Postsynaptic clustering of γ-aminobutyric acid type A receptors by the γ3 subunit *in vivo*

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Synaptic localization of γ-aminobutyric acid type A ($GABA_A$) receptors is a prerequisite for synaptic inhibitory function, but the mechanism by which different receptor subtypes are localized to postsynaptic sites is poorly understood. The γ2 subunit and the postsynaptic clustering protein gephyrin are required for synaptic localization and function of major $GABA_A$ receptor subtypes. We now show that transgenic overexpression of the γ3 subunit in γ2 subunit-deficient mice restores benzodiazepine binding sites, benzodiazepine-modulated whole cell currents, and postsynaptic miniature currents, suggesting the formation of functional, postsynaptic receptors. Moreover, the γ3 subunit can substitute for γ2 in the formation of $GABA_A$ receptors that are synaptically clustered and colocalized with gephyrin *in vivo*. These clusters were formed even in brain regions devoid of endogenous γ3 subunit, indicating that the factors present for clustering of γ2 subunit-containing receptors are sufficient to cluster γ3 subunit-containing receptors. The $GABA_A$ receptor and gephyrin-clustering properties of the ectopic γ3 subunit were also observed for the endogenous γ3 subunit, but only in the absence of the γ2 subunit, suggesting that the γ3 subunit is at a competitive disadvantage with the γ2 subunit for clustering of postsynaptic $GABA_A$ receptors in wild-type mice.

Nervous system signaling depends on the coordinated function of a variety of cell surface ion channels and receptors that are typically concentrated at specific subcellular domains, such as postsynaptic sites, axon terminals, or nodes of Ranvier (1, 2). Clustering, postsynaptic localization, and synapse-specific targeting of receptors are prerequisites for synaptic function and appear to characterize essential steps in synapse formation during neuronal development. In addition, modulation of synaptic clustering and localization of neurotransmitter receptors provide a potential mechanism for rapid adaptation of synaptic efficacy in mature neurons. This is particularly evident for fast neurotransmission mediated by receptors of the ligand-gated ion channel family.

Inhibitory neurotransmission in the brain is mediated mainly by γ-aminobutyric acid (GABA) acting at heteropentameric GABA type A ($GABA_A$) receptor chloride channels. $GABA_A$ receptors comprise an ill-defined number of structurally and pharmacologically distinct receptor subtypes, which emerge by differential expression and assembly of a large number of subunits classified according to their primary structure as α, β, γ, δ, ε, π, or ρ subunits (3–5). Most $GABA_A$ receptors are composed of variant α and β subunits together with the γ2 subunit and are concentrated at postsynaptic sites. Specific receptor subtypes might be targeted to different types of synapses in distinct cellular domains. For example, in hippocampal pyramidal cells, $GABA_A$ receptors containing the α1 subunit are found in all types of GABAergic synapses (on the soma, proximal, and distal dendrites, and the axon initial segment), whereas receptors containing the α2 subunit are selectively localized in the axon initial segment, which is innervated by axo-axonic interneurons (6, 7). Although the γ2 subunit is largely dispensable for assembly and surface expression of $GABA_A$ receptors *in vivo*, it is required for normal channel conductance, postsynaptic clustering, and postsynaptic function of major $GABA_A$ receptor subtypes (8–10). Moreover, the γ2 subunit is essential for proper aggregation of the clustering molecule gephyrin at sites apposed to GABAergic terminals (9).

The γ2 and γ3 subunits are 64% identical at the level of their primary structure. They form recombinant receptors with similar unitary channel conductance (11), and both can contribute to the benzodiazepine (BZ) binding site (11, 12). However, the γ3 subunit-containing $GABA_A$ receptors are characterized by a very low affinity for the BZ agonist zolpidem (13–15). Because of the low abundance of the γ3 subunit in the brain (11, 13, 14), a functional analysis of the corresponding receptors *in vivo* has been quite limited. In particular, it has remained elusive whether the γ3 subunit contributes to receptors at postsynaptic sites. To determine whether the γ3 subunit can substitute for the γ2 subunit in the formation of functional postsynaptic $GABA_A$ receptors *in vivo*, a transgenic mouse strain overexpressing the γ3 subunit throughout the brain has been established in a γ2 subunit-deficient background. The results show that ectopically overexpressed γ3 subunit partially restores the formation of BZ-modulated $GABA_A$ receptors *in vivo* and promotes the synaptic localization of gephyrin and $GABA_A$ receptors that contribute to miniature postsynaptic currents, similarly to the γ2 subunit.

Methods

Generation of γ3 Transgenic Mice. The murine γ3 cDNA (16), including the entire γ3 subunit coding region, 23 bp of 5′ flanking sequence, and 115 bp of 3′ flanking sequence, was reconstructed from two partial cDNAs generously provided by N. Walter and J. M. Sikela (University of Colorado, Denver). The cDNA was cloned into pBAP (17) in between 5′ genomic sequences from the human β-actin gene and a 3′ poly(A) signal from simian virus 40. The expression cassette was separated from vector sequences and injected into the pronuclei of fertilized eggs of B6D2F1 hybrid donor mice, and the eggs were transferred into foster mothers as described (18). Transgenic founder mice were identified by PCR analyses of tail biopsies and expanded into lines by crossing with mice that are heterozygous for the $GABA_A$ receptor γ2 subunit gene ($\gamma2^{0/+}$) (8) on a 129/Ola × C57BL/6 background. The offspring were screened for expression of the transgene by Western blotting of brain membranes. One transgenic line was found to express substantial amounts of the γ3 subunit and was subsequently crossed with $\gamma2^{0/+}$ mice. Offspring that were hemizygous for the γ3 transgene and heterozygous for the null allele of the γ2 subunit ($\gamma3^{tg}$/

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Abbreviations: GABA, γ-aminobutyric acid; $GABA_A$, GABA type A; BZ, benzodiazepine; IR, immunoreactivity; mIPSCs, miniature inhibitory postsynaptic currents; P*n*, postnatal day *n*; RTN, reticular thalamic nucleus; wt, wild type.

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$\gamma 2^{0/+}$) were crossed with $\gamma 2^{0/+}$ mice to obtain $\gamma 3^{tg}/\gamma 2^{0/0}$ homozygous mutants. This breeding scheme maintains hemizygosity at the transgene locus, thus eliminating possible effects due to insertion mutagenesis. The presence of the transgene and the genotype at the $\gamma 2$ locus were determined by PCR analyses of tail biopsies or embryonic tissue, using the primers 5′-CCGGC CCGGC TTCCT TTGTC C-3′ ($\gamma 3^{tg}$ upper primer) and 5′-TGCCT AATGT TGTTC TTGCT GGTGT CG-3′ (lower primer) and those described (8) for the $\gamma 2$ gene locus.

Immunoprecipitation. Affinity-purified $\alpha 1$ subunit-specific Abs were coupled to protein A-agarose beads and incubated overnight at 4°C with deoxycholate membrane extracts prepared from three postnatal day 10–13 (P10–13) brains per genotype as described (13). Following extensive washing with 10 mM Tris·HCl (pH 7.5)/150 mM NaCl/1 mM EDTA/0.1 mM PMSF/0.2% Triton X-100, the immunoprecipitates were subjected to Western blot analysis with antibodies against the $\alpha 1$, $\beta 2,3$, $\gamma 2$, and $\gamma 3$ subunits (13).

Autoradiography. Cryostat brain sections (12 μm) of P14 mice were incubated with 6 nM [^{3}H]flumazenil in the absence or presence of 10 μM zolpidem as described (19). Quantification in the hippocampal region was performed by densitometry in three mice per genotype with an MCID imaging system (Imaging Research, St. Catherine's, ON, Canada). For each mouse, data were averaged from nine measurements of the CA1 region in three parasagittal sections.

Immunohistochemistry. Cryostat brain sections (12 μm) of P14 mice were processed for double-immunofluorescence staining as described (7, 9). $GABA_A$ receptor subunit expression was investigated with polyclonal antisera raised in guinea pig ($\alpha 1$, $\alpha 2$, $\alpha 3$, $\gamma 3$) or rabbit ($\gamma 3$) (13, 20). Gephyrin was detected with the mAb 7a (Connex, Martinsried, Germany) (21). Following overnight incubation with a mixture of two primary Abs, the sections were incubated for 30 min in the corresponding secondary Abs coupled to Cy3 (Jackson ImmunoResearch) or Oregon Green (Molecular Probes), washed, and coverslipped with buffered glycerol.

Data Analysis. Fluorescent images were captured with a confocal laser scanning microscope (TCS 4D; Leica, Deerfield, IL). For quantification of the number of synaptic receptor clusters, digital images were analyzed with the MCID software using a threshold segmentation algorithm for detection of clusters as described (9). For each mouse (n = 3–4 per genotype), measurements were done in triplicate from three distinct sections. Results are given as mean ± SD. The size of clusters was averaged from individual measurements (n = 500–1300 per genotype).

Electrophysiological Recordings and Neuronal Cultures. Miniature inhibitory postsynaptic currents (mIPSCs) and currents evoked by pulse application of GABA were recorded with the whole-cell patch-clamp method applied to neocortical neurons from $\gamma 2^{+/+}$, $\gamma 2^{0/0}$, and $\gamma 3^{tg}/\gamma 2^{0/0}$ embryos cultured for 2–3 wk, as previously described (9).

Results

A transgenic mouse line overexpressing the $\gamma 3$ subunit under control of the human β-actin promoter was established. In the adult brain, the endogenous $\gamma 3$ subunit gene is expressed primarily in the olfactory bulb, striatum, and a subset of thalamic nuclei, but is absent, for example, in the cerebellum (11). Hence, to test for ectopic expression of the $\gamma 3$ subunit in transgenic mice, Western blots were performed using cerebellar and forebrain membrane preparations (Fig. 1*A*). Whereas no $\gamma 3$ subunit signal was present in cerebellum of nontransgenic, control mice, ectopic $\gamma 3$ subunit was readily detected as an intense band in $\gamma 3^{tg}$ cerebellum. The electrophoretic mobility and band pattern of the transgene-encoded $\gamma 3$ subunit were indistinguishable from those of endogenous $\gamma 3$ subunit in forebrain, indicating that the protein was properly translated and efficiently processed. The expression level of the $\gamma 3$ subunit in forebrain was 5–10 times higher in transgenic mice than in controls, indicating that it was expressed throughout the brain (Fig. 1*A*). The transgene-encoded $\gamma 3$ subunit was assembled with endogenous $\alpha 1$ and $\beta 2,3$ subunits, as shown by Western blot analysis of $GABA_A$ receptors isolated by immunopurification (Fig. 1*B*). In the absence of the

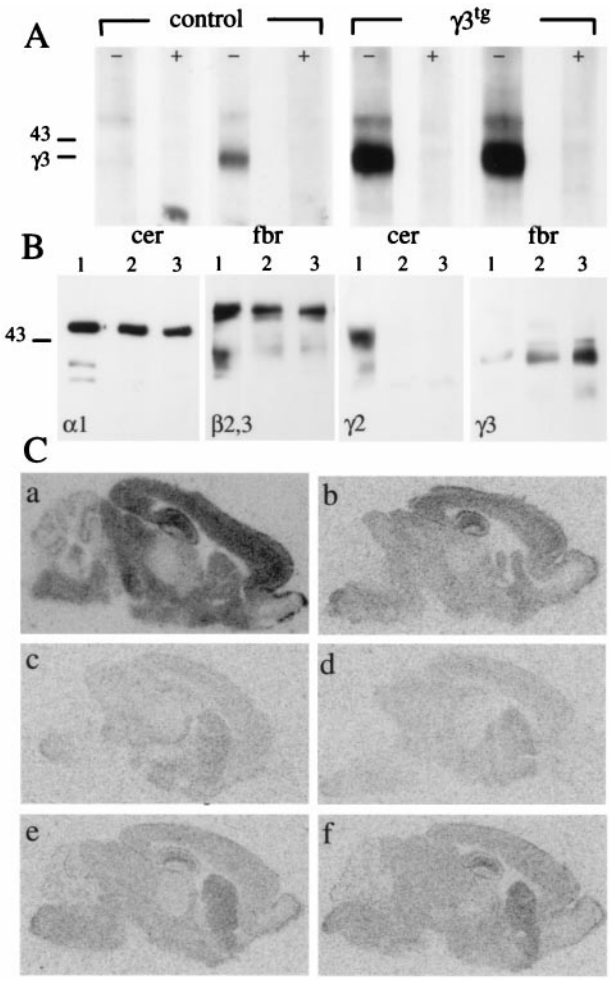

Fig. 1. Expression of $\gamma 3$ subunit and reconstitution of BZ binding sites in $\gamma 3^{tg}/\gamma 2^{0/0}$ brain. (*A*) Western blot of $\gamma 3$ subunit in membranes from cerebellum (cer) and forebrain (fbr) of adult $\gamma 2^{+/+}$ (control) and $\gamma 3^{tg}/\gamma 2^{+/+}$ ($\gamma 3^{tg}$) mice using a $\gamma 3$ subunit-specific antiserum. Lanes marked with + indicate competition by antigenic peptide (10 μg/ml). Note the low level of $\gamma 3$ subunit expression in forebrain and the lack of a signal in wt cerebellum. In contrast, the $\gamma 3$ subunit is abundant in both parts of transgenic brain. The molecular mass (in kDa) of a standard and the position of the $\gamma 3$ subunit are indicated. (*B*) Western blot of $GABA_A$ receptors immunoprecipitated from brain membranes with an $\alpha 1$ subunit antiserum. Lanes from $\gamma 2^{+/+}$ (1), $\gamma 2^{0/0}$ (2), and $\gamma 3^{tg}/\gamma 2^{0/0}$ (3) animals were labeled with the Abs indicated. Note the graded up-regulation of the $\gamma 3$ subunit in $\gamma 2^{0/0}$ and $\gamma 3^{tg}/\gamma 2^{0/0}$ mice. (*C*) Distribution of total and zolpidem-insensitive BZ binding sites in $\gamma 2^{+/+}$ (*a* and *b*), $\gamma 2^{0/0}$ (*c* and *d*), and $\gamma 3^{tg}/\gamma 2^{0/0}$ brain (*e* and *f*) as seen by autoradiography with [^{3}H]flumazenil (*a*, *c*, and *e*) or with [^{3}H]flumazenil and 10 μM zolpidem (*b*, *d*, and *f*). Note that nearly all BZ binding sites remaining in $\gamma 2^{0/0}$ mice are zolpidem-insensitive (*d*), suggesting that they represent $GABA_A$ receptors containing the $\gamma 3$ subunit. In $\gamma 3^{tg}/\gamma 2^{0/0}$ mice, there is a marked increase in BZ binding sites that are likewise zolpidem-insensitive (*e* and *f*).

$\gamma 2$ subunit, an increased $\gamma 3$ subunit expression was noted, which was further increased in the presence of the $\gamma 3$ subunit transgene.

To test whether ectopic $\gamma 3$ subunit would compensate for loss of function in $\gamma 2$ subunit-deficient mice, $\gamma 3^{tg}$ mice were crossed with $\gamma 2^{0/+}$ mice (8) and the $\gamma 3^{tg}/\gamma 2^{0/+}$ offspring were subsequently crossed with $\gamma 2^{0/+}$ mice to obtain $\gamma 3$-transgenic offspring lacking the $\gamma 2$ subunit ($\gamma 3^{tg}/\gamma 2^{0/0}$). Similar to the $\gamma 2^{0/0}$ phenotype (8), $\gamma 3^{tg}/\gamma 2^{0/0}$ mice displayed perinatal or postnatal lethality with no survivors beyond the third postnatal week, indicating that the $\gamma 3$ subunit could not fully substitute for the $\gamma 2$ subunit. The few $\gamma 2^{0/0}$ mice ($\gamma 3^{tg}$ and nontransgenic) that survived to P14 were used for analysis. The $\gamma 3^{tg}$ mice also displayed a phenotype in the $\gamma 2^{+/+}$ and $\gamma 2^{0/+}$ backgrounds, with a significant number of animals remaining smaller than their nontransgenic littermates [20 of 143 $\gamma 3^{tg}/\gamma 2^{0/+}$ and $\gamma 3^{tg}/\gamma 2^{+/+}$ mice compared with 3 of 214 $\gamma 2^{+/+}$ and $\gamma 2^{0/+}$; $\chi^2\,(1) = 20.48$, $P < 0.001$]. In addition, fertility was reduced, and spontaneous or handling-induced seizures were sometimes observed in $\gamma 3^{tg}/\gamma 2^{0/+}$ mice, and more of these mice than $\gamma 2^{0/+}$ mice died unexpectedly (18.3% of $\gamma 3^{tg}/\gamma 2^{0/+}$ compared with <1% in $\gamma 2^{0/+}$ mice). Thus, ectopic $\gamma 3$ subunit expression in both $\gamma 2^{0/+}$ and $\gamma 2^{+/+}$ mice resulted in a functional deficit indicative of a dominant-negative effect.

Restoration of BZ Binding Sites in $\gamma 3$-Transgenic Mice. $GABA_A$ receptors containing the $\gamma 3$ subunit are characterized by zolpidem-insensitive [^{3}H]flumazenil binding sites, similar to receptors containing the $\alpha 5$ subunit in conjunction with the $\gamma 2$ subunit (11, 22). In $\gamma 2^{0/0}$ and $\gamma 3^{tg}/\gamma 2^{0/0}$ brains, the $\alpha 5\beta\gamma 2$ receptors are missing, and zolpidem-insensitive flumazenil sites are indicative of $\gamma 3$ subunit-containing $GABA_A$ receptors. To determine whether the $\gamma 3$ subunit can substitute for the $\gamma 2$ subunit *in vivo*, the total number of BZ sites was analyzed by [^{3}H]flumazenil autoradiography of P14 brain sections (Fig. 1*C*) and quantified in hippocampus. Compared with wild type (wt), the number of [^{3}H]flumazenil binding sites in $\gamma 2^{0/0}$ hippocampus was markedly reduced to 8.4 ± 1.6% ($n = 3$) in agreement with previous observations (8). The large majority of the $\gamma 2^{0/0}$ [^{3}H]flumazenil sites (73.8%, corresponding to 6.2 ± 1.8% of total wt BZ sites) were insensitive to competition with zolpidem, suggesting that they represented receptors containing the endogenous $\gamma 3$ subunit (Fig. 1*C*). In hippocampus of $\gamma 3^{tg}/\gamma 2^{0/0}$ mice, [^{3}H]flumazenil binding was restored to 31.9 ± 2.3% of the wt level, and nearly all of these sites were insensitive to zolpidem (31.5 ± 3.1% of wt level). A 5- to 10-fold increase in $\gamma 3$ subunit steady-state levels in $\gamma 3^{tg}/\gamma 2^{0/0}$ compared with $\gamma 2^{0/0}$ brain resulted in a corresponding 5-fold increase in hippocampal BZ sites. Thus, the transgene-encoded $\gamma 3$ subunit assembles with endogenous α and β subunits similarly to endogenous $\gamma 3$ subunit and restores a substantial proportion of BZ site-containing $GABA_A$ receptors. These display the zolpidem-insensitive pharmacology characteristic of $\gamma 3$ subunit-containing receptors.

A similar analysis was carried out on cerebellum. Loss of the $\gamma 2$ subunit resulted in complete loss of [^{3}H]flumazenil sites in this location (Fig. 1*C*) as expected, because the cerebellum is normally devoid of $\gamma 1$ and $\gamma 3$ subunits. In contrast, BZ binding sites were clearly detectable in the cerebellum of $\gamma 3^{tg}/\gamma 2^{0/0}$ mice, albeit at a significantly lower level than in wt cerebellum. Thus, the transgene-encoded $\gamma 3$ subunit can contribute to $GABA_A$ receptors in brain regions where it does not naturally occur.

The $\gamma 3$ Subunit Directs Functional $GABA_A$ Receptors to Postsynaptic Sites. To determine whether the increased number of BZ binding sites in $\gamma 3^{tg}/\gamma 2^{0/0}$ mice was reflected electrophysiologically, we first analyzed the effect of flunitrazepam on GABA-evoked responses in recordings from cultured cortical neurons (14–21 days *in vitro*). In $\gamma 2^{0/0}$ neurons, the GABA-gated currents were unaffected by flunitrazepam in most cases, or very slightly potentiated, as expected from the loss of most BZ sites (Fig. 2*A*). In striking contrast, $\gamma 3^{tg}/\gamma 2^{0/0}$ neurons displayed a potentiation by flunitrazepam similar to that observed in wt. Thus, the transgene-encoded $\gamma 3$ subunit contributes to the formation of BZ-sensitive $GABA_A$ receptors in $\gamma 2$ subunit-deficient neurons.

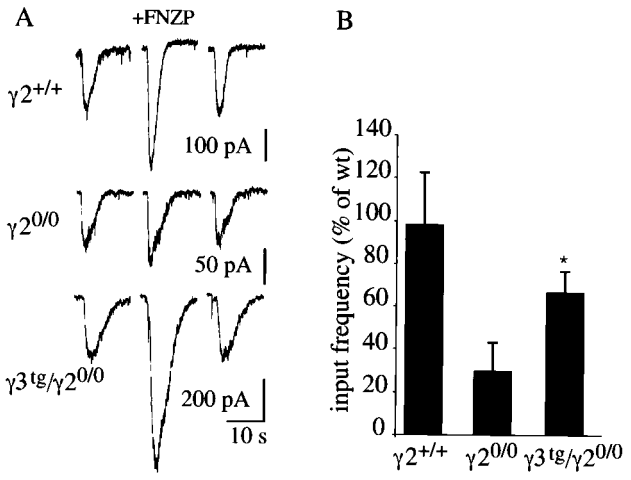

Fig. 2. Restoration of functional BZ-modulated and synaptic $GABA_A$ receptors in cultured cortical $\gamma 3^{tg}/\gamma 2^{0/0}$ neurons. (*A*) Representative recordings illustrating the effect of flunitrazepam (1 μM) on GABA-evoked currents (GABA 1 μM, 2 s) recorded from $\gamma 2^{+/+}$, $\gamma 2^{0/0}$, and $\gamma 3^{tg}/\gamma 2^{0/0}$ cortical neurons (18 days *in vitro*). (*B*) Relative frequency of GABAergic mIPSCs in cortical neurons cultured from $\gamma 2^{+/+}$, $\gamma 2^{0/0}$, and $\gamma 3^{tg}/\gamma 2^{0/0}$ embryos. GABAergic mIPSCs were identified based on current decay kinetics and pharmacological sensitivity (9). The input frequency was reduced to 33 ± 13.1% of $\gamma 2^{+/+}$ in $\gamma 2^{0/0}$ neurons and restored to 67 ± 8.9% by overexpression of the $\gamma 3$ subunit in $\gamma 3^{tg}/\gamma 2^{0/0}$ neurons. Error bars, standard error.

Postsynaptic localization of $GABA_A$ receptors in cultured cortical neurons requires the $\gamma 2$ subunit (9). Consequently, the input frequency of GABAergic mIPSCs in $\gamma 2^{0/0}$ neurons was reduced to 33 ± 13.1% ($n = 19$) of wt (Fig. 2*B*), as described (9). In $\gamma 3^{tg}/\gamma 2^{0/0}$ neurons, the input frequency was restored to 67 ± 8.9% ($n = 21$) of the wt level (Fig. 2*B*). Thus, in the absence of the $\gamma 2$ subunit, ectopic $\gamma 3$ subunit not only assembles with endogenous α and β subunits but also contributes to the formation of a major population of functional postsynaptic $GABA_A$ receptors.

Ectopic $\gamma 3$ Subunit Promotes Synaptic Clustering of $\alpha 1$ and $\alpha 2$ Subunit-Containing $GABA_A$ Receptors *in Vivo*. A major fraction of hippocampal $GABA_A$ receptors is characterized by the presence of the $\alpha 2$ subunit. These receptors are clustered at gephyrin-rich, presumably postsynaptic sites, as indicated by the punctate and colocalized immunoreactivity (IR) for the $\alpha 2$ subunit and gephyrin in the CA1 region (Fig. 3 *a* and *d*) (9, 23). In the absence of the $\gamma 2$ subunit, punctate staining for the $\alpha 2$ subunit and gephyrin was drastically reduced, indicating a major loss of $\alpha 2$ subunit-containing $GABA_A$ receptors and gephyrin at postsynaptic sites (Fig. 3 *b* and *e*) (9). Little staining was detectable for the endogenous $\gamma 3$ subunit. Upon overexpression of the $\gamma 3$ subunit, a substantial recovery of punctate $\alpha 2$ subunit IR was observed in $\gamma 3^{tg}/\gamma 2^{0/0}$ littermates. Moreover, $\alpha 2$ subunit IR colocalized with punctate $\gamma 3$ subunit staining (Fig. 3*c*), indicating that the two subunits were located in the same synapse, and perhaps in the same $GABA_A$ receptor complex. This pattern was seen in all animals analyzed ($n = 4$–6 per genotype). Although the $\gamma 3$ subunit was expressed under control of a ubiquitously active promoter, no $\gamma 3$ subunit IR was detected in nonneural cells, which is consistent with the notion that the $\gamma 3$ subunit requires α and β subunits for assembly into stable complexes. The number

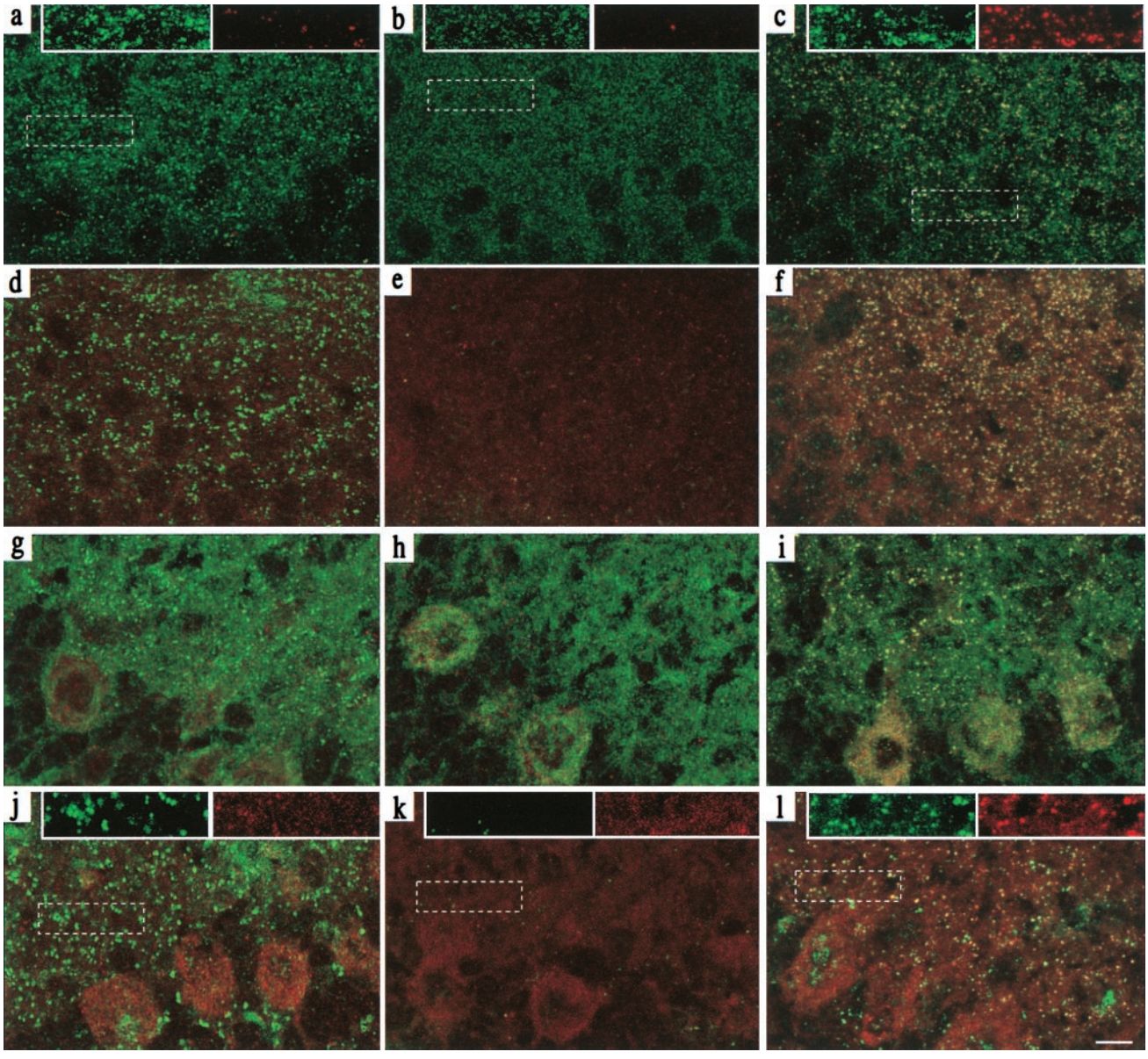

Fig. 3. Clustering of $\gamma 3$ subunit-containing receptors and gephyrin in $\gamma 3^{tg}/\gamma 2^{0/0}$ hippocampus and cerebellum. Parasagittal sections through the CA1 region of P14 hippocampus (*a–f*) ($n = 4–6$ per genotype) were stained with Abs specific for the $\alpha 2$ and $\gamma 3$ subunits (*a–c*; $\alpha 2$ green, $\gamma 3$ red) and for gephyrin and the $\gamma 3$ subunit (*d–f*; gephyrin green, $\gamma 3$ red) and visualized by confocal microscopy. The images were digitally superimposed to illustrate the presence or absence of colocalization between the markers used (yellow puncta). The punctate $\alpha 2$ (*a*) and gephyrin (*d*) staining, which is not colocalized with the scarce $\gamma 3$ subunit IR in wt mice, was completely lost in $\gamma 2^{0/0}$ mice (*b* and *e*) and largely restored in $\gamma 3^{tg}/\gamma 2^{0/0}$ mice (*c* and *f*). In the latter, it was extensively colocalized with the $\gamma 3$ subunit, as shown in yellow. Parasagittal sections through the molecular layer of P14 cerebellum (*g–l*) were stained for the $\alpha 1$ (green) and $\gamma 3$ subunit (red; *g–i*) and for gephyrin (green) and the $\gamma 3$ subunit (red; *j–l*). The punctate $\alpha 1$ subunit (*g*) and gephyrin (*j*) staining in wt brain was completely lost in $\gamma 2^{0/0}$ (*h* and *k*) and largely restored in $\gamma 3^{tg}/\gamma 2^{0/0}$ brain (*i* and *l*). Whereas specific $\gamma 3$ subunit IR was absent in wt and $\gamma 2^{0/0}$ cerebellum, it was readily detected and extensively colocalized with the $\alpha 1$ subunit in $\gamma 3^{tg}/\gamma 2^{0/0}$ cerebellum. The data were reproduced with four to six animals per genotype. *Insets* at the top of panels *a–c* and *j–l* show enlargements of the boxed areas to illustrate the colocalization of clustered $GABA_A$ receptor and gephyrin IR in color-separated images. (Scale bar, 10 μm.)

of $\alpha 2$ subunit clusters in $\gamma 3^{tg}/\gamma 2^{0/0}$ hippocampus (542 ± 18.1 clusters per 10^4 μm^2) was as high as in wt (483.7 ± 22.3), and the average size of $\alpha 2$ subunit clusters [0.198 $\mu m^2 \pm 0.104$ (wt) and 0.194 ± 0.115 ($\gamma 3^{tg}/\gamma 2^{0/0}$)] was also restored. Interestingly, $\gamma 3$ subunit-containing receptors were colocalized with punctate IR for gephyrin (Fig. 3*f*), similar to $\gamma 2$ subunit-containing receptors in wt hippocampus. Thus, in $\gamma 3^{tg}/\gamma 2^{0/0}$ hippocampus, the $\gamma 3$ subunit substitutes for the $\gamma 2$ subunit in the formation of clustered, presumably postsynaptic receptors. The two types of receptors are indistinguishable with regard to their colocalization with gephyrin clusters.

A similar analysis was conducted in the molecular layer of the cerebellum, which is largely devoid of $\alpha 2–6$, $\gamma 1$, $\gamma 3$, ε, and δ subunits, so that virtually all $GABA_A$ receptors conform to the $\alpha 1\beta\gamma 2$ subunit composition (20, 24, 25). Upon double immunofluorescence staining for the $\alpha 1$ and $\gamma 3$ subunit in wt mice, strong punctate $\alpha 1$ subunit IR was seen in the molecular layer of the cerebellum, probably representing postsynaptic receptors on dendrites of Purkinje, stellate, and basket cells (Fig. 3*g*). In addition, diffuse staining of the neuropil was evident, indicating extrasynaptic receptors containing the $\alpha 1$ subunit. No $\gamma 3$ subunit IR was detected in the cerebellum of nontransgenic mice (Fig.

3 *g*, *h*, *j*, and *k*). In corresponding sections from $\gamma2^{0/0}$ littermates, the punctate $\alpha1$ subunit staining was lost entirely, whereas the diffuse $\alpha1$ subunit staining appeared unaffected, as described (9). Finally, in sections from $\gamma3^{tg}/\gamma2^{0/0}$ littermates, the punctate $\alpha1$ staining was largely reestablished and was extensively colocalized with punctate IR for the $\gamma3$ subunit (Fig. 3*i*). Recovery of $GABA_A$ receptor clusters in the cerebellum was again paralleled by a corresponding gain in gephyrin clusters that were colocalized with the $\gamma3$ (Fig. 3 j–l) and $\alpha1$ subunit IR (data not shown). However, the number of $\alpha1$ subunit clusters in cerebellum was slightly reduced in $\gamma3^{tg}/\gamma2^{0/0}$ compared with wt tissue (243.8 ± 97.8 in $\gamma3^{tg}/\gamma2^{0/0}$ compared with 274 ± 9.8 clusters per 10^4 μm^2 in $\gamma2^{+/+}$), together with a reduction in the mean cluster size [0.28 ± 0.20 μm^2 ($\gamma3^{tg}/\gamma2^{0/0}$) and 0.35 ± 0.32 μm^2 (wt)]. Thus, ectopically expressed $\gamma3$ subunit can substitute for the $\gamma2$ subunit in the formation of clustered $\alpha1$ subunit-containing $GABA_A$ receptors in the cerebellar molecular layer, but the size and number of clusters do not reach wt levels.

$GABA_A$ Receptor Clustering Mediated by the Endogenous $\gamma3$ Subunit. Detailed examination of the [^{3}H]flumazenil autoradiograms revealed that the reticular thalamic nucleus (RTN) and the striatum of $\gamma2^{0/0}$ P14 brain displayed increased levels of zolpidem-insensitive BZ binding sites compared with the surrounding tissue (Fig. 1*B*). This indicated that loss of BZ sites was not uniform across the brain, possibly because of higher than average levels of endogenous $\gamma3$ subunit in some brain regions. In RTN of P14 $\gamma2^{0/0}$ brain, staining for the $\gamma3$ subunit revealed prominent punctate IR, indicative of synaptically clustered receptors. Moreover, $\gamma3$ subunit staining was colocalized with the $\alpha3$ subunit (Fig. 4*a*), the most abundant α subunit in the RTN (20), and with gephyrin (Fig. 4*b*). Thus, in $\gamma2^{0/0}$ RTN, the endogenous $\gamma3$ subunit appears to contribute to postsynaptic $GABA_A$ receptors containing the $\alpha3$ subunit, thereby preventing the loss of $GABA_A$ receptor clusters.

In the wt RTN, the $\alpha3$ subunit and gephyrin IR were also colocalized (Fig. 4*d*). However, the $\gamma3$ subunit IR was distinctly weaker than in $\gamma2^{0/0}$ RTN, forming smaller puncta that were only partially colocalized with the $\alpha3$ subunit (Fig. 4*c*). These findings suggest that the $\gamma3$ subunit contributes to only a fraction of postsynaptic $GABA_A$ receptors in wt RTN, the majority presumably being clustered by the $\gamma2$ subunit. Thus, $GABA_A$ receptor clustering can be mediated by both the endogenous and the ectopic $\gamma3$ subunit, a property best seen in the absence of the $\gamma2$ subunit.

Discussion

The $\gamma2$ subunit and gephyrin are essential for postsynaptic localization of major $\gamma2$ subunit-containing $GABA_A$ receptor subtypes. We now report that the $\gamma3$ subunit is functionally equivalent to the $\gamma2$ subunit in its contribution to postsynaptic localization and function of $\gamma3$ subunit-containing $GABA_A$ receptors *in vivo* and for recruitment of gephyrin to GABAergic synapses. In transgenic mice, these receptors were formed and clustered, even in brain regions devoid of endogenous $\gamma3$ subunit, indicating that all the factors needed for clustering of $\gamma3$ subunit-containing receptors are present and can be recruited in these neurons.

In $\gamma2^{0/0}$ mice, the $\gamma3$ subunit-containing receptor appeared up-regulated (Fig. 1*B*). A partial compensation for the lack of $\gamma2$ subunit was even observed in the RTN, where the endogenous $\gamma3$ subunit contributed to postsynaptic clusters containing the $\alpha3$ subunit and gephyrin. Because $\gamma3$ subunit-containing clusters were much fewer in wt RTN, these data suggest that the $\gamma3$ subunit is unable to displace the $\gamma2$ subunit during formation of synaptically localized receptors. This might be due, in part, to structural differences or to a lower level of expression of the $\gamma3$ subunit compared with the $\gamma2$ subunit in wt RTN.

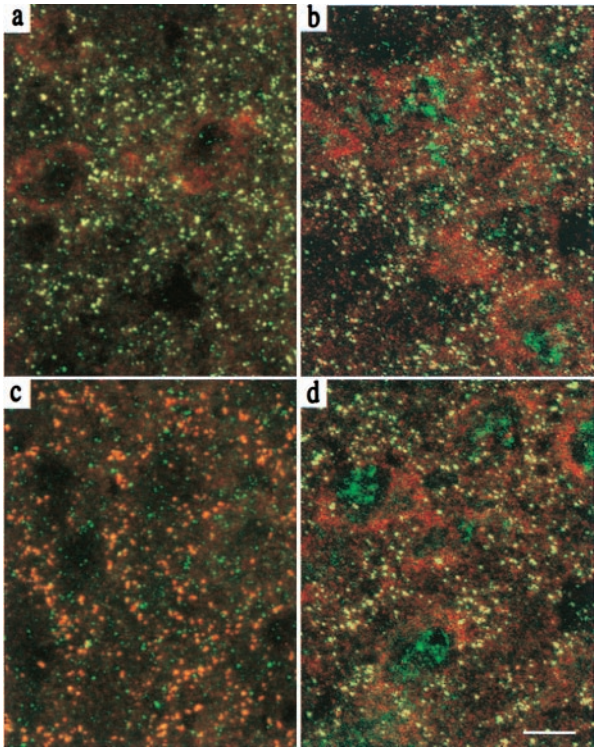

Fig. 4. Clustering of $GABA_A$ receptors mediated by the endogenous $\gamma3$ subunit. The $\gamma3$ subunit in the RTN promotes gephyrin and $GABA_A$ receptor $\alpha3$ subunit clustering in $\gamma2^{0/0}$ mice. Parasagittal sections through the RTN of P14 mice ($n = 4–6$ per genotype) were double stained for the $\alpha3$ and $\gamma3$ subunit (*a* and *c*; $\alpha3$ red, $\gamma3$ green) or the $\alpha3$ subunit and gephyrin (*b* and *d*; $\alpha3$ red, gephyrin green) and visualized by confocal microscopy. Yellow puncta in the superimposed red and green images illustrate colocalization of the markers used. In $\gamma2^{0/0}$ RTN, the $\alpha3$ subunit IR was colocalized with strong IR for the $\gamma3$ subunit (*a*), as well as for gephyrin (*b*), whereas in $\gamma2^{+/+}$ RTN, there was only a partial colocalization of the $\alpha3$ subunit IR and the weaker $\gamma3$ subunit IR (*c*), but a prominent colocalization of the gephyrin and $\alpha3$ subunit IR (*d*). (Scale bar, 10 μm.)

Despite the apparent functional similarity between the $\gamma2$ and $\gamma3$ subunits for the formation of postsynaptic receptors, the overexpressed $\gamma3$ subunit did not rescue $\gamma2^{0/0}$ mice from a lethal phenotype. Crossing of transgenic lines overexpressing the $\gamma2$ subunit under control of the same ubiquitous promoter used here for the $\gamma3$ transgene allowed full rescue of mice without an overt behavioral phenotype (K.B., C.E., S. Balsiger, M. J. Wick, R. A. Harris, J.M.F., and B.L., unpublished work). This indicates that the level of transgene expression with this promoter can be sufficient to fully restore $GABA_A$ receptor function and that ectopic expression of a γ subunit is not in itself toxic. However, the ectopic expression of $\gamma3$ subunit appeared to exert a dominant-negative effect, as seen in some $\gamma2^{+/0}$ and $\gamma2^{+/+}$ transgenic animals.

The phenotype of $\gamma3^{tg}/\gamma2^{0/0}$ mice cannot be attributed simply to inefficient assembly of the $\gamma3$ subunit with endogenous α and β subunits. In line with previous findings on $GABA_A$ receptors immunoprecipitated from brain extracts (13, 14), the $\gamma3$ subunit was found to assemble with α and β subunits (Fig. 1*B*). In the hippocampus, a 5- to 10-fold increase in $\gamma3$ subunit expression resulted in a roughly 5-fold increase in BZ binding sites. Furthermore, $\gamma3$ subunit-containing receptors show a unitary channel conductance similar to that of $\gamma2$ subunit-containing receptors (11). However, synaptic $GABA_A$ receptor and gephyrin clusters appeared fully restored in hippocampus but not in cerebellum. Incomplete restoration of synaptic receptors might

in part be because of insufficient expression of the $\gamma 3$ subunit in the cerebellum and possibly other brain regions, possibly contributing to the lack of rescue of $\gamma 2^{0/0}$ mice by the $\gamma 3$ transgene.

Mechanisms of $GABA_A$ Receptor Clustering. The clustering of glycine receptors at postsynaptic sites requires both receptor activation and interaction with gephyrin (2, 26, 27). It is still unknown, however, whether this is also the case for $GABA_A$ receptors (9, 28), or whether protein interactions, involving at least the $\gamma 2$ or $\gamma 3$ subunit and gephyrin, are sufficient for clustering at postsynaptic sites. In the absence of the $\gamma 2$ subunit, $GABA_A$ receptors are characterized by a markedly reduced channel conductance (8), which might also account for the lack of clustering. The $\gamma 3$ subunit can substitute for the $\gamma 2$ subunit with respect to the conductance of the corresponding $GABA_A$ receptors (11). Thus, if receptor activation were required for clustering of $GABA_A$ receptors, then it would be the gain in conductance conferred by the presence of the $\gamma 3$ subunit that would trigger clustering of $\gamma 3$ subunit-containing $GABA_A$ receptors.

Independent of the outcome of further experiments addressing this issue, the $\gamma 3$ subunit-containing $GABA_A$ receptors were colocalized with gephyrin in $\gamma 3^{tg}/\gamma 2^{0/0}$ mice in a manner very similar to the $\gamma 2$ subunit-containing receptors in wt mice. Therefore, if protein interactions are important for this process, it is very likely that similar domains and factors are involved for both subunits. As the $\gamma 2$ and $\gamma 3$ subunits exhibit considerable heterogeneity in their intracellular cytoplasmic loop, it is remarkable that the gephyrin-clustering capability is also present in the $\gamma 3$ subunit. At glycinergic synapses, gephyrin is thought to mediate clustering of glycine receptors by direct protein–protein interaction with the receptor β subunit. Although gephyrin is also required for postsynaptic localization of $GABA_A$ receptors (9), there is so far no evidence for a direct interaction between gephyrin and any $GABA_A$ receptor subunit. A candidate protein (GABARAP) that interacts directly with the $\gamma 2$ subunit and might mediate interaction between the $\gamma 2$ subunit and gephyrin has recently been identified (29). Interestingly, the portion of the putative $\gamma 2$ subunit cytoplasmic loop that interacts with GABARAP (residues 394–411) is highly conserved between all γ subunits, indicating that GABARAP might also interact with $\gamma 3$ subunit-containing receptors (29). These residues might be crucial for protein interactions underlying the clustering of $GABA_A$ receptors at postsynaptic sites.

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