Selective changes in single cell $GABA_A$ receptor subunit expression and function in temporal lobe epilepsy

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Temporal lobe epilepsy is the most prevalent seizure disorder in adults. Compromised inhibitory neurotransmitter function in the hippocampus contributes to the hyperexcitability generating this condition, but the underlying molecular mechanisms are unknown. Combining patch-clamp recording and single-cell mRNA amplification (aRNA) techniques in single dentate granule cells, we demonstrate that expression of $GABA_A$ receptor subunit mRNAs is substantially altered in neurons from epileptic rats. These changes in gene expression precede epilepsy onset by weeks and correlate with profound alterations in receptor function, indicating that aberrant $GABA_A$ receptor expression and function has an essential role in the process of epileptogenesis.

Fast synaptic inhibition in the forebrain is mediated mostly by the neutral amino acid GABA interacting with post-synaptic $GABA_A$ receptors (GABARs). GABARs are heteromeric protein complexes composed of multiple subunits that form ligand-gated anion-selective channels that are modulated by barbiturates, benzodiazepines, ethanol, volatile anesthetics and the anesthetic steroids[1,2]. Subunit composition varies in different brain regions and cell types, and this confers distinct functional and pharmacological properties to the resulting receptors[1,2,3]. Impaired GABAergic function contributes to some forms of epilepsy[4,5], and may result, in part, from normal GABARs participating in abnormal circuitry because of epilepsy-associated cell death and activity-dependent secondary synaptic plasticity. Less-studied are epilepsy-associated alterations in the function and molecular composition of the post-synaptic GABARs in surviving hippocampal neurons. Such changes may result in receptors with altered functional properties that contribute to the process of epileptogenesis and compromise the efficacy of anti-epileptic drugs that act at GABARs. Long-lasting alterations in post-synaptic GABAR function have been demonstrated in temporal lobe epilepsy[6,7] (TLE), but the underlying molecular basis has not been determined. Epilepy-associated changes in GABAR function might result from altered subunit composition, as other mechanisms such as changes in phosphorylation seem less likely to produce such permanent alterations in GABAR function. Altered transcriptional control of the production of the various GABAR subunit mRNAs could result in alterations in subunit composition. If this occurred, the steady-state levels of these GABAR subunit messages should change substantially in epileptic neurons. Such changes might precede the onset of epilepsy and participate in the process of epileptogenesis. Long-term alterations in gene expression[8], including changes in GABAR subunit mRNA levels[9–13], occur in some animal models of epilepsy, but have not been correlated with functional changes at a cellular level. To address these questions, we examined GABAR function and subunit mRNA expression in single dentate granule cells (DGCs) in an animal model of temporal lobe epilepsy.

Results

We combined the techniques of whole-cell patch-clamp recording and single-cell mRNA (aRNA) amplification[14,15] to analyze post-synaptic GABAR function and subunit mRNA expression in individual DGCs isolated from control and pilocarpine-treated adult rats before and after the onset of temporal lobe epilepsy. All rats included in the pilocarpine-treated groups had status epilepticus lasting 1–5 hours (class 3–5 behavioral seizures[16]) immediately after injection. These seizures were terminated with diazepam, the rats recovered for 2 weeks and then they began to have recurrent spontaneous seizures of limbic origin[6,17]. Rats were considered epileptic after two or more documented spontaneous seizures, beginning 2 or more weeks after recovery from pilocarpine injection. On average, epileptic rats were studied 1–4 months after pilocarpine-induced status epilepticus. To minimize any immediate effects of seizures on $GABA_A$ receptor properties, epileptic rats were video-monitored in the 24 hours before use to ensure that no seizures occurred.

Altered GABAR function in DGCs from chronically epileptic rats

Representative analysis combining whole-cell patch-clamp recording and aRNA expression profiling of a single DGC from an epileptic rat is shown in Figure 1. This cell exemplifies the substantially altered GABAR function and pharmacology in DGCs from epileptic rats, with minimal augmentation of the GABA response by zolpidem and substantial blockade of the GABA response by zinc. Similar analysis of multiple DGCs from control rats ('control DGCs') and epileptic rats ('epileptic

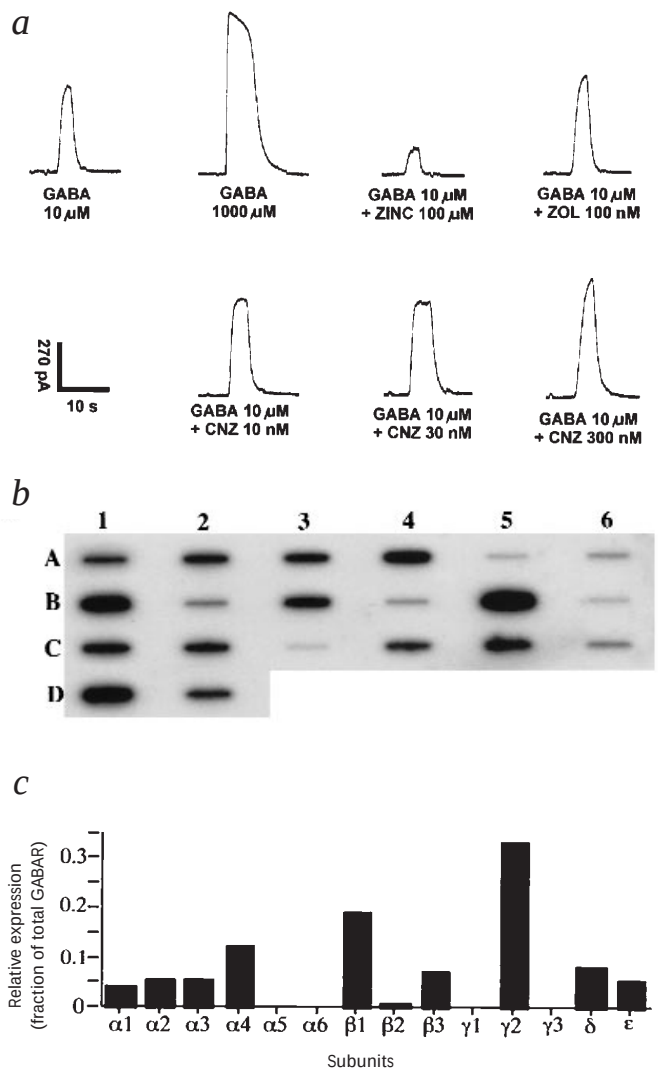

Fig. 1 Whole-cell patch-clamp recordings and aRNA expression profiling in a single dentate granule neuron from a rat with temporal lobe epilepsy after pilocarpine injection. ***a***, Responses of a representative DGC isolated from a chronically epileptic rat to concentration-clamp application of GABA (10 and 1,000 μM), and modulation of the 10-μM GABA response by co-applied zolpidem (ZOL), zinc, and clonazepam (CNZ). There was very little effect of ZOL on the GABA response (10% augmentation), whereas there was a considerable blockade of the GABA response by zinc (70% block). ***b***, Slot-blot demonstrating hybridization intensities of GABAR subunit mRNAs for the cell in *a*. Radiolabeled aRNA probe from the DGC is hybridized against a slot-blot containing cDNAs encoding GABAR subunit α1–α6 (A1–6), β1–β3 (B1–3), γ1–γ3 (B4–6), δ and ε (C1–2), glial fibrillary acidic protein (C3), neurofilament-L (C4), β-actin (C5), pBluescript (C6), and GAD 65 and GAD 67 (D1–2). The value for the slot containing plasmid cDNA (pBluescript) is considered background; neurofilament-L expression is a marker for neuronal phenotype; glial fibrillary acidic protein expression is a control for glial contamination; and β-actin expression is an internal reference value. ***c***, Relative expression of each GABAR subunit in this same cell calculated as hybridization signal for the single subunit divided by the sum of hybridization signals for all GABAR subunits within the cell.

DGCs') showed profound differences in postsynaptic GABAR properties between the two groups. Mean GABA receptor response efficacy (assessed from cellular responses to application of a saturating (1 mM) concentration of GABA.) in epileptic DGCs was increased to 159% of control values ($P < 0.04$, Mann Whitney Rank Sum test), with no alteration in GABA potency (Fig. 2*a*) or cell capacitance between groups (7.2 ± 0.5 for epileptic compared with 8.6 ± 0.4 pF for control DGCs; not significant). Sensitivity of GABARs to block by zinc was enhanced in epileptic DGCs to 171% of controls ($P < 0.001$, Mann-Whitney Rank Sum test), whereas sensitivity to augmentation by the BZ1-selective benzodiazepine agonist zolpidem was decreased to 18% of control values ($P < 0.001$, Mann-Whitney Rank Sum test; Fig.2*b* and *c*).

Changes in subunit mRNAs correlate with altered GABAR function

After whole-cell patch-clamp recordings were made, cell contents were aspirated into the patch electrode, and relative expression of GABAR mRNAs in control and chronically epileptic DGCs was analyzed using single-cell aRNA amplification to determine whether changes occurred that were consistent with and might contribute to the observed functional alterations. The aRNA amplification technique results in synthesis of aRNA in amounts linearly proportional to the amounts of individual cellular mRNAs[14,15]. GABAR subunit mRNAs were successfully amplified and analyzed in a total of 16 control and 23 epileptic DGCs (from a minimum of five different rats for each group) in which GABAR properties were examined in patch experiments. Successful aRNA amplification was achieved in 95% of DGCs analyzed electrophysiologically. Studies of recombinant heterotrimeric GABARs (α_x, β_y and γ_z) have shown that receptors containing α1 subunit demonstrate increased augmentation by zolpidem (BZ1 receptors) compared with GABARs containing other α subunits (BZ2 receptors)[18]. Given the substantially decreased augmentation by zolpidem in epileptic DGCs (Fig. 2*b*), these cells should express relatively lower amounts of α1 mRNA compared with mRNA for other α subunits. The ratio of α1 mRNA expression to expression of all other α-subunit mRNAs ('α1 ratio') within individual epileptic DGCs was, in fact, decreased to 54% of control values (0.91 ± 0.13 for control compared with 0.49 ± 0.08 for epileptic DGCs; $P < 0.003$, *t*-test), correlating well with the decreased zolpidem augmentation of GABAR responses evident in these cells (Fig. 3*a*). Mean relative expression of α1-subunit mRNA (as a fraction of total GABAR mRNA expression) in epileptic DGCs was decreased to 65% of controls ($P < 0.02$, *t*-test), whereas the relative expression of α4 mRNA in epileptic DGCs was increased to 175% of control values ($P < 0.007$, *t*-test), with no significant change in relative expression of α2-, α3-, α5- or α6-subunit mRNAs.

Sensitivity to blockade of GABA-evoked currents by zinc was considerably increased in epileptic compared with control DGCs (Fig. 2*c*). The decrease in the relative expression of α1 compared with other α subunits in epileptic DGCs may contribute to this increased zinc potency, as recombinant GABARs containing α4, α5 or α6 subtypes with a β and γ subunit are more sensitive to zinc inhibition than those containing an α1 subtype[19–23]. Zinc sensitivity can also be affected, however, by changes in the γ, δ or ε subunits. Recombinant GABARs consisting of only α and β subunits are potently blocked by zinc, whereas addition of a γ or ε subunit substantially reduces zinc sensitivity[24,25]. Replacement of a γ subunit with a δ subunit in α,β-containing recombinant GABARs confers increased zinc sensitivity[26]. Given the results of these recombinant receptor studies, alterations might be expected in γ-subunit expression in epileptic neurons. In fact, there were no significant changes in relative expression of any of the γ-subunit mRNAs (Fig. 3*b*). In contrast, relative expression of δ-

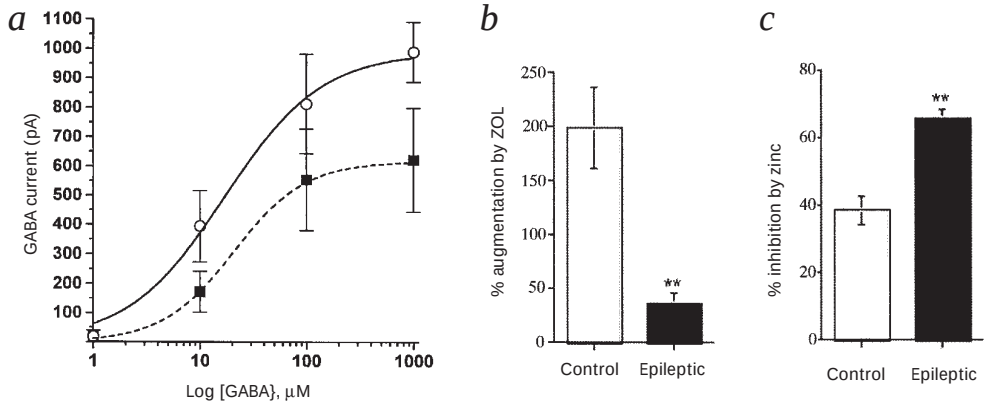

Fig. 2 Functional alterations in $GABA_A$ receptor response properties of dentate granule neurons isolated from epileptic rats. ***a***, Concentration–response plot comparing GABA-evoked responses in 10 control DGCs and 8 DGCs from chronically epileptic pilocarpine-treated rats for which aRNA amplification profiles were subsequently done. Epileptic DGCs (○) had considerably larger amplitude GABA-evoked responses at a given GABA concentration compared with those of controls (■), with no difference in GABA potency. Curves are generated from a best fit sigmoidal dose–response curve, using the Marquardt-Levenberg nonlinear least squares algorithm. Best fit parameters were K_d = 17.0 ± 5.4 uM, maximal effect = 991 ± 70.2 pA, and Hill coefficient 1.0 ± 0.24 for epileptic DGCs; and K_d = 22.6 ± 2.3 uM, maximal effect = 464 ± 10.4 pA, and Hill coefficient 1.4 ± 0.13 for control DGCs. ***b***, Mean percent augmentation of the 10-μM GABA response by 100 nM zolpidem for control (n = 10) and epileptic (n = 18) DGCs. The level of zolpidem augmentation in epileptic DGCs was decreased to 18% of controls. ***c***, Mean percent blockade of the 10-μM GABA response by 100 μM zinc for control (n = 20) and epileptic (n = 21) DGCs. There was an increase in the level of zinc block of GABA responses in the epileptic DGCs to 171% of controls. All values are mean ± s.e.m. (**, P < 0.01, t-test).

and ε-subunit mRNAs in epileptic DGCs were increased to 225% (P < 0.005, Mann-Whitney Rank Sum test) and 233% (P < 0.01, Mann-Whitney Rank Sum test) of control values, respectively (Fig. 3*b*). Given that variations in levels of mRNA expression may result in alterations in protein expression, this shift in the relative amounts of δ- and ε-containing receptors compared with γ-containing receptors may also contribute to the substantially increased zinc sensitivity in epileptic DGCs.

Significant changes also occurred in β-subunit expression in epileptic DGCs (Fig.3*b*). The amount of β1-subunit expression in epileptic DGCs was decreased to 59% of control expression (P < .01, t-test), whereas β3 expression was increased to 267% of control expression (P < 0.001, Mann-Whitney Rank Sum test). The nature of the β subtype has been shown to affect channel properties[27], benzodiazepine efficacy[28], affinity for GABA analogues, and efficacy of allosteric modulation by barbiturates, steroids, zinc and loreclezole[29,30]. An additional potential consequence of the observed β-subunit switch could be a shift in the nature of GABAR responses to phosphorylation[31], which could substantially alter neuronal excitability in the setting of high calcium loads, as occurs during seizure activity and rapid neuronal firing.

To determine whether an overall increase in GABAR subunit mRNA expression occurs in epileptic neurons that might correlate with the considerable increase in GABA efficacy seen in these cells, we analyzed the expression of all GABAR subunit mRNAs relative to β-actin expression within individual cells. Using this calculation, a trend towards increased relative expression of total GABAR mRNA expression was apparent, but did not reach statistical significance (7.4 ± 0.9 for control compared with 8.1 ± 0.9 for epileptic DGCs). These finding must be interpreted with caution because of possible epilepsy-associated alterations in β-actin expression, but indicates that increased GABAR density may not be solely attributable to increased transcription, and raises the question of whether enhanced translation of GABAR mRNA to protein may also occur during epileptogenesis. GABAR subunit mRNA and membrane protein levels do not always demonstrate a one-to-one correspondence[32].

Alterations in GABAR properties precede the onset of epilepsy

Next we determined whether alterations in GABAR subunit expression and function might precede development of spontaneous recurrent seizures, and thus potentially be involved in the

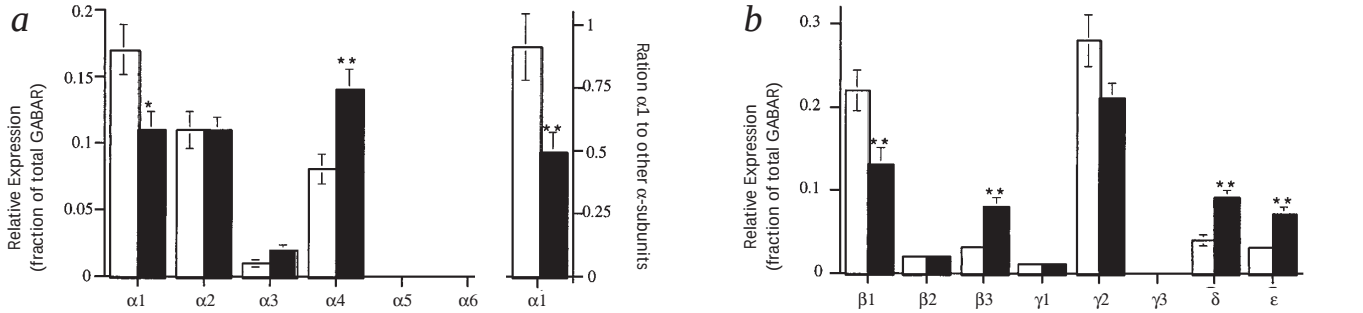

Fig. 3 Relative expression of GABAR subunit mRNAs in isolated DGCs from control rats (open bars) and chronically epileptic rats in which temporal lobe epilepsy has been elicited by pilocarpine injection (filled bars). ***a***, Relative expression (mean ± s.e.m.) of the six different α-subunit mRNAs (left) and ratio of expression of α1 to all other α-subunit mRNAs (right). Scale of vertical axis changes for ratio on right. Expression of α1-mRNA decreases significantly compared with expression of other α-subunits in epileptic compared with control DGCs. ***b***, Relative expression (mean ± s.e.m.) of GABAR β-, γ-, δ- and ε-subunit mRNAs. Relative expression of δ- and ε-subunit mRNAs increase in epileptic compared with control DGCs. Relative expression is defined as hybridization signal for a single GABAR subunit divided by the sum of hybridization signals for all GABAR subunits within an individual cell. Values based on expression profiling of 16 control and 23 epileptic DGCs using the technique of single-cell aRNA amplification. (*, P < 0.05; **, P < 0.01)

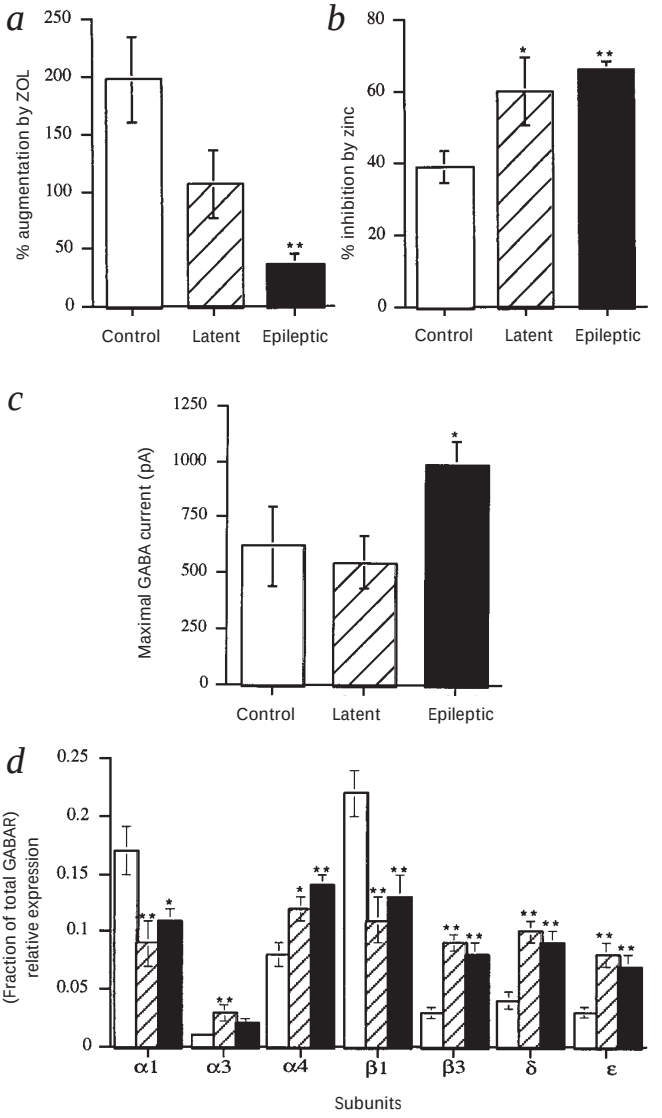

Fig. 4 Comparative changes in GABAR expression and function in isolated DGCs from control (open bars), latent-period (hashed bars) and chronically epileptic pilocarpine-treated (solid bars) rats. ***a***, Zolpidem modulation of GABA-evoked currents. Mean (± s.e.m.) efficacy of zolpidem (100 nM) in augmenting GABA responses in latent-period DGCs (106 ± 29.2%) is intermediate between (but not statistically significantly different from) control (197 ± 37.5%) and chronically epileptic (36.2 ± 8.8%) DGCs. Data represent 10 control, 5 latent-period and 18 epileptic DGCs. ***b***, Inhibition of GABA-evoked currents by zinc. Mean (± s.e.m.) blockade of GABA responses by 100 μM zinc in latent-period DGCs (59.6 ± 9.3%) is significantly greater than in control DGCs (38.5 ± 4.3%; $P < 0.04$, *t*-test) but is not different from epileptic DGCs (65.7 ± 2.6%). Data represent 20 control, 5 latent-period and 21 epileptic DGCs. ***c***, Maximal GABA-evoked currents. Mean (± s.e.m.) maximal currents evoked by GABA (1 mM) in latent-period DGCs is not significantly different from control DGCs (622 ± 178 pA for controls; 549 ± 113 pA for latent-period DGCs ($n = 10$), but is significantly less than that seen in chronically epileptic DGCs (990 ± 102%; $P < 0.02$, Mann-Whitney Rank Sum test). ***d***, Relative expression of GABAR subunit mRNAs. Relative expression of α1, α3, α4, β1, β3, δ and ε-subunit mRNAs are significantly different in latent-period compared with control DGCs. Relative expression is defined as hybridization signal for a single GABAR subunit divided by the sum of hybridization signals for all GABAR subunits within an individual cell. Data represent 16 control, 14 latent-period and 23 epileptic DGCs. (*, $P < 0.05$; **, $P < 0.01$).

process of epileptogenesis. We therefore analyzed DGCs from rats killed 24 hours after pilocarpine-induced status epilepticus, which is usually at least 2 weeks before the onset of spontaneous seizures ('latent-period' rats). Alterations in GABAR pharmacology in 'latent-period DGCs' paralleled those seen in epileptic DGCs. Increased sensitivity to blockade by zinc was evident in latent-period DGCs compared with control DGCs, and there was a nonsignificant trend towards decreased augmentation by zolpidem (Fig. 4*a* and *b*). However, GABA efficacy was not enhanced in latent-period DGCs relative to controls, and was significantly decreased to 55% of GABA efficacy evident in epileptic DGCs ($P < 0.02$, Mann-Whitney Rank Sum test; Fig. 4*c*). Thus, the increased GABA efficacy evident in DGCs from chronically epileptic rats may be compensatory rather than epileptogenic, as it may only appear after the onset of spontaneous seizures.

GABAR subunit expression profiles in latent-period DGCs ($n = 14$ cells from three different rats) were also similar in many respects to those in the epileptic DGCs. The ratio of α1 mRNA expression to that of other α subunits in latent-period DGCs was decreased to 40% of control values ($P < 0.003$, *t*-test), with a decrease in relative expression of α1-subunit mRNA to 53% of control values (Fig. 4*d*, $P < 0.005$, *t*-test), and an increase in relative expression of α4 mRNA to 150% of control values ($P < 0.04$, *t*-test). Relative expression of β1 mRNA in latent-period DGCs was decreased to 50% of controls ($P < 0.001$, *t*-test), whereas the relative expressions of α3, β3, δ and ε mRNAs were each increased more than twofold in latent-period compared with control DGCs ($P < 0.001$, Mann-Whitney Rank Sum test). Latent-period and epileptic DGCs differed by the presence of low levels of α6-mRNA expression in a small number of latent-period DGCs. There was expression of α6 mRNA in 3 of 14 latent-period DGCs, compared with 0 of 16 control DGCs and 1 of 23 epileptic DGCs. Although α6-subunit expression is thought to be restricted to cerebellar granule neurons *in vivo*[3], α6 expression has been demonstrated in cultured embryonic hippocampal neurons[33]. This expression is inhibited by blockade of the NMDA-selective glutamate receptor, indicating it may be related to elevated levels of extracellular glutamate present in the culture system (Jin, H. *et al.* Expression of α6 subunit mRNA in cultured hippocampal neurons. *Soc. Neurosci. Abstr.* **23**, 100 (1997)). Increased glutamate release has been found after seizures in some animal models[34] and may contribute to the enhanced α6 expression seen in some of these latent-period DGCs. Furthermore, α6 expression in cerebellar granule neurons has also recently been shown to be induced by electrical activity independent of glutamate receptor stimulation[35]. If a similar regulatory mechanism exists in hippocampal neurons, the recurrent excitatory discharges associated with pilocarpine-induced status epilepticus may contribute to the unexpected transient expression of α6 mRNA seen in a subset of latent-period DGCs.

Alterations in pre-synaptic GABAergic function in TLE

To examine other aspects of GABAergic function, we evaluated expression of glutamic acid decarboxylase (GAD65 and GAD67) mRNAs in these DGCs. Expression of GAD65 and GAD67 mRNAs have been demonstrated to be present in excitatory hippocampal neurons using aRNA amplification and reverse transcriptase PCR[36], and the presence of GAD67 immunoreactivity has been shown in dentate granule cells[37]. As in those studies, we observed expression of both GAD65 and GAD67 mRNA in control DGCs (Fig. 5). We also found that relative expression (compared with expression of

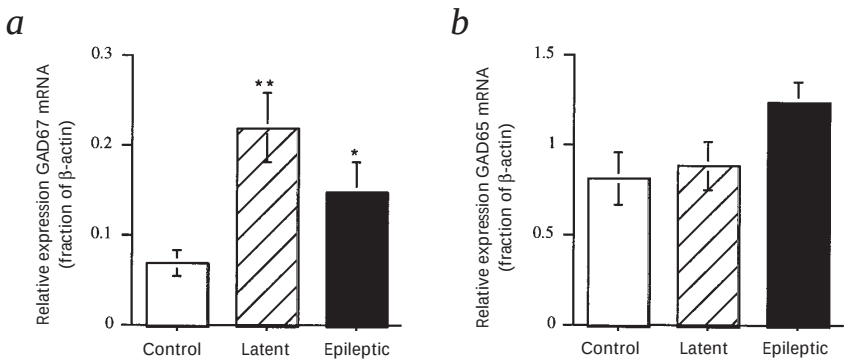

Fig. 5 Relative expression of GAD67 and GAD65 mRNAs in isolated DGCs from control rats, latent-period rats and chronically epileptic rats in which temporal lobe epilepsy has been elicited by pilocarpine injection. Histograms demonstrating mean (± s.e.m.) relative expression of GAD67 (***a***) and GAD65 (***b***) mRNAs from 16 control, 14 latent-period and 23 epileptic DGCs. Relative expression is defined as hybridization signal for GAD 67 or 65 cDNA divided by the hybridization signal for β-actin cDNA from the same cell. Mean relative expression of GAD67 mRNA is increased threefold in latent-period and twofold in epileptic compared with control DGCs (ANOVA, $P < 0.02$) (*, $P < 0.05$; **, $P < 0.01$; post-hoc *t*-test).

β-actin) of GAD67 was significantly increased in pilocarpine-treated compared with control DGCs (Fig. 5*a*). Relative expression of GAD67 mRNA increased more than threefold in latent-period and more than twofold in epileptic compared with control DGCs (ANOVA, $P < 0.006$). In contrast, relative expression of GAD65 was not increased in latent-period compared to control DGCs, and had a nonsignificant 1.7-fold increase in epileptic DGCs (Fig. 5*b*). These findings are consistent with previous *in situ* hybridization and immunohistochemistry studies demonstrating an acute increase in GAD67 expression after perforant pathway stimulation[37] and seizures induced by kainic acid[38]. Those studies, however, did not demonstrate long-term changes in GAD expression as demonstrated here. These results indicate that multiple alterations in GABAergic mechanisms (presynaptic and postsynaptic) may be occurring during epileptogenesis.

Discussion

Temporal lobe epilepsy is a symptomatic condition characterized by the delayed development of spontaneous seizures after injury to a previously normal brain. The finding that epilepsy-associated alterations in GABAR subunit expression are persistent and precede the onset of spontaneous seizure activity indicates that these changes are temporally positioned to participate in the process of epileptogenesis. What might be the functional relevance of these GABAR alterations in epileptic hippocampus? The enhanced zinc sensitivity of these 'epileptic' GABARs may be essential, given the circuit rearrangements in the epileptic hippocampus. Mossy fiber terminals contain high concentrations of zinc in addition to their excitatory neurotransmitters. This zinc can be released after repetitive synaptic stimulation[39], and released zinc can achieve concentrations of 100–300 μM in the extracellular space in the hippocampus[40]. Control DGCs do not innervate themselves, and have GABARs that are relatively zinc-insensitive. In contrast, epileptic DGCs in sclerotic hippocampi send zinc-containing mossy-fiber collaterals back onto the inner molecular layer and re-innervate the proximal dendrites of DGCs (refs. 17,41,42). This pathological auto-innervation provides a zinc-release mechanism onto DGCs in the epileptic hippocampus. These DGCs now express GABARs with heightened zinc sensitivity, setting the stage for a catastrophic failure of inhibition[6,7], particularly during repetitive activation (for example, during seizure initiation). The essential role of axonal sprouting in this process may explain why recurrent spontaneous seizures do not occur until several weeks after pilocarpine-induced status epilepticus despite the rapid alterations in GABAR function.

Previous studies of regional changes in GABAR subunit expression in epileptic dentate gyrus have yielded variable results. *In situ* hybridization and immunohistochemistry studies have demonstrated increased expression of multiple GABAR subunits in the dentate gyrus after kainate-induced status epilepticus in rat[9,10]. Such diffuse increases in multiple subunits indicate a generalized increase in GABAR expression that correlates well with functional studies that have demonstrated augmentation of inhibition in dentate gyrus from 'kindled' animals[7] and increases in GABAR current density in DGCs from epileptic pilocarpine-treated animals seen in this and other studies[6] (Fig. 2*a*). *In situ* hybridization studies in the dentate gyrus after kindling epileptogenesis have demonstrated both decreased[11] and increased[12] expression of the same subunits (for example, α1 and γ2). *In situ* hybridization studies specifically using the pilocarpine model are limited, but have demonstrated increased α5-subunit mRNA in the dentate gyrus of epileptic rats[13]. Some of the variability in findings between different studies may result from differences in the epilepsy model used, but another confounding factor may be the heterogeneity of cell types sampled when the entire dentate gyrus is examined. Cellular specificity may be particularly essential to the study of GABAR subunit expression, which varies between neuronal subtypes and is high in glial cells[43]. In addition, when generalized increases in subunit expression are occurring, immunocytochemical and *in situ* hybridization studies may miss changes in expression of subunits relative to one another within individual neurons. This study addresses these issues of cellular specificity and changes in subunit expression relative to each other by examining expression of multiple GABAR subunits simultaneously in individual neurons of only a single cell type. Differences in GABAR subunit mRNA expression between cell subtypes may also explain, at least in part, the lower levels of expression of the β2 and α5 subunits seen in DGCs in this study compared with the relatively higher levels seen regionally in the dentate gyrus by *in situ* hybridization[3].

Epilepsy-associated changes in GABAR structure and function have important implications for the therapy of patients with temporal lobe epilepsy. The mechanisms of action of several commonly used anti-epileptic drugs involve modulation of GABAergic function by direct effects on the $GABA_A$ receptor (barbiturates and benzodiazepines), or by altering synaptic GABA levels (vigabatrin and tiagabine). The presently understood mechanism of action of these drugs, however, is based on their activity on normal brains with acutely induced seizures[44]. Changes in the molecular composition and function of the $GABA_A$ receptor associated with chronic epilepsy may alter the activity and efficacy of these agents. For example, our findings indicate that chronically epileptic DGCs may be less sensitive to the effects of certain benzodiazepines because of decreased expression of α1 subunits and increased expression of α4 subunits

(Fig. 3). Understanding epilepsy-associated, regionally distinct changes in GABARs in different hippocampal neurons should permit development of new anti-epileptic drugs that specifically target receptor subtypes predominating on epileptic neurons. Such selectivity may increase efficacy and diminish toxicity by targeting drug action more specifically to epileptic cells. In addition, these findings indicate important new therapies to prevent epilepsy might specifically target regulation of GABAR subunit expression.

Methods

Pilocarpine injections. Pilocarpine injections were done according to published protocols[6,17]. Adult rats, approximately 60–90 days postnatal, were injected first with scopolamine methyl nitrate (1 mg/kg; intraperitoneal) to minimize the peripheral effects of pilocarpine, and then injected 30 min later with pilocarpine (350 mg/kg; intraperitoneal). Pilocarpine injection triggered seizures of long duration (more than 30 min) within 10–30 min after injection. Rats that did not have behavioral seizures (class 3 or higher on scale of Racine[16]) within 1 h of pilocarpine injection were injected with a second dose of pilocarpine (175 mg/kg; intraperitoneal). Diazepam (4 mg/kg; intraperitoneal) in 50% propylene glycol was administered 1 h after the onset of status epilepticus to stop seizure activity, and again 3 h and 5 h after onset of seizure as needed. Control rats were treated identically to pilocarpine-injected rats, except that a subconvulsive dose of pilocarpine (35 mg/kg) was administered. Rats were video-monitored beginning 2 weeks after pilocarpine injection to document at least two spontaneous seizures (class 3 or higher) before being diagnosed as epileptic. To minimize any immediate effects of seizures on $GABA_A$ receptor properties, epileptic rats were assessed by video monitoring to ensure that no seizures occurred in the 24 h before use.

Isolation of neurons. Neurons were isolated using published protocols[6]. Brains were dissected in chilled, oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid solution (201 mM sucrose, 3 mM KCl, 1.25 mM $NaHPO_4$, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM dextrose). Hippocampal slices 450 μm in thickness were cut on a vibratome and incubated for 1 h in an oxygenated medium containing 120 mm NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM glucose and 20 mM PIPES, pH adjusted to 7.0 with NaOH at 32 °C. Slices were enzymatically digested 30–60 min in 3 mg/ml Protease XXIII (Sigma) 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, then cells were mechanically dissociated and plated onto 35-mm culture dishes in HEPES medium (155 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 3 mM $CaCl_2$, 500 nM tetrodotoxin, and 10 mM HEPES-sodium, pH adjusted to 7.4 with NaOH).

Voltage-clamp recordings in isolated neurons. Using the whole-cell variant of the patch-clamp technique, neurons were voltage-clamped at –20 mV using a pipette solution containing 100 mM Trizma phosphate (dibasic), 28 mM Trizma base, 11 mM EGTA, 2 mM MgCl2, 0.5 mM CaCl2, 4 mM Mg^{2+}-ATP and 1 U/ul 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 using an Axopatch 200A amplifier (Axon Instruments, Foster City, California) and filtered at 5 kHz before storage on a PCM device at 44 kHz (Neurodata Instruments, New York, New York). Electrode glass was autoclaved, and all solutions were prepared using 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 using a 14-barrel 'sewer pipe' perfusion system, with a solution change time of 100–200 ms. GABA, clonazepam and zinc were obtained from Sigma, and zolpidem, from RBI (Natick, Massachusetts). CNZ and ZOL were dissolved as stock solutions in DMSO. DMSO at concentrations similar to final dilutions (0.01%) had no effect on cell properties or GABA responses. For statistical analyses, significance was tested using the Student's unpaired *t*- test or the Mann-Whitney Rank Sum test for groups with unequal variance. Curves were fitted using the Marquardt-Levenberg nonlinear least-squares algorithm (ORIGIN; Microcal Software, Northhampton, Massachisetts). Recording duration was limited to 10–15 min as this seemed to facilitate success of subsequent aRNA amplification.

mRNA measurement. Relative expression of GABAR mRNAs within individual isolated DGCs were measured using the technique of single-cell aRNA amplification[14,15] modified as described[45]. After patch-clamp recording, neuronal contents were aspirated into the micropipette. The contents of each microelectrode were expelled into a microcentrifuge tube, and first-strand cDNA synthesis was done using 1mM deoxynucleotide triphosphates (dNTPs), 0.5 unit/μl avian myeloblastosis virus reverse transcriptase (AMVRT; Seikagaku America, Ijamsville, Maryland) and 2 ng/μl oligonucleotide-T7 primer (5'–AAACGACGGCCAGTGAATTGTAATACGACCAC-TATAGGCGCT$_{24}$–3') at 42 °C for 60–90 minutes. After phenol–chloroform extraction and ethanol precipitation with 1 μg *E. coli* tRNA as carrier, double-stranded DNA was made by incubation at 14 °C for 14–18 h with dNTPs (1 mM), T4 DNA polymerase (1 U) and the Klenow fragment of DNA Polymerase I (1U). The single-stranded hairpin loop was removed with S1 nuclease (1U), the ends of the double-stranded template were blunted with T4 DNA polymerase (0.5U) and the Klenow fragment of DNA Polymerase I (0.5U) at 37 °C for 2 h, then cDNA was drop-dialyzed for 4 h against RNAse-free water to remove unincorporated dNTPs. A portion (25%) of the cDNA recovered from the filter was used for synthesis of amplified RNA (aRNA) in 40 mM Tris (pH 7.4), 10 mM NaCl, 10 mM $MgCl_2$ and 5 mM dithiothreitol, with addition of 250 μM ATP, GTP and UTP, 50μM CTP, 15 pmol of α-[^{32}P]-CTP (3000 Ci/mmol; Amersham), 20 U RNAasin and 2000 U T7 RNA polymerase (Epicentre Technologies, Madison, Wisconsin) at 37 °C for 4 h. aRNA was then 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.

Slot-blot preparation and expression profiles. Fourteen $GABA_A$ receptor subunit cDNAs (α1–6, β1–3, γ1–3, δ and ε), GAD65, GAD 67, β-actin (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. $GABA_A$ receptor cDNAs were obtained from the D. Pritchett, except εcDNA, which was provided by E. Kirkness. Identity of all $GABA_A$ receptor subunit cDNAs were confirmed by sequencing. GAD65 and GAD 67 clones were provided by M. Dichter and A. Tobin. All GABAR and GAD cDNAs include the full coding region except α2, α4, γ1 and δ. α2 is a 100-bp fragment from the distal 3' end of the coding region (bp 1206–1306). α4, γ1 and δ cDNAs are each fragments greater than 1 kb including the distal 3' coding region (α4, bp 694–1725; γ1, bp 445–1483; δ, bp 524–1580). β-actin cDNA is a 460-bp fragment (bp1236–1694). GFAP and NF-L clones were provided by J. Eberwine. Each blot was pre-hybridized for 12 h at 42 °C in 5 ml of pre-hybridization 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), then hybridized with the radiolabeled aRNA probe from an individual cell for 48 h at 42 °C. The blots were washed to a final concentration of 0.2× SSC at 42 °C for 30 min, then directly exposed for 2 h to a Phosphor-Image screen (Molecular Dynamics, Sunnyvale, California) with a linear dynamic range over five orders of magnitude. All hybridization signals were well within this dynamic range.

Quantitation and statistical analysis. Intensity of the autoradiographic signal was measured by three-dimensional laser scanning densitometry utilizing Image-Quant software (Molecular Dynamics, Sunnyvale, California). For each blot, the relative abundance of each subunit mRNA was calculated as the hybridization signal for that subunit cDNA divided by the total hybridization signal of all GABAR subunit cDNAs on the blot. This unit was chosen to optimally compare changes in subunit expression relative to one another. All statistically significant differences between control and epileptic neurons calculated this way were also statistically significant when relative expression of subunits were calculated as the hybridization signal for an individual subunit divided by the hybridization

signal for β-actin within that cell. Presence of a subunit mRNA was defined as hybridization signal above background by greater than or equal to 1% of the total hybridization signal for all GABAR subunits on the blot. This value was selected because it represents 1 standard deviation (s.d.) of the estimated variability in background noise (based on differences in hybridization signal for bluescript plasmid cDNA and GFAP cDNA). ANOVA analysis was used for statistical comparison of the mean relative expression for each subunit in each of the three treatment groups (control, latent-period and epileptic), and for subunits that had a statistically significant difference between treatment groups and controls on ANOVA, post-hoc *t*-tests or Mann-Whitney Rank Sum test (for groups with unequal variances) were done between control and individual treatment groups.

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