with the relative expression of α1 and γ2 mRNA and negatively with α4 and δ mRNA levels within each cell demonstrate that structure–function relationships seen in recombinant studies are consistent with the findings in native human GABARs.

A second finding of interest that arises from examining the structure–function correlations of the native GABAR is the presence of two distinct populations of DGCs in the epileptic human hippocampus. These populations differ in mean augmentation by clonazepam, overall level of GABAR mRNA expression (total expression of GABAR subunit mRNAs as compared with expression of mRNA for cyclophyllin), and the ratio of α1 mRNA expression to the expression of all other α-subunits (α1 ratio). One group of cells demonstrated low total GABAR subunit mRNA expression, low mean augmentation by clonazepam (13.6 ± 4.2%), and an increased α1 ratio. The second group of cells shows a nearly twofold higher total GABAR subunit mRNA expression, a more than threefold higher mean augmentation by clonazepam (51.3 ± 12.9%), but a twofold lower α1 ratio. How can the presence of two distinct populations of cells be explained? It is tempting to speculate that seizure-induced increased DGC neurogenesis might be a contributing factor. Neurogenesis in the dentate gyrus has been documented to continue through adulthood in rodents (Kaplan and Hinds, 1977; Bayer and Yackel, 1982; Cameron et al., 1993; Kuhn et al., 1996) and humans (Eriksson et al., 1998), and prolonged seizure activity has been shown to stimulate DGC neurogenesis in rodents (Parent et al., 1997). The "later-born" DGCs might exhibit unique GABAergic properties as compared with neurons that matured earlier, and there is recent evidence in human studies for two functionally distinct populations of DGCs in epileptic human hippocampus (Dietrich et al., 1999). Our group previously has demonstrated epilepsy-associated alterations in GABAR subunit mRNA expression and function in rat DGCs, including a decrease in α1 mRNA expression, increased GABAR current density, and increased augmentation of the GABA response by clonazepam (Gibbs et al., 1997; Brooks-Kayal et al., 1998b). Augmented GABAergic inhibition (Buhl et al., 1996) and an increased number of synaptic GABARs (Nusser et al., 1998) in rat DGCs also have been demonstrated after kindling. Many of these changes in GABAR expression and function occur as soon as 24 hr after prolonged seizures (Brooks-Kayal et al., 1998b), suggesting that they are occurring in DGCs that are already present when the seizure occurs rather than in resultant "newly born" cells. These distinct effects of prolonged seizure activity on DGC neurogenesis and GABAR expression together may explain the finding of two cell populations in epileptic human dentate gyrus. The group of cells demonstrating higher total GABAR subunit mRNA expression and augmentation by clonazepam, but lower expression of α1-mRNA as compared with other α-subunits, may represent DGCs that were present at the time of epileptogenesis in these patients, whereas the group of cells with lower total levels of GABAR expression, CNZ augmentation, and higher α1-mRNA expression could have been born during or after epileptogenesis. Confirmation of this hypothesis will require studies combining the labeling of newly born cells (with bromodeoxyuridine, for example) with an examination of single-cell GABAR expression and function before, during, and after epileptogenesis.

In conclusion, these data demonstrate that specific GABAR subunit mRNAs exhibit coordinated control of expression in individual human DGCs, which has a significant impact on inhibitory function in individual human dentate granule cells. This coordinated expression also is observed in DGCs from control and epileptic rats and does not appear to be related to the location of the subunit genes in a "chromosomal cluster" nor to be species-specific or secondary to epileptogenesis. Additional studies are required to delineate the molecular mechanisms that may control this coordinated subunit expression and to determine whether it occurs in other neuronal cell types.

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