Research report

Distribution of α1, α4, γ2, and δ subunits of GABA$_A$ receptors in hippocampal granule cells

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Abstract

GABA$_A$ receptors are pentamers composed of subunits derived from the α, β, γ, δ, θ, ε, and π gene families. α1, α4, γ2, and δ subunits are expressed in the dentate gyrus of the hippocampus, but their subcellular distribution has not been described. Hippocampal sections were double-labeled for the α1, α4, γ2, and δ subunits and GAD65 or gephyrin, and their subcellular distribution on dentate granule cells was studied by means of confocal laser scanning microscopy (CLSM). The synaptic versus extrasynaptic localization of these subunits was inferred by quantitative analysis of the frequency of colocalization of various subunits with synaptic markers in high-resolution images. GAD65 immunoreactive clusters colocalized with $26.24 \pm 0.86\%$ of the α1 subunit immunoreactive clusters and $32.35 \pm 1.49\%$ of the γ2 subunit clusters. In contrast, only $1.58 \pm 0.13\%$ of the α4 subunit immunoreactive clusters and $1.92 \pm 0.15\%$ of the δ subunit clusters colocalized with the presynaptic marker GAD65. These findings were confirmed by studying colocalization with immunoreactivity of a postsynaptic marker, gephyrin, which colocalized with $27.61 \pm 0.16\%$ of the α1 subunit immunoreactive clusters and $23.45 \pm 0.32\%$ of the γ2 subunit immunoreactive clusters. In contrast, only $1.90 \pm 0.13\%$ of the α4 subunit immunoreactive clusters and $1.76 \pm 0.10\%$ of the δ subunit clusters colocalized with gephyrin. These studies demonstrate that a subset of α1 and γ2 subunit clusters colocalize with synaptic markers in hippocampal dentate granule cells. Furthermore, all four subunits, α1, α4, γ2, and δ, are present in the extrasynaptic locations.
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1. Introduction

The GABA$_A$ receptor gene family includes seven major classes of subunits with multiple subtypes: six types of α subunit, four types of β subunit, three types of γ subunit, one type of δ, one type each of θ, ε, and π subunit [2,27]. It is known that certain subunits cluster on postsynaptic membranes and mediate fast synaptic inhibition in the brain. For example, electron microscopic examination of GABAergic synapses in cerebellum and hippocampus reveals aggregates of α1, β2/3, and γ2 subunits at GABAergic synapses [16,19]. Conversely, post-embedding immunogold electron microscopic studies of cerebellar granule cells have demonstrated that α6 and δ subunits are expressed in the extrasynaptic membrane [11,19].

In addition to cerebellar granule cells, dentate granule cells in the hippocampus also express the δ subunit mRNA and polypeptide [3,32]. The α6 subunit is not expressed in these neurons and the δ subunit is believed to combine with the α4 subunit to form functional receptors [34]. A recent study of mouse dentate granule cells demonstrated that GABA$_A$ receptors containing the δ subunit are expressed in the perisynaptic region [35]. In addition to the α4 and δ subunits, dentate granule cells express α1 and γ2 subunits.

Previous studies suggest that the $\alpha1$ subunit is expressed at synapses [16]. Recent electrophysiological studies in hippocampal dentate granule cells suggest the existence of synaptic and extrasynaptic $GABA_A$ receptors in these neurons [14,33]. These studies suggested that $GABA_A$ receptors with distinct subunit compositions are present in either synaptic or extrasynaptic locations. Taken together, these studies suggest that in dentate granule cells, the distribution of $\alpha1$, $\gamma2$ subunit-containing receptors is distinct from that of receptors containing $\alpha4$, δ subunits; receptors containing $\alpha1$, $\gamma2$ subunits are expressed at synapses, and $\alpha4$, δ subunits are expressed at extrasynaptic membrane.

While the distribution of $\alpha1$, $\alpha4$, $\gamma2$, and δ subunits in dentate gyrus has been described using light microscopy, few studies have compared the distribution of these subunits using high-resolution confocal laser scanning microscopy (CLSM) [29]. Furthermore, the colocalization of these subunits with GABAergic synaptic markers, GAD65 and gephyrin, in hippocampal dentate granule cells has not been assessed. We used double-label fluorescence immunohistochemistry of the hippocampus visualized with CLSM, to study the distribution of $\alpha1$, $\alpha4$, $\gamma2$, and δ subunits in hippocampal dentate granule cells. High-resolution images of granule cell layer in sections double-labeled for various subunits and synaptic markers GAD65 or gephyrin were analyzed quantitatively.

2. Experimental procedures

2.1. Animal and tissue preparation and immunohistochemistry

Experiments were performed using 11 male adult Sprague–Dawley rats weighing 250–350 g. Six animals were used to study the colocalization of $GABA_A$ receptor subunits with GAD; and five animals were used to study colocalization with gephyrin. Animals were housed at 22 °C, two per cage, on a standard light–dark schedule with free access to food and water. Animals were handled according to NIH Animal Care and Use Guidelines and a protocol approved by the University of Virginia Animal Care and Use Committee.

Animals were injected with a lethal dose of pentobarbitone sodium (220 mg/kg, i.p.) and perfused through the ascending aorta with 50–100 ml 0.9% NaCl, followed by 300 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4); for the gephyrin study, 2% paraformaldehyde was used [28]. The brains were removed and post-fixed in the same fixative for 1 h at 4 °C. After overnight incubation in 30% sucrose in 0.1 M PB for cryoprotection, the brains were frozen by immersion in -70 °C isopentane and sectioned on a cryostat. Coronal sections (40 µm) containing dorsal hippocampus were processed for free floating immunohistochemistry. Sections from each animal were collected in seven vials in sequence, for control, $\alpha1$, $\alpha4$, $\gamma2$, and δ subunits immunohistochemistry, GAD and gephyrin. Following three washes in 0.1 M PB and preincubation with blocking solution containing 5% normal goat serum (Jackson Immunoresearch Laboratories, West Grove, PA) and 0.1% Triton X-100 in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 1 h, tissue sections were incubated with the primary antibodies at 4 °C for 72 h on a shaker. The primary and secondary antibodies were diluted in PBS containing 2% normal goat serum, 0.2% bovine serum albumin (Jackson Immunoresearch Laboratories). Subsequently, the sections were incubated in secondary antibodies of goat anti-rabbit conjugated with Alexa fluor 488 and goat anti-mouse conjugated with Alexa fluor 594 (concentration used: 5µg/ml, Molecular Probes, Eugene, OR) for 1 h on a shaker at room temperature in darkness. Sections were then mounted on slides with Gel/Mount* (Foster City, CA) and air dried, the edge of each coverslip was sealed with clear nail polish, and slides were stored at -20 °C. Controls in which the primary antibody was omitted provided only very weak nonspecific staining.

2.2. Antibodies

The antibody against the $GABA_A$ receptor subunit $\alpha1$ (1–16) was obtained from Alomone labs (Israel). The antibody against $\alpha4$ subunit (1–14) has been characterized previously [1,12,32]. The antibodies against the $\gamma2$ (1–33) and δ (1–44) subunit of the $GABA_A$ receptor have been characterized extensively in the past by immunoprecipitation, Western blot, and immunohistochemistry [11,19–22,32]. The antibodies against $\alpha1$, $\alpha4$, $\gamma2$ and δ were characterized by Western blot. Membrane proteins were collected from whole hippocampus, and Western blot was performed and visualized by chemiluminescence system. Each antibody recognized a single band (Fig. 1). The antibody anti-glutamate decarboxylase (GAD) was obtained from Chemicon (Clone GAD-6, MAB351R, Temecula, CA) and characterized [5,31,37]. Anti-gephyrin was obtained from BD Transduction Laboratories (clone 45, Franklin Lakes, NJ); this antibody has been characterized by Western blot. The different concentrations of primary antibodies were titrated to arrive at the final concentrations (1.5 µg/ml for $\alpha1$; 2.0 µg/ml for $\gamma2$ and GAD; 2.5 µg/ml for gephyrin; 5 µg/ml for $\alpha4$ and δ) used in this study.

2.3. Image acquisition and analysis

Sections were studied on a Nikon PCM2000 confocal microscope system equipped with a Nikon TE-200 inverted epifluorescence microscope (Nikon, Melville, NY) with an Argon/HeNe laser. Images were acquired with an Orca-1 CCD Camera (Hamamatsu Photonics System, Middlesex, NY). Confocal and camera-based image acquisition and processing were driven by SimplePCI software (version 4.0.6, Compix, Cranberry Township, PA). Argon-ion and
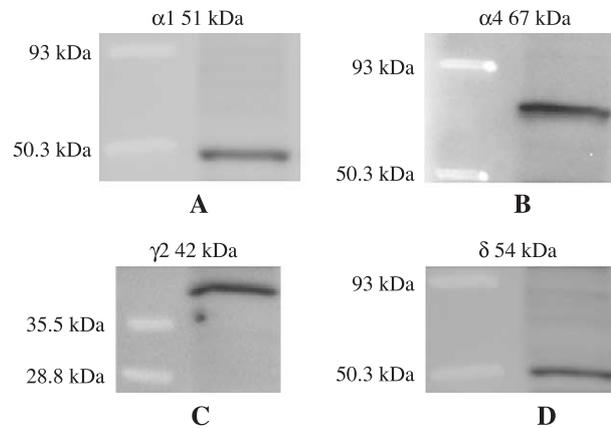
Fig. 1. Characterization of GABA$_A$ receptor subunit α1, γ2, α4, and δ by Western blot. (A) A single 51 kDa band of α1. (B) A 67 kDa band of α4. (C) A 42 kDa band of γ2 was seen. (D) A 54 kDa δ band was shown.

He/Ne lasers were used to visualize the fluorochromes, Alexa Fluor 488 and Alexa Fluor 594, respectively. Double-labeled sections were scanned by the simultaneous application of both excitation wavelengths. The intensity of each laser was optimized for the sections of each animal and each combination of antibodies. The parameters were kept constant for each animal. Controls included dual-channel recording of sections without antigen labeling and single antigen labeling to detect non-specific staining and bleed-through phenomena. All CLSM was performed at the W.M. Keck Center for Cellular Imaging at the University of Virginia.

Each hippocampal section was examined with a low-magnification objective lens (40×) in order to locate the dentate gyrus and the granule cell layer. In preliminary experiments, dentate gyrus sections were labeled with antibody against calbindin to identify granule cells; previous studies have shown that calbindin-like immunoreactivity was present in all dentate granule cells [30]. Sections were visualized using a Nikon plan Apo 40×/1.0 NA oil immersion objective; the molecular layer, granule cell layer, and hilar border were included in the images. For quantitative analysis of GABA$_A$ receptor immunoreactive clusters and their colocalization with GAD and gephyrin, high resolution digitized images (50 nm/pixel) were acquired using a Nikon plan Apo 100×/1.40 NA oil immersion objective, and an electronic zoom factor 2.3. All settings were kept constant throughout the analysis to yield unbiased measurements for each set of comparisons.

The immunoreactivity of GABA$_A$ receptor subunits and synaptic markers in dorsal hippocampal sections were analyzed with MetaMorph 6.03 software (Universal Imaging, PA, USA). The same threshold was applied to all images acquired from the sections of one brain in each experiment. Most of these antigens were revealed as individual clusters, which were considered as individual objects in binary images. The degree of colocalization was evaluated by comparing binary images for two antigens with a "logical and" function. An aggregation of 20 to 1000

pixels (0.05–2.5 μm^2) of intensity at least twice the background intensity was considered a cluster. Preliminary analysis of the images in this study also demonstrated that clusters smaller than 0.05 μm^2 rarely colocalized with synaptic markers. Data were analyzed using Prism 3.0 software (GraphPad Software, San Diego, CA). Unless specified otherwise, all values are reported as mean±S.E.M. The digital images selected for publication were processed in PhotoShop 6.0 (Adobe, San Jose, CA). For each antibody, a contrast/brightness setting was selected that yielded a high-resolution image for both bright and dim sections without exceeding a maximal pixel intensity of 255.

3. Results

3.1. Distribution of α1 and γ2 subunits

The distribution of GABA$_A$ receptor α1 subunit immunoreactivity in hippocampal dentate gyrus of adult rats was studied using CLSM. Interneurons at the hilar margin of the dentate gyrus were more intensely stained by anti-α1 subunit antibody than neurons in the granule cell layer (Fig. 2A, arrowhead). Previous studies have demonstrated that a subset of GABAergic interneurons expresses the α1 subunit at a higher level than the principal neurons [8,10]. Only moderately intense immunoreactivity was found in the granule cell layer, which consisted of punctate staining outlining the soma of granule cells, and dendrites passing through the granule cell layer (Fig. 2A). The molecular layer was more intensely stained than the granule cell layer, with distinct clusters of α1 subunit immunoreactivity extending along dendrites of granule cells (Fig. 2A).

The distribution of the γ2 subunit immunoreactivity was similar to that of the α1 subunit (Fig. 2B). At the hilar border of the granule cell layer, the γ2 subunit immunoreactivity was present in somata of interneurons (Fig. 2B, arrowhead). The granule cell layer was stained moderately
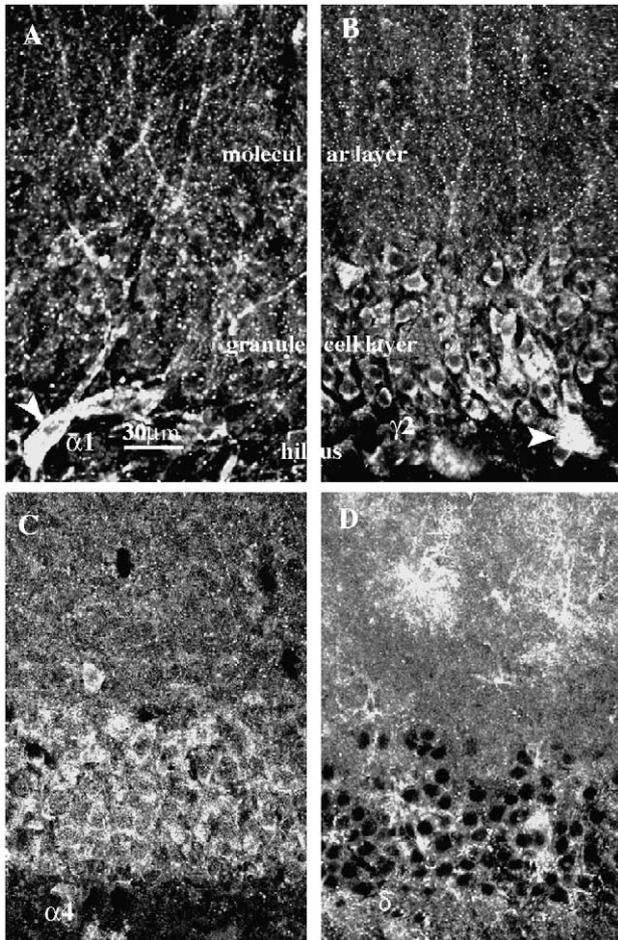
Fig. 2. Distribution of GABA$_A$ receptor α1, γ2, α4, and δ subunit immunoreactivity in hippocampal dentate granule cells acquired by CLSM, 40× objective lens, scale bar = 30 μm (shown in A). Sections were labeled with GABA$_A$ subunit isoform-specific antibodies and immunoreactivity was assessed in the form of clusters that outlined dentate granule cell soma. The immunoreactivity of α1 (A) and γ2 (B) subunits was similar, the somata and dendrites of interneurons near the hilar border were strongly labeled (A, B; arrowhead); fewer clusters were found in granule cell somata, molecular, and granule cell layers. The immunoreactivity of α4 (C) and δ (D) subunits was more diffuse and clusters were less obvious. Some interneurons in the molecular layer stained intensely for the δ subunit. Intense staining of the δ subunit was found in the molecular layer.

with γ2 subunit immunoreactivity distributed circumferentially around granule cells; clusters of γ2 subunit immunoreactivity were present along the dendrites in the molecular layer, too (Fig. 2B).

3.2. Distribution of α4 and δ subunit

The distribution of α4 subunit immunoreactivity in the dentate gyrus was quite distinct from that of the α1 and γ2 subunits (Fig. 2C). It was distributed diffusely over the dentate granule cell layer and the molecular layer. The immunoreactivity was more intense in the granule cell layer and the inner molecular layer, and less intense in the outer molecular layer. Neither distinct clusters of immunoreactiv-

ity nor outlines of cell body dendrites of granule cells were obvious at this magnification.

δ subunit immunoreactivity appeared to outline dentate granule cell bodies (Fig. 2D). Some clusters of the δ subunit were present but these were far sparser than that seen for the α1 and γ2 subunits. In contrast to the α4 subunit, the δ subunit immunoreactivity was more intense in the molecular layer than in the granule cell layer. Some interneurons in the molecular layer were intensely immunoreactive for the δ subunit but not for the α4 subunit. A detailed characterization of δ subunit expression in the interneurons was not performed.

3.3. Distribution of GAD65 and gephyrin

We wished to describe the colocalization of GABA$_A$ receptor subunit clusters with the synaptic markers, GAD65 and gephyrin. Hippocampal sections were stained for GAD65, the synthetic enzyme for GABA, which is known to be concentrated in the synaptic terminals of GABAergic neurons [4,23,26]. Punctate GAD65 immunostaining formed a dense network in the dentate granule cell layer and inner molecular layer, and less staining in the outer molecular layer of the dentate gyrus.

We tested whether gephyrin, which is present at GABAergic synapses [6,13,25], was also present in the dentate granule cell layer. Hippocampal sections were stained for gephyrin and the dentate gyrus was studied by CLSM. The most intense gephyrin staining occurred in the granule cell layer and in the cell bodies of interneurons in the hilus (Fig. 3B). Less intense staining was present in the molecular layer. In the cell soma of the dentate granule cells,
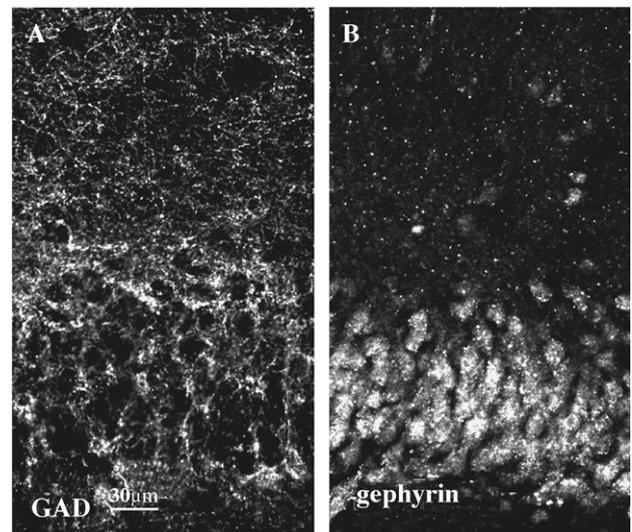

Fig. 3. Distribution of GAD65 (A) and gephyrin (B) immunoreactivity in the dentate gyrus, scale bar = 30 μm. There was intense punctate GAD65 staining in the granule cell layer and in the inner molecular layer, which formed a net of clusters (A). In the somata of dentate granule cells, there was diffuse low intensity gephyrin immunoreactivity and more intense punctate staining (B).

there was diffuse low-intensity immunostaining and more intense punctate staining for gephyrin. This suggested that both pre- and postsynaptic markers of GABAergic synapses were present in the granule cell layer and molecular layer.

3.4. Colocalization of α1, γ2 subunit clusters with synaptic markers in high-resolution images

High magnification images of granule cells in hippocampal sections stained for α1 subunit immunoreactivity were examined (Fig. 4A,G). Clusters of α1 subunit immunoreactivity appeared to outline the cell somata of

dentate granule cells and were of various sizes (0.05 to 1.0 μm^2). There were more small clusters than large clusters. Similar to the α1 subunit clusters, the γ2 subunit clusters were distributed in the periphery of granule cell soma and both small and large clusters were present (Fig. 4D,J). Similar to the α1 subunit immunoreactive clusters, small γ2 subunit clusters were more common than larger clusters.

In the same sections, the distribution of GAD65 clusters was studied. Clusters of GAD65 immunoreactivity outlined the somata of granule cells. The GAD65 clusters were larger in size and fewer than the α1 and γ2 subunit clusters. The median size of GAD65 clusters was 0.15 μm^2 and the mean
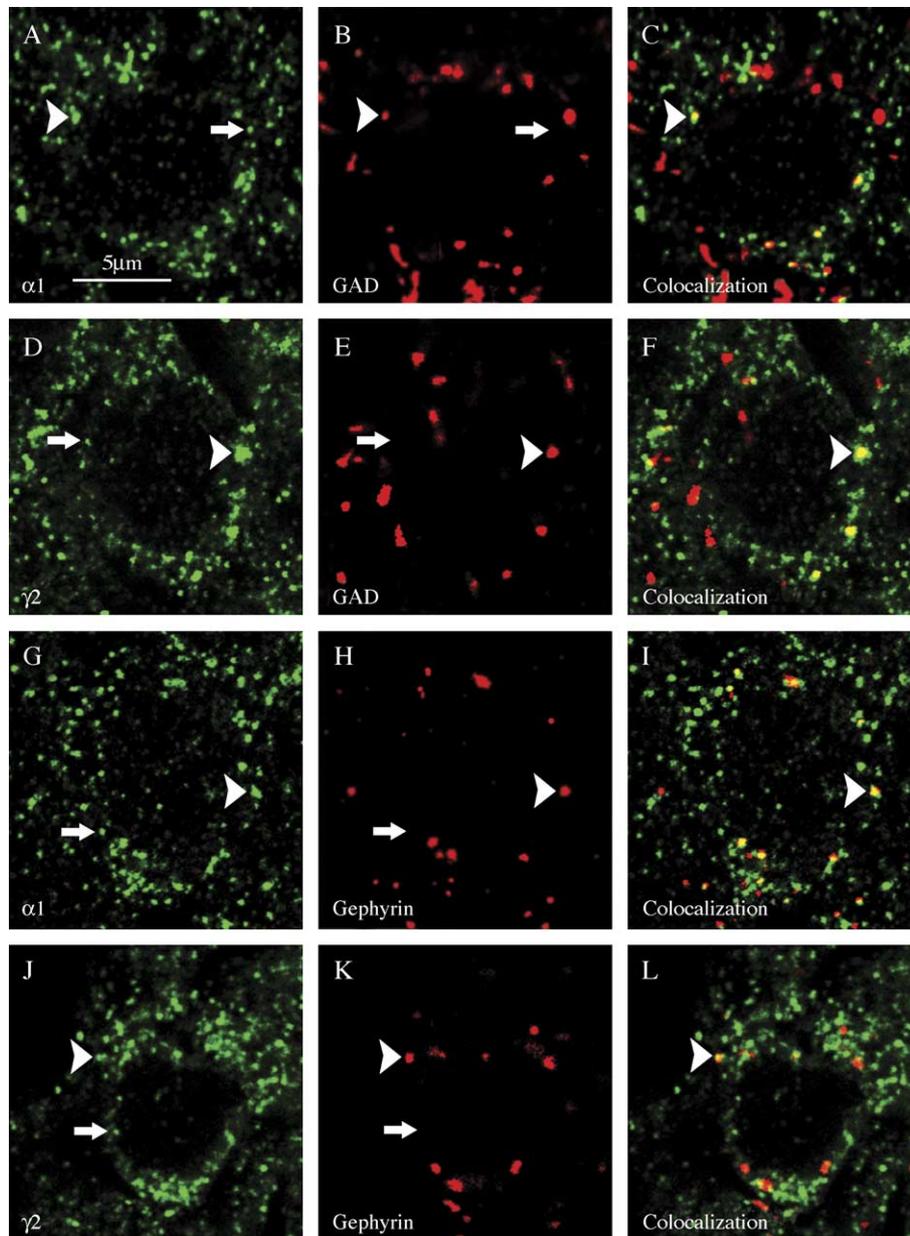

Fig. 4. Large clusters of α1 or γ2 GABA$_A$ receptor subunits frequently colocalized with synaptic makers (arrowhead), whereas most small clusters did not (arrow). Hippocampal dentate granule cells were double-labeled with the presynaptic marker GAD (B, E) or the postsynaptic marker gephyrin (H, K) and GABA$_A$ subunit isoform-specific antibodies to α1 (A, G) or γ2 (D, J). Merged images of the markers and subunits are shown in C, F, I, and L. GABA$_A$ subunits were visualized by secondary antibody conjugated with Alexa Fluor 488 (green) and synaptic markers with Alexa Fluor 594 (red). Scale bar = 5 μm.

size was 0.212 μm^2. In high magnification images, the distribution of gephyrin immunoreactivity in dentate granule cell somata was similar to that of GAD65. Gephyrin formed large clusters that outlined somata of dentate granule cells. Previous studies have described similar punctate distribution of gephyrin in dentate granule cells [28].

We examined colocalization of the immunoreactivity of GAD65 with clusters of $\alpha1$ and $\gamma2$ subunit immunoreactivity (Fig. 5A). The degree of colocalization of GABA$_A$ receptor $\alpha1$ subunit clusters was $26.24 \pm 0.86\%$ ($n=6$). Similar to the $\alpha1$ subunit, that of $\gamma2$ subunit clusters colocalized with GAD65was $32.35 \pm 1.49\%$ ($n=8$). The majority of GABA$_A$ receptor subunits did not colocalize with the presynaptic marker GAD65 in hippocampal dentate granule cells of the adult rat. To further confirm the finding, double-labeling of gephyrin and the GABA$_A$ receptor subunits $\alpha1$ or $\gamma2$ was studied (Fig. 5B). The degree of colocalization of the $\alpha1$ subunit clusters with gephyrin was $27.61 \pm 1.64\%$ ($n=5$) while that of $\gamma2$ subunit clusters was $23.45 \pm 0.32\%$ ($n=8$).

3.5. Cluster size and colocalization with synaptic markers

We examined whether GABA$_A$ receptor clusters of different sizes had different associations with synaptic markers by analyzing the correlation of subunit cluster size with the degree of colocalization with GAD65. Large $\alpha1$ subunit clusters commonly colocalized with GAD (arrowhead, Fig. 4A–C), whereas small clusters did not (arrow, Fig. 4A–C). There was a strong correlation between the degree of colocalization and $\alpha1$ subunit cluster size (nonparametric Spearman correlation coefficient $r=0.92$, $p<0.0001$). Similar to the $\alpha1$ subunit, most of the large $\gamma2$ subunit clusters colocalized with GAD (arrowhead in Fig. 4D–F), whereas small clusters did so rarely (arrow, Fig. 4D–F). The fraction of clusters colocalizing with GAD increased with the cluster size (nonparametric Spearman correlation coefficient $r=0.88$, $p<0.0001$).

The preferential colocalization of large clusters of $\alpha1$ and $\gamma2$ subunit immunoreactivity was confirmed by analyzing colocalization with gephyrin. Gephyrin clusters colocalized with large $\alpha1$ subunit clusters commonly (arrowhead, Fig. 4G–I), but did not colocalize with small ones (arrow, Fig. 4G–I). Similar to $\alpha1$ subunit colocalization with gephyrin, large $\gamma2$ subunit clusters colocalized commonly with gephyrin (arrowhead in Fig. 4J–L), but small ones did so rarely (arrow, Fig. 4J–L). There was a linear correlation between the degree of colocalization and $\gamma2$ subunit cluster size (nonparametric Spearman correlation coefficient $r=0.74$, $p<0.001$). The mean area of $\alpha1$ subunit clusters
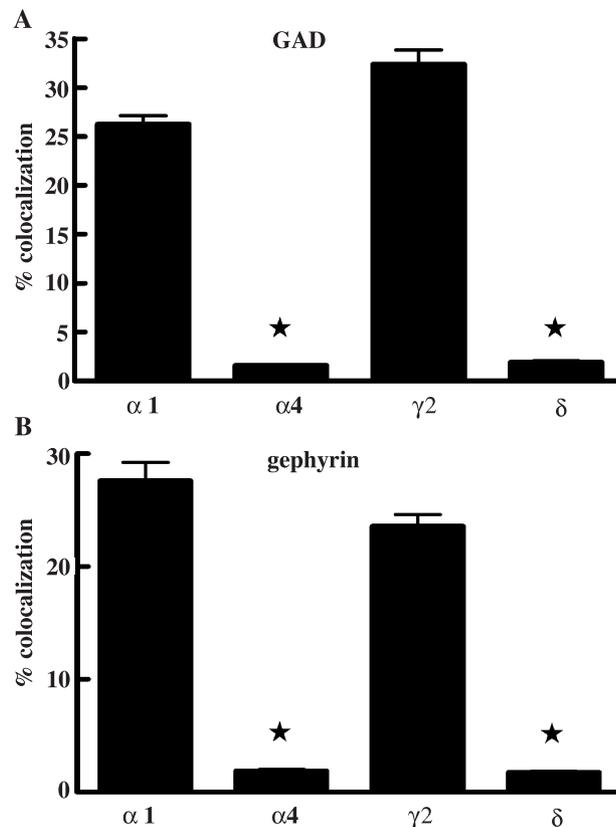

Fig. 5. Percent colocalizaton of different GABA$_A$ receptor subunits with the synaptic markers GAD65 and gephyrin is demonstrated. The percent fraction colocalization of GABA$_A$ receptor $\alpha1$ or $\gamma2$ subunits with GAD65 was significantly higher than those of $\alpha4$ or δ subunits, respectively (*$p<0.0001$, A). The percent fraction of colocalization of GABA$_A$ receptor $\alpha1$ or $\gamma2$ with gephyrin are significantly higher than those of $\alpha4$ or δ subunits, respectively (*$p<0.0001$, B).

that colocalized with GAD65 (0.122±0.010 μm^2) was similar to the size of α1 subunit clusters that colocalized with gephyrin (0.133±0.008 μm^2, $p=0.3876$, grouped t-test). Similarly, the mean area of γ2 subunit clusters that colocalized with GAD65 (0.124±0.006 μm^2) was similar to the size of γ2 subunit clusters that colocalized with gephyrin (0.128±0.008 μm^2, $p=0.694$, t-test). In addition, all four measures of the cluster size that colocalized with the synaptic marker were compared using a two-way ANOVA test; and there was no significant difference between any two pairs of values. While the absence statistical difference does not prove similarly and the possibility of type II error remains, these comparisons suggested that large clusters of

α1 and γ2 subunit immunoreactivity that colocalized with synaptic markers were a distinct subset of all clusters.

3.6. Relationship of α4 and δ subunit immunoreactivity to synaptic markers

The distribution of α4 subunit clusters and GAD65 clusters in the granule cell layer was examined from double-labeled hippocampal sections. The α4 clusters were distributed circumferentially around the granule cell (Fig. 6A–C). The colocalization of α4 subunit clusters and GAD65 clusters was analyzed. Using the same criteria used to define colocalization of α1 and γ2 subunits with synaptic
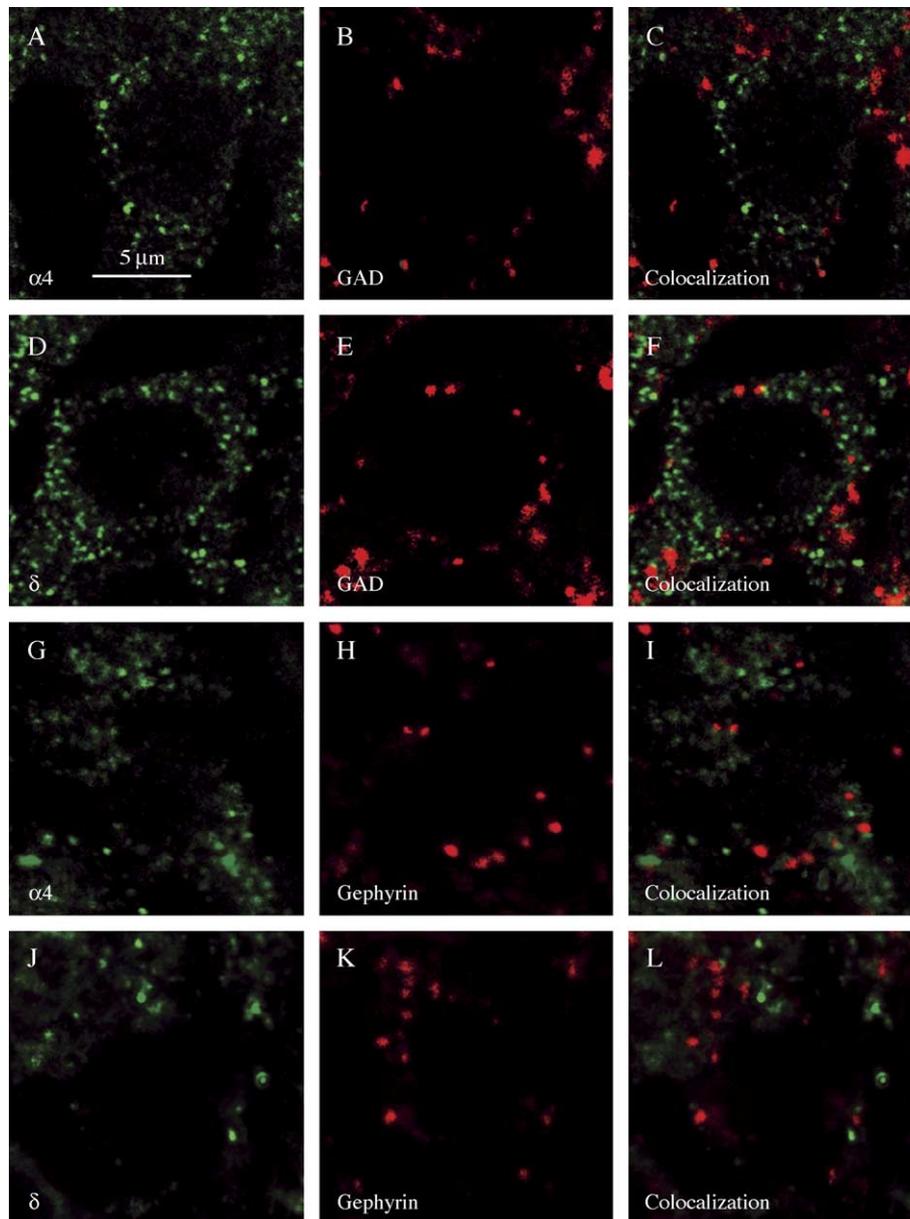

Fig. 6. Clusters of α4 and δ GABA$_A$ receptor subunits rarely colocalized with synaptic makers. Hippocampal dentate granule cells were double-labeled with the presynaptic marker GAD (B, E) or the postsynaptic marker gephyrin (H, K) and GABA$_A$ subunit isoform-specific antibodies to α4 (A, G) or δ (D, J). GABA$_A$ subunits were visualized by secondary antibody conjugated with Alexa Fluor 488 (green) and synaptic markers with Alexa Fluor 594 (red). Merged images of synaptic markers and subunits are shown in C, F, I, and L. Scale bar=5 μm.

markers, we found that $1.58 \pm 0.13\%$ ($n=5$) of all $\alpha 4$ subunit clusters colocalized with GAD65 clusters (Fig. 5A), which was significantly lower than the degree of colocalization of $\alpha 1$ subunit clusters ($n=6$), $26.24 \pm 0.86\%$ (grouped t-test, $p<0.0001$).

The distribution of δ subunit clusters and GAD65 clusters in the granule cell layer was examined from double-labeled hippocampal sections. The δ subunit clusters were distributed circumferentially around the granule cell. The colocalization of δ subunit and GAD65 clusters was analyzed (Fig. 6D–F). Only $1.92 \pm 0.15\%$ ($n=7$) of δ subunit clusters colocalized with GAD65 clusters (Fig. 5A), which was significantly lower than the degree of colocalization of $\gamma 2$ clusters ($n=8$), $32.35 \pm 1.49\%$ (t-test, $p<0.0001$).

These findings suggested that $\alpha 4$ and δ subunit clusters rarely colocalized with the synaptic marker GAD65. This was confirmed using the synaptic marker gephyrin. As demonstrated in Fig. 6G–I, $\alpha 4$ subunit clusters rarely colocalized with gephyrin clusters. The fraction of $\alpha 4$ subunit clusters that colocalized with gephyrin was $1.90 \pm 0.13\%$ ($n=7$) (Fig. 5B), which was significantly less than the fraction of $\alpha 1$ clusters ($n=5$) colocalized with gephyrin $27.61 \pm 0.16\%$ (t-test, $p<0.0001$). Similar to the $\alpha 4$ subunit clusters, δ subunit clusters rarely colocalized with gephyrin clusters (Fig. 6J–L). The fraction of δ subunit clusters colocalized with gephyrin was $1.76 \pm 0.10\%$ ($n=8$) (Fig. 5B). In contrast, $23.45 \pm 0.32\%$ ($n=8$) of $\gamma 2$ subunit clusters colocalized with gephyrin. The difference was significant (t-test, $p<0.0001$).

4. Discussion

The distribution of GABA$_A$ receptor $\alpha 1$, $\alpha 4$, $\gamma 2$, and δ subunits and synaptic markers GAD65 and gephyrin in rat hippocampal dentate gyrus was described using high-resolution CLSM. Quantitative analysis of high-resolution images of the granule cell layer in sections double-labeled for various subunits and the synaptic markers revealed that a distinct fraction of $\alpha 1$ and $\gamma 2$ immunoreactivity colocalized with synaptic markers, which was in the form of large clusters. In contrast, the $\alpha 4$ and δ subunit immunoreactivity rarely colocalized with the synaptic markers. This study further suggested that $\alpha 1$ and $\gamma 2$ subunits are also expressed in extrasynaptic locations in hippocampal dentate granule cells.

4.1. Subcellular distribution of α1 and γ2 subunits

The cellular distribution of the $\alpha 1$ subunit in the dentate gyrus was similar to that described in detail in the past [8,10,16,29,32]. The distribution of the $\gamma 2$ subunit was similar to that of the $\alpha 1$ subunit and it is possible that the two antibodies detected similar sets of GABA$_A$ receptors on granule cells. The $\gamma 2$ subunit commonly co-assembles with the $\alpha 1$ subunit to form the BZ type I benzodiazepine site in

GABA$_A$ receptors [36]. The $\alpha 1$ subunit has been localized to GABAergic synapses in dentate granule cells [16] and the $\gamma 2$ subunit is essential for clustering of GABA$_A$ receptors at synapses [9]. While the distribution of $\alpha 1$ and $\gamma 2$ subunits was similar, the current study did not determine whether these subunits co-assemble into functional receptors.

Quantitative analysis revealed that large clusters of $\alpha 1$ and $\gamma 2$ subunits detected by CLSM are present at synapses. The mean area of $\alpha 1$ clusters that colocalized with the presynaptic marker GAD65 was similar to that colocalized with postsynaptic marker gephyrin, and similar to the mean area of $\gamma 2$ subunit clusters that colocalized with GAD65 and gephyrin, ranging from 0.12 to 0.13 μm^2 in size. The size of synaptic clusters of GABA$_A$ receptors determined by the current study was within the range of that determined by electron microscopy. Post-embedding immunogold electron microscopic studies of cerebellar interneurons suggested that the area of GABAergic synapses is 0.152 μm^2 and the size distribution is skewed [17]. In hippocampal dentate granule cells, quantitative immunogold studies estimated the area of GABAergic synapses on the cell soma to be 0.044 μm^2, and in the axon initial segment, 0.042 μm^2 [18]. Cluster size is not the only criterion for making the distinction between synaptic and extrasynaptic receptors because not all large clusters of $\alpha 1$ and $\gamma 2$ subunits colocalized with the synaptic markers, and $\alpha 4$ and δ subunit immunoreactivity did not colocalize with the synaptic markers, regardless of the cluster size. These studies suggest that the most reliable criterion for detecting synaptic clusters of GABA$_A$ receptors when using CLSM is to determine colocalization with GAD65 and gephyrin clusters. Recent studies using double-label immunohistochemistry combined with CLSM have also demonstrated that large clusters of $\alpha 1$ and $\gamma 2$-subunit colocalize with synaptic markers. In one study, gephyrin was found to colocalize extensively with GABA$_A$ receptor clusters larger than 0.1 μm^2 [25]. These results are similar to those of our study, which found extensive colocalization of gephyrin with large GABA$_A$ receptor clusters. While this and other studies have found colocalization of gephyrin with GABA$_A$ receptors, this colocalization does not arise from direct binding of two molecules. While gephyrin is involved in GABA$_A$ receptor clustering it does not directly bind to the receptor protein [24].

Larger clusters may have colocalized more often with synaptic markers than smaller clusters because of their size. However, frequency of occurrence of specific clusters would also impact on likelihood of random colocalization. In fact, small clusters were much more common than larger clusters. We did not construct a mathematical model to test the impact of cluster size and frequency of colocalization, qualitatively it is possible that these two effects cancel each other. In addition, low degree of colocalization of $\alpha 4$ and δ subunits with synaptic markers suggests the colocalization event is not purely random.

A substantial fraction of $\alpha 1$ and $\gamma 2$ subunit clusters did not colocalize with synaptic markers. These aggregates of

immunoreacitvity were smaller than those that colocalized with synaptic markers. Previous electron microscopic studies of cat and rat dentate granule cells have reported extrasynaptic GABA$_A$ receptors containing α1 and β2/3 subunits [16]. A quantitative immunogold study compared the relative density of α1 and β2/3 subunits in synaptic and extrasynaptic membrane of cerebellar granule cells [15] and found that these subunits are concentrated in synapses but are present also in the extrasynaptic membrane. Studies using double-label immunohistochemistry combined with CLSM have also demonstrated that many clusters of α1 and γ2 subunits do not colocalize with synaptic markers. In one study [7], colocalization of α2 or γ2 subunit clusters with gephyrin was evaluated in the inferior olivary nucleus; 22% of α2 subunit clusters and 36% of γ2 subunit clusters did not colocalize with gephyrin clusters.

4.2. Distribution of α4 and δ subunits

Quantitative evaluation of high-resolution images revealed that α4 and δ immunoreactivity rarely colocalized with the synaptic markers GAD65 and gephyrin. This colocalization degree was far lower than that of α1 and γ2 subunits suggesting that α4 and δ subunit containing receptors were not present at synapses. The colocalization of the α4 or δ subunit with synaptic markers has not been investigated by means of CLSM in the past but a recent ultrastructural study demonstrated that immunogold-labeled δ subunits were located in the perisynaptic region in the molecular layer of dentate granule cells [35]. Furthermore, electrophysiological studies demonstrate that the properties of tonic inhibition are similar to the properties of GABA$_A$ receptors containing α4 and δ subunits. Other ultrastructural studies in cerebellar granule cells have demonstrated that the δ subunit is located on the extrasynaptic membrane.

GABAergic synapses have been studied by means of electron microscopy in the past but only a limited number of GABA$_A$ receptor subunits, synapses, and cell types have been studied with this technique. Recently, CLSM has been used to study the spatial distribution of GABA$_A$ receptors and gephyrin [25,29]. CLSM offers the advantage that a large number of neurons can be sampled using immunohistochemical techniques. However, the use of CLSM also has certain limitations. The resolution of confocal microscopy is insufficient to distinguish between the receptors expressed on the cell membrane from those expressed in the intracellular compartments. In addition, hippocampal sections were treated with a detergent to improve antibody penetration, further preventing the separation of surface receptors from intracellular receptors.

In summary, these studies demonstrate that a subset of GABA$_A$ receptor α1 and γ2 subunit clusters colocalize with synaptic markers in hippocampal dentate granule cells. Furthermore, all four subunits, α1, α4, γ2, and δ, are present in the extrasynaptic locations. CLSM combined with double label immunohistochemistry offers a method for localizing neurotransmitter receptors to synapses.

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