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Cover photo. Cultured hippocampal neuron expressing the $\beta 2/3$ subunits of the GABAA receptor (Immunocytochemistry with the bd17 antibody 17 days after plating). The clusters of GABAA receptors (bright puncta) are above a diffuse staining of the somatodendritic compartment. This study shows that GABAA receptor clusters are associated with gephyrin at synaptic and extrasynaptic loci. See the article by Danglot et al. in this issue.



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Association of gephyrin with synaptic and extrasynaptic GABA_A receptors varies during development in cultured hippocampal neurons

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Abstract

Several studies have reported extrasynaptic clusters of GABA_A receptors in hippocampal neurons. Yet their functional relevance as well as their evolution in relation with gephyrin during synaptogenesis remain unknown. We have analyzed the expression pattern of the main proteins of the GABAergic synapses during synaptogenesis in cultured hippocampal neurons. We found that GABAergic terminals, characterized by VIAAT and GAD-65 expression, differentiated 3 to 7 days after the glutamatergic endings. At the postsynaptic side, the GABA_A β 3 subunit was first diffuse and then clustered when GABAergic terminals differentiated and gephyrin formed large clusters. Colocalization of these proteins was high and increased with development. At later stages, GABA_A β 3 clusters colocalized with gephyrin at synaptic but also at extrasynaptic sites. GABA_A γ 2 subunits were directly expressed as clusters which were first extrasynaptic and not associated with gephyrin. Subsequently, the GABA_A γ 2 subunits associated with gephyrin at synaptic and/or extrasynaptic sites. Our data indicate that formation of GABA_A γ 2 subunit clusters is gephyrin independent.

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Introduction

Extrasynaptic clusters of GABA_A receptors (GABA_ARs) have been reported in hippocampal neurons (Banks and Pearce, 2000, *in situ*; Christie et al., 2002; Kannenberg et al., 1999; Nusser et al., 1995, *in situ*; Scotti and Reuter, 2001) but their involvement in the construction of the postsynaptic domains remained unexplored. It is established that inhibition of gephyrin expression leads to a dramatic decrease in GABA_A clusters (Essrich et al., 1998; Feng et al., 1998; Kneussel et al., 1999). Conversely, mice knocked-out for the GABA_A γ 2 subunits show significant reduction of gephyrin clustering (Essrich et al., 1998). As a direct binding of gephyrin to the GABA_AR could not be demonstrated (Meyer et al., 1995), it has been proposed that gephyrin mediates clustering and/or synaptic anchoring

(Craig et al., 1996; Kneussel et al., 1999) of GABA_AR through indirect binding to its γ 2 and maybe β 3 subunits (Kirsch et al., 1995). Surprisingly, however, the association of GABA_AR and gephyrin at extrasynaptic sites remains poorly studied. Moreover, only few data concerning the association of these proteins during development are available (Dumoulin et al., 2000). The comparison of the expression pattern of gephyrin with that of GABA_A γ 2 and β 3 subunits during synaptogenesis is necessary to understand the role of each partner. Detailed study of GABAergic synaptogenesis has been performed in spinal interneurons (Dumoulin et al., 2000), but in this system, only few extrasynaptic clusters of GABA_ARs are detected. In addition, inhibitory synapse maturation in spinal interneurons is achieved within 10–12 days (Dumoulin et al., 2000), while it needs about 21 days in cultured hippocampal neurons (Craig et al., 1996; Levi et al., 2002). Christie et al. (2002) have studied the effect of GABAergic innervation on GABA_ARs clustering in cultured hippocampal neurons, but the developmental aspects were not investigated, and the relationships between synaptic localization of GABA_AR clusters and gephyrin association have not been assessed. In

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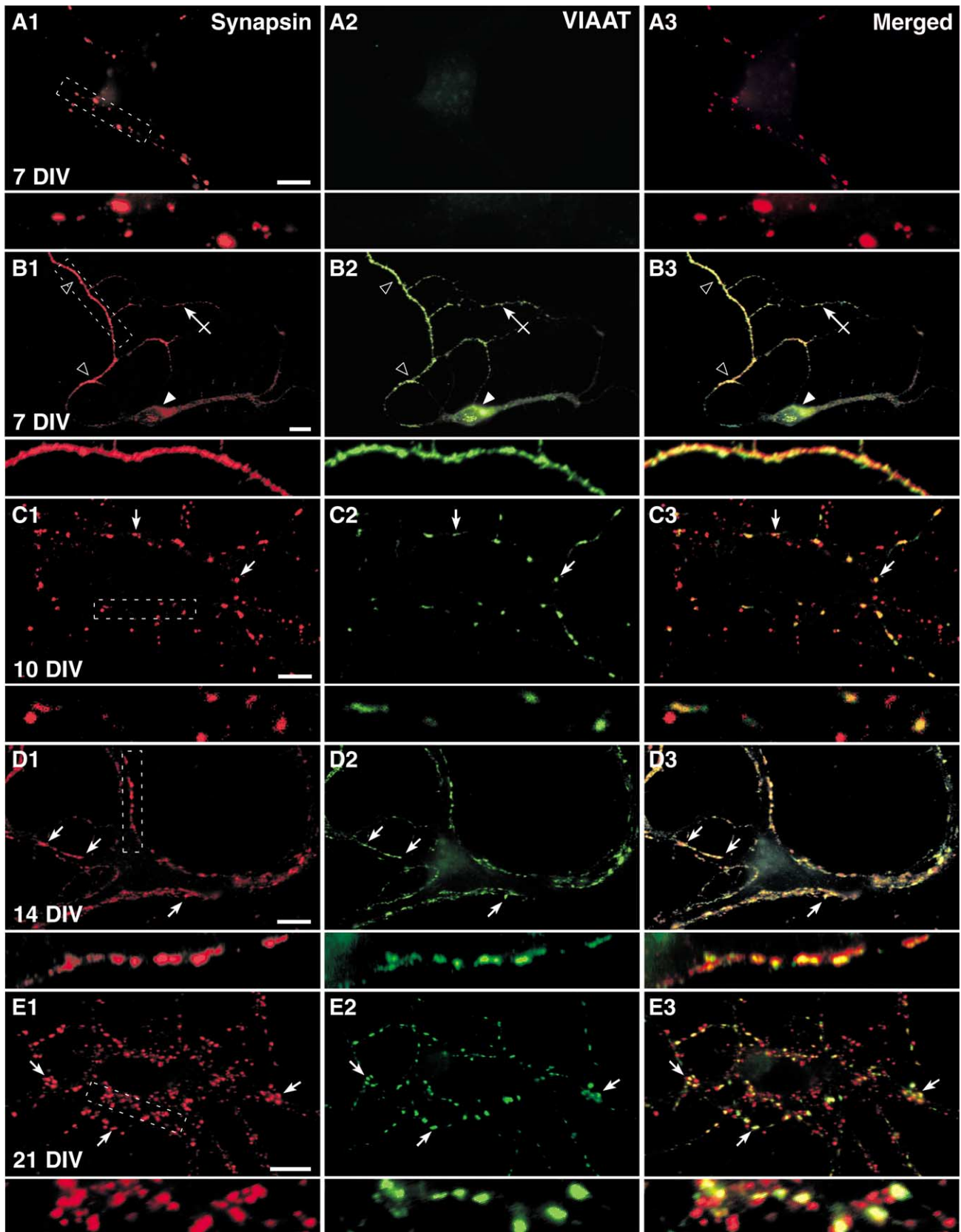


Fig. 1. Distribution patterns of synapsin and VIAAT in inhibitory presynaptic elements during the development of hippocampal neurons in culture. Double detection of synapsin (red) and VIAAT (green) at 7 (A and B), 10 (C), 14 (D), and 21 (E) days in culture. Lower panels are magnifications of the boxed windows. At 7 DIV, synapsin appeared as puncta in most neurons (A1) without expression of VIAAT (A2). Some neurons (B1) displayed diffuse synapsin-IR in the soma (arrowhead) and axonal compartment (open arrowheads), colocalizing with VIAAT-IR (B2 and 3). Note that in these neurons, small puncta of VIAAT-IR colocalizing with synapsin-IR were detected (crossed arrow). During maturation (C–E), diffuse VIAAT-IR progressively disappeared while the number of VIAAT puncta colocalizing with synapsin (arrows) increased. Scale bars, 10 μ m.

contrast, in the same system, Scotti and Reuter (2001) have analyzed the localization of GABA_AR clusters at some developmental stages, but the relation of these clusters with gephyrin was not investigated.

We have now analyzed the development of inhibitory synapse components in cultured hippocampal neurons. The presynaptic inhibitory elements were identified by the presence of the GABA synthesizing enzyme GAD-65 and/or the vesicular inhibitory amino acid transporter immunoreactivity (VIAAT-IR, characterized in *Caenorhabditis elegans* as Unc47/VGAT) (McIntire et al., 1997; Sagne et al., 1997). On the postsynaptic side, we have followed the GABA_AR $\gamma 2$ and $\beta 3$ subunits and gephyrin-IR. This allowed us to unravel unexpected stages in the sequence of GABAergic synaptogenesis. We observed that during synaptic maturation GABA_AR $\gamma 2$ and $\beta 3$ subunits behave differently and that these subunits are able to associate with synaptic and extrasynaptic clusters of gephyrin. Our data are compatible with a role of gephyrin in GABA_AR synaptic stabilization rather than in clusters formation.

Results

Inhibitory boutons differentiate after excitatory ones

In cultured hippocampal neurons, 6 to 7% of neurons are GABAergic interneurons, whereas the remainder are pyramidal glutamatergic cells (Benson et al., 1994). To investigate the formation of inhibitory presynaptic elements, we double-labeled rat hippocampal neurons cultured from 3 up to 21 days in vitro (DIV) with antibodies against VIAAT and synapsin. The time course and pattern of synapsin expression were comparable to those described in other studies (e.g., Fletcher et al., 1991). A diffuse synapsin staining appeared by 3 DIV in the cell body and processes, and by 5 DIV staining became punctiform and restricted to the sites of contact between the axon and other cells. These synapsin puncta will be referred to as boutons. At 7 DIV, an expected synapsin punctiform labeling was observed on most neurons; and these puncta were VIAAT-negative (Figs. 1A1–3). Some other neurons displayed a diffuse synapsin labeling of neurites (Fig. 1B1, open arrowheads). These neurites were axons (not shown), as they were stained with an anti-dephosphorylated tau antibody (Mandell and Banker, 1996). This diffuse axonal synapsin-IR colocalized with a diffuse VIAAT staining (Figs. 1B2 and 3), suggesting that these neurons were most probably inhibitory interneurons. VIAAT-IR displayed a diffuse to punctated staining pattern transition comparable to that observed for synapsin between 3 and 5 DIV, but later on. More precisely, even though weak VIAAT-IR could be detected in few neurons at early stages, VIAAT staining was detected over an important number of neurons only by 7 DIV. VIAAT-IR was first diffuse in the cell body and the axon (see Fig. 1B2, solid and open arrowheads). It is only between 10 and 14 DIV that the diffuse VIAAT immunofluorescence decreased whereas

bright puncta appeared over the neuronal somata and dendrites (Figs. 1C2–D2). Between 10 and 21 DIV, the proportion of synaptic VIAAT-IR puncta increased significantly (Mann–Whitney $P < 0.005$; 10 DIV: $84.5 \pm 3.0\%$, $n = 531$ puncta, 15 neurons; 14 DIV: $89.1 \pm 2.1\%$, $n = 1351$, 16 neurons; 17 DIV: $89.3 \pm 1.8\%$, $n = 1060$, 16 neurons; 21 DIV: $95.5 \pm 0.6\%$, $n = 1376$, 11 neurons; means \pm SEM). These results indicate that despite a delayed expression, most VIAAT-IR profiles correspond to synaptic boutons defined by their synapsin-IR.

The expression of VIAAT-IR was then compared with that of GAD-65 (Fig. 2). At 7 DIV, GAD-IR was diffuse in the cell body (Fig. 2A2), whereas it was detected as puncta by 10 DIV (Fig. 2B2). Between 14 and 21 days, most neurons showed a bright staining pattern (Figs. 2C2 and D2). VIAAT and GAD displayed a similar time course of expression and distribution. The proportion of GAD-65-IR boutons colocalizing with VIAAT-IR was high during all development (10 DIV: $97.1 \pm 0.8\%$, $n = 708$ clusters, 12 neurons; 14 DIV: $96.6 \pm 0.8\%$, $n = 1582$, 15 neurons; 17 DIV: $99.3 \pm 0.4\%$, $n = 926$, 12 neurons; 21 DIV: $97.7 \pm 0.8\%$, $n = 3128$, 17 neurons; means \pm SEM. The differences are not significant except between 14 and 17 DIV; Mann–Whitney test $P < 0.05$). However, the number of VIAAT-IR boutons devoid of GAD-IR was higher than expected (data not shown). This difference could be due to a higher sensitivity for VIAAT than for GAD-65 detection. Alternatively, this could result from the expression of another GAD isoform (Stone et al., 1999). Lastly, VIAAT being a GABA and glycine transporter (Sagne et al., 1997), one cannot exclude that few synapses might be glycinergic and not GABAergic (Malosio et al., 1991) even though no glycinergic IPSC has been recorded in the hippocampus.

The observed interval between VIAAT/GAD-65 and synapsin expressions in boutons is striking. One may hypothesize that the inhibitory boutons are formed after the excitatory ones. Alternatively, synapsin might be recruited first at all endings prior to recruitment of the specific proteins such as VIAAT, GAD-65, or the vesicular glutamate transporter (VGLUT) at the excitatory synapses (Bellocchio et al., 2000; Takamori et al., 2000). To distinguish between these two hypotheses, we examined the expression of the VGLUT1. At 7 DIV, a stage at which VIAAT-IR is still diffuse (Figs. 1B1 and 2A1), VGLUT1-IR was already punctiform (Fig. 2E1) and widely colocalized with synapsin (Fig. 2E2). Altogether, our data suggest that glutamatergic presynaptic elements differentiate earlier than the GABAergic ones. Further, the colocalization of VIAAT and synapsin in axons at 7 DIV shows that these major presynaptic proteins are expressed at the same time in interneurons.

The onset of inhibitory terminal differentiation coincides with clustered expression of gephyrin–GABA_AR $\beta 3$ complexes

It has been proposed that gephyrin is involved in the postsynaptic clustering of GABA_AR (Essrich et al., 1998;

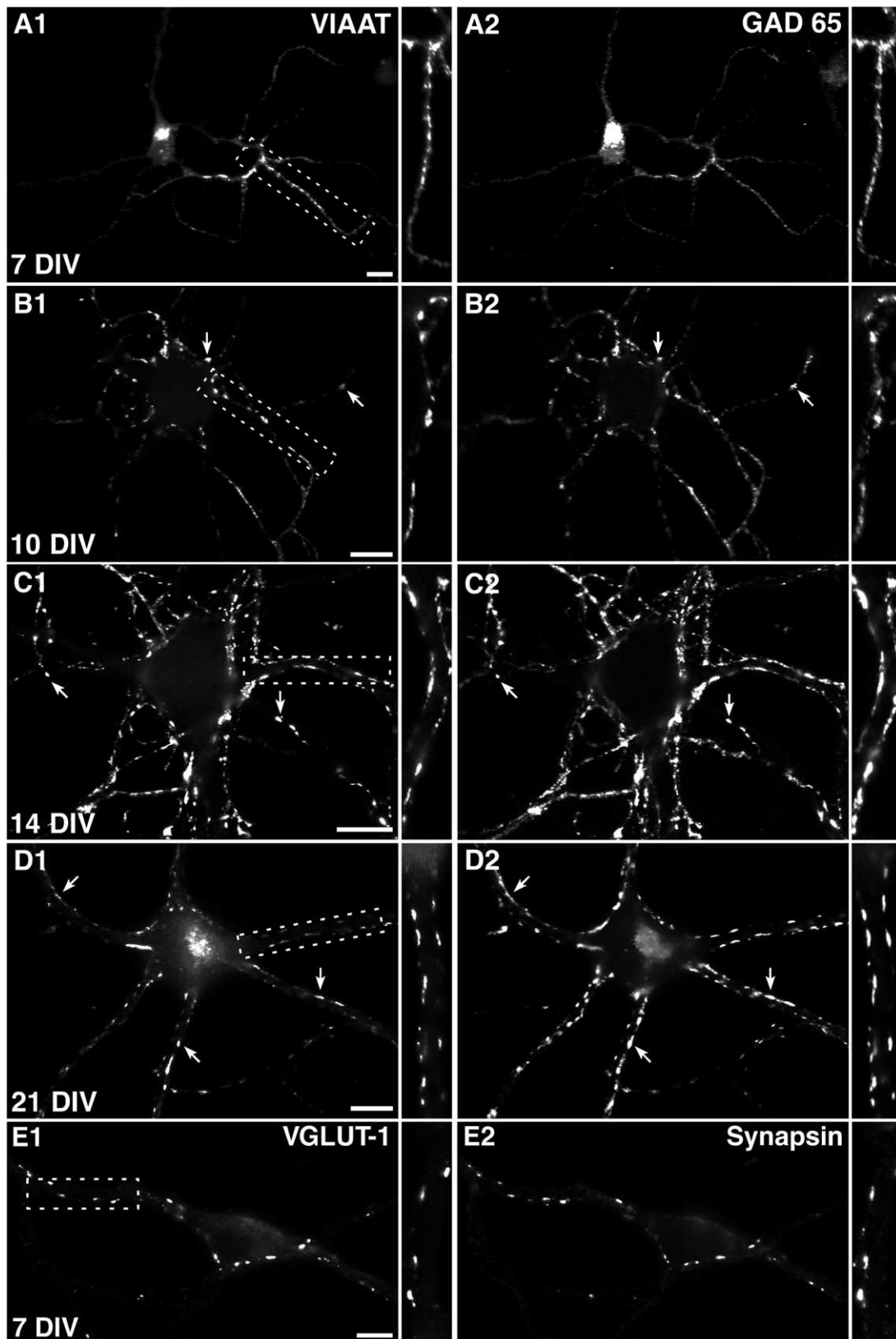


Fig. 2. Distribution pattern of VIAAT, GAD-65, and VGLUT1 in presynaptic elements during the development of hippocampal neurons in culture. Panels on the right are magnifications of the boxed windows. GAD-IR, which consistently colocalized with VIAAT-IR (arrows), was diffusely distributed at 7 DIV (A), and formed puncta at 10 (B), 14 (C), and 21 days (D). VGLUT1 (E1) and synapsin (E2) formed puncta and colocalized after 7 DIV, whereas VIAAT-IR was still diffuse (See A1 and Fig. 1B). Scale bars, 10 μ m.

Kneussel et al., 1999), via an indirect interaction with the GABA A $\gamma 2$ and maybe $\beta 3$ subunits (Kirsch et al., 1995), which are the most abundantly expressed subunits in hippocampal neurons (Fritschy and Mohler, 1995). We first analyzed the GABA A $\beta 2/3$ subunit distribution at synaptic and extrasynaptic sites. Synaptic sites are defined as being in front of synaptic boutons, characterized here by the VIAAT-IR. From 3 to 7 DIV, the GABA A $\beta 2/3$ -IR was diffusely distributed over the entire cell, interspersed with brighter regions of staining around the soma (Fig. 3A1, crossed arrows). At 10 DIV, the GABA A $\beta 2/3$ (Fig. 3A2) or $\beta 3$ (Fig. 3B2) subunit pattern of expression displayed remarkable changes: a punctate labeling appeared on the soma and dendrites, superimposed on a diffuse one. The clusters were occasionally small and aggregated, being thus difficult to distinguish from the diffuse staining. However, we have quantified their evolution. At 10 DIV, up to 61.5% of GABA A $\beta 2/3$ clusters were localized at extrasynaptic sites (Fig. 4B). Interestingly, the proportion of extrasynaptic clusters decreased during development but remained significantly high, even in mature neurons (Fig. 3A4, arrowheads; 33.4% at 21 DIV in Fig. 4B).

The distribution pattern of the GABA A $\beta 3$ subunits vs that of the gephyrin scaffold protein was investigated at the same stages of development. At 3 DIV, only rare clusters of gephyrin displaying very low fluorescence intensity could be detected in a few neurons (not shown). After 7 DIV, more neurons were labeled, but the clusters remained small with low fluorescence intensity (Figs. 3B1 and 5B1). They were not colocalized with the GABA A $\beta 3$ subunit-IR, which still displayed a diffuse staining (Fig. 3B1). At 10 DIV, as the punctate labeling of GABA A $\beta 3$ subunit-IR appeared, small gephyrin clusters were still observed, but consistently brighter and larger gephyrin clusters were detected (Figs. 3B2 and 5B2). At this stage the proportion of GABA A $\beta 3$ subunit clusters colocalizing with gephyrin was already as high as 63% (Fig. 4A1). During maturation, this proportion increased regularly (Figs. 3B3 and 4), reaching 85.6% (Fig. 4A1) by 17 DIV. The proportion of synaptic GABA A $\beta 2/3$ subunit clusters (Fig. 4B) was always lower than that of GABA A $\beta 3$ clusters associated with gephyrin (Fig. 4A1). This indicates that some clusters of the GABA A $\beta 3$ subunits that were associated with gephyrin were extrasynaptic.

The relationship between gephyrin clusters and synaptic boutons was quantitatively analyzed with a simultaneous detection of VIAAT and gephyrin (Figs. 4C and 5). At 10 DIV, only 58% of the gephyrin clusters were detected at synaptic sites. In contrast, up to 90% of these clusters were synaptic in 21 DIV neurons (Fig. 4C). The proportion of synaptic gephyrin clusters (Fig. 4C) was always higher than the proportion of gephyrin associated with GABA A $\beta 3$ subunit (Fig. 4A2). These quantitative data indicate that a proportion of synaptic gephyrin clusters are not associated with GABA A $\beta 3$ -IR.

Gephyrin and GABA A $\gamma 2$ subunit clusters associate at extrasynaptic sites

The synaptic gephyrin clusters not associated with the GABA A $\beta 3$ clusters might have been associated with other GABA A subunits. We therefore analyzed the colocalization of the GABA A $\gamma 2$ subunit and gephyrin at the synapse during synaptogenesis using triple labelling (see examples in Fig. 6 and quantitative analysis in Fig. 7). At all stages examined and in contrast to the GABA A $\beta 3$ -IR, clusters of GABA A $\gamma 2$ -IR could be detected in all neurons even at 3 and 7 DIV (Fig. 6A), i.e. before GABAergic bouton differentiation (see Figs. 1A and B and 2A) at stages when gephyrin-IR was still weak (Figs. 6A2 and 5B1). These GABA A $\gamma 2$ subunit clusters were first detected at extrasynaptic sites and were not associated with gephyrin (Fig. 6, open arrowheads). Upon maturation, the proportion of GABA A $\gamma 2$ subunit clusters associated with gephyrin increased (Fig. 6, double open-closed arrowheads) and were detected at synaptic sites (Fig. 6, triple-crossed arrows). Yet, even at later stages, extrasynaptic clusters of GABA A $\gamma 2$ subunits associated with gephyrin were consistently detected. We then analyzed the association of gephyrin and GABA A $\gamma 2$ subunit clusters in relation to their synaptic localization at 10, 14, 17, and 21 DIV (exemplified in Fig. 6 and quantified in Fig. 7). During synaptogenesis, the evolution of the number of GABA A $\gamma 2$ subunit clusters closely paralleled that of gephyrin clusters (Fig. 7A). At 10 DIV, 69.4% of the GABA A $\gamma 2$ clusters were extrasynaptic (Fig. 7B1). From 14 DIV the proportion of synaptic clusters became more abundant, reaching 82.2% at 21 DIV (Fig. 7B1). Thus, even at later stages, a significant proportion of the GABA A $\gamma 2$ subunit clusters remained extrasynaptic (Fig. 7B1; see also Fig. 6D, open and double arrowheads). We then examined whether a differential association with gephyrin could account for this synaptic vs extrasynaptic localization of GABA A . For this purpose, we quantified (Fig. 7B2) the evolution of the four pools of GABA A $\gamma 2$ clusters as synaptic or extrasynaptic, and associated or not with gephyrin. This led to the notion that during synaptogenesis, GABA A $\gamma 2$ subunit clusters were first extrasynaptic and not associated with gephyrin (Figs. 6A and B, open arrowheads). This indicates that gephyrin is not required for GABA A $\gamma 2$ subunit clustering. In addition, a significant proportion of GABA A $\gamma 2$ clusters remained extrasynaptic but colocalized with gephyrin (25.4% at 10 DIV and 11.6% at 21 DIV; Fig. 7B2; also see Fig. 6, double open-closed arrowheads). This suggests that gephyrin alone is not sufficient to accumulate the GABA A $\gamma 2$ subunit clusters at synaptic sites. However, since the number of synaptic GABA A $\gamma 2$ subunit–gephyrin complexes markedly increased during maturation, the extrasynaptic GABA A $\gamma 2$ –gephyrin complexes could correspond to kinetic intermediates en route to synaptic sites.

During maturation, the proportion of GABA A $\gamma 2$ subunit clusters not colocalized with gephyrin significantly

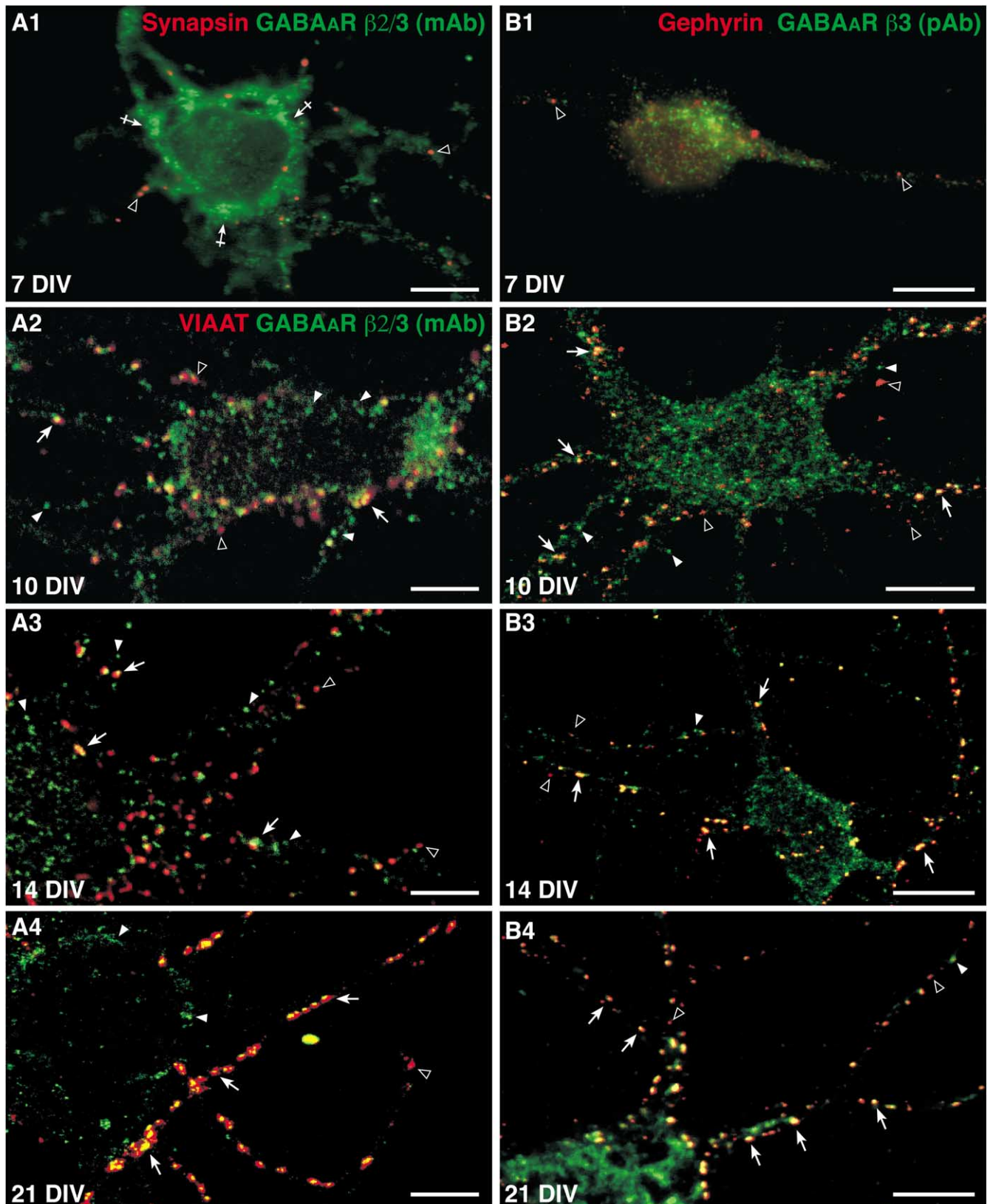


Fig. 3. Association of GABAAR $\beta 3$ subunits (green) with synaptic boutons and gephyrin. (A1–4) Double detection of GABAAR $\beta 2/3$ subunits (green, with the bd17 antibody) and synapsin (red; A1) or VIAAT (red; A2–4) after 7 (A1), 10 (A2), 14 (A3), and 21 (A4) days in culture. At 7 DIV, GABAAR $\beta 2/3$ displayed a diffuse immunoreactivity (IR) in the soma (A1) with brighter immunoreactive blobs (crossed arrows). At 10 days (A2), GABAAR $\beta 2/3$ -IR clusters emerged from the diffuse-IR. The number of GABAAR $\beta 2/3$ -IR clusters associated with VIAAT-IR (arrows) increased with maturation, but some single-labeled GABAAR $\beta 2/3$ clusters (arrowheads) persisted even at later stages (A4) while boutons that were not facing GABAAR $\beta 2/3$ -IR were detected at all stages (open arrowheads). (B1–4) Double detection of GABAAR $\beta 3$ subunit-IR (green, with a polyclonal antibody; Todd et al., 1996) and gephyrin-IR (red; B1–4) after 7, 10, 14, and 21 days in culture. At 7 DIV, gephyrin formed small clusters (open arrowheads) in some neurons while GABAAR $\beta 3$ -IR was diffused. At 10 (B2), 14 (B3), and 21 (B4) DIV, an increasing number of GABAAR $\beta 3$ -IR clusters colocalized with gephyrin-IR clusters (arrows). At all stages, some GABAAR $\beta 3$ -IR clusters were not associated with gephyrin (arrowheads) and some gephyrin clusters were not associated with GABAAR (open arrowheads). Scale bars, 10 μm .

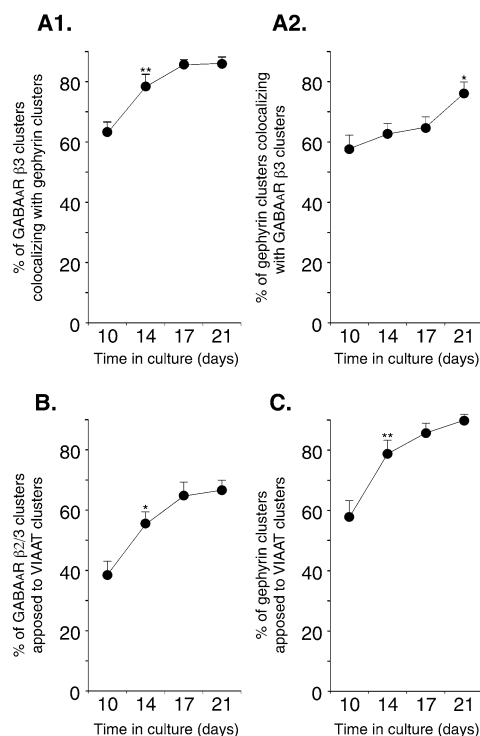


Fig. 4. Quantitative analysis of GABAAR β 3-IR, gephyrin-IR, and VIAAT-IR colocalization during synaptogenesis at 10, 14, 17, and 21 days in culture. Asterisks indicate significant differences between the point and the preceding one (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (A1) Percentage of clusters of GABAAR β 3 subunit colocalizing with gephyrin clusters (number of GABAAR β 3 clusters colocalizing with gephyrin clusters vs total number of GABAAR β 3 clusters). (A2) Percentage of gephyrin clusters colocalizing with GABAAR β 3 clusters vs total number of gephyrin clusters; 785, 2425, 1827, and 2766 GABAAR β 3 clusters and 835, 2970, 2485, and 3280 gephyrin clusters were counted on 15 different neurons at each stage). (B) Percentage of GABAAR β 2/3 clusters colocalizing with VIAAT clusters (number of GABAAR β 2/3 clusters colocalizing with VIAAT-IR puncta vs total number of GABAAR β 2/3 clusters; 973, 1402, 1134, and 3098 GABAAR β 2/3 clusters were counted in 11, 11, 19, and 20 neurons respectively). (C) Percentage of gephyrin clusters apposed to VIAAT puncta (number of gephyrin clusters apposed to VIAAT-IR puncta vs total number of gephyrin clusters; 1298, 2970, 1892, and 2454 gephyrin clusters were counted in 14, 15, 15, and 15 different neurons, respectively). Values are means \pm SEM.

decreased both at synaptic and extrasynaptic sites. Actually, the proportion of extrasynaptic GABAAR γ 2 subunit clusters without gephyrin-IR decreased from 44.0% at 10 DIV to 6.2% at 21 DIV (diamonds in Fig. 7B2; also see Fig. 6, open arrowheads), while the proportion of synaptic GABAAR γ 2 subunit clusters without gephyrin, which remained consistently low, decreased from 7.9 to 1.8% between 10 and 21 DIV (circles in Fig. 7B2; also see Fig. 6C, single-crossed arrows). In contrast, the synaptic GABAAR γ 2 subunit-gephyrin colocalization increased to 80.4% at 21 DIV (triangles in Fig. 7B2; also see Fig. 6, triple-crossed arrows and double arrowheads), whereas the proportion of extrasynaptic clusters colocalizing with gephyrin decreased and reached a stable level upon maturation (12.4 and 11.6%

at 17 and 21 DIV; squares in Fig. 7B2; also see Fig. 6, double arrowheads). These observations favor the notion that GABAAR γ 2 subunit clusters might be stabilized by their association with gephyrin, independently of their synaptic localization.

A symmetric analysis was performed to evaluate the influence of gephyrin–GABAAR γ 2 subunit association in the stabilization of the gephyrin (Fig. 7C). The evolution from 10 DIV to 21 DIV of the gephyrin clusters was compared to that of GABAAR γ 2 subunit and synapsin. At 10 DIV, 49.1% of the gephyrin clusters were synaptic and 50.9% were extrasynaptic (Fig. 7C1). As for the GABAAR γ 2 subunit clusters, the synaptic localization of gephyrin increased during maturation (86.7% at 21 DIV; Fig. 7C1), whereas the proportion of extrasynaptic clusters decreased from 50.9% to 15.9 and 13.3% at 17 and 21 DIV, respectively. As for the GABAAR γ 2 clusters, a significant proportion of extrasynaptic gephyrin clusters colocalized with GABAAR γ 2 subunit clusters (from 30.9% at 10 DIV to 12.0% at 21 DIV; squares in Fig. 7C2; also see Fig. 6, double arrowheads). This favors the notion that association of GABAAR γ 2 subunit clusters with gephyrin is not sufficient to direct the complex to the synapse. We also observed that the proportion of gephyrin not associated with GABAAR γ 2 subunit clusters decreased dramatically during maturation (from 40.2 to 4.6% at 10 and 21 DIV; sum of diamonds and circles in Fig. 7C2) independently of their synaptic location. The proportion of nonsynaptic gephyrin colocalized with GABAAR γ 2 subunit clusters remained relatively high at later stages of maturation (13.3 and 12.0% at 17 and 21 DIV, respectively; squares in Fig. 7C2). This suggests that gephyrin clusters are stabilized by their association with GABAAR γ 2 clusters independently of their synaptic localization.

We also compared the proportion of GABAAR γ 2–gephyrin complexes to that of GABAAR γ 2 clusters not containing gephyrin (Fig. 7D) at synaptic sites. The comparison stage by stage was very instructive. At 10 DIV, 48.4% of the GABAAR γ 2–gephyrin complexes were located at synaptic sites (triangles). In contrast, at the same stage, only 15.5% of the GABAAR γ 2 subunit clusters without gephyrin were synaptic (squares). At 21 DIV, 87.3% of the GABAAR γ 2–gephyrin complexes were synaptic, whereas only 25.6% of the GABAAR γ 2 subunit clusters without gephyrin were synaptic. We next compared the proportion of gephyrin clusters not associated with GABAAR γ 2 but located at synaptic sites (circles) with that of gephyrin associated with GABAAR γ 2 detected at synaptic sites (triangles). Even in the absence of GABAAR γ 2-IR, 52.1% and up to 72.8% of gephyrin clusters not associated with GABAAR γ 2 were detected at synaptic sites after 10 and 21 DIV, respectively (Fig. 7D). These comparisons indicate that association with gephyrin favors the synaptic localization of GABAAR γ 2 clusters but that the synaptic localization of the gephyrin clusters is independent of their association with GABAAR γ 2 clusters.

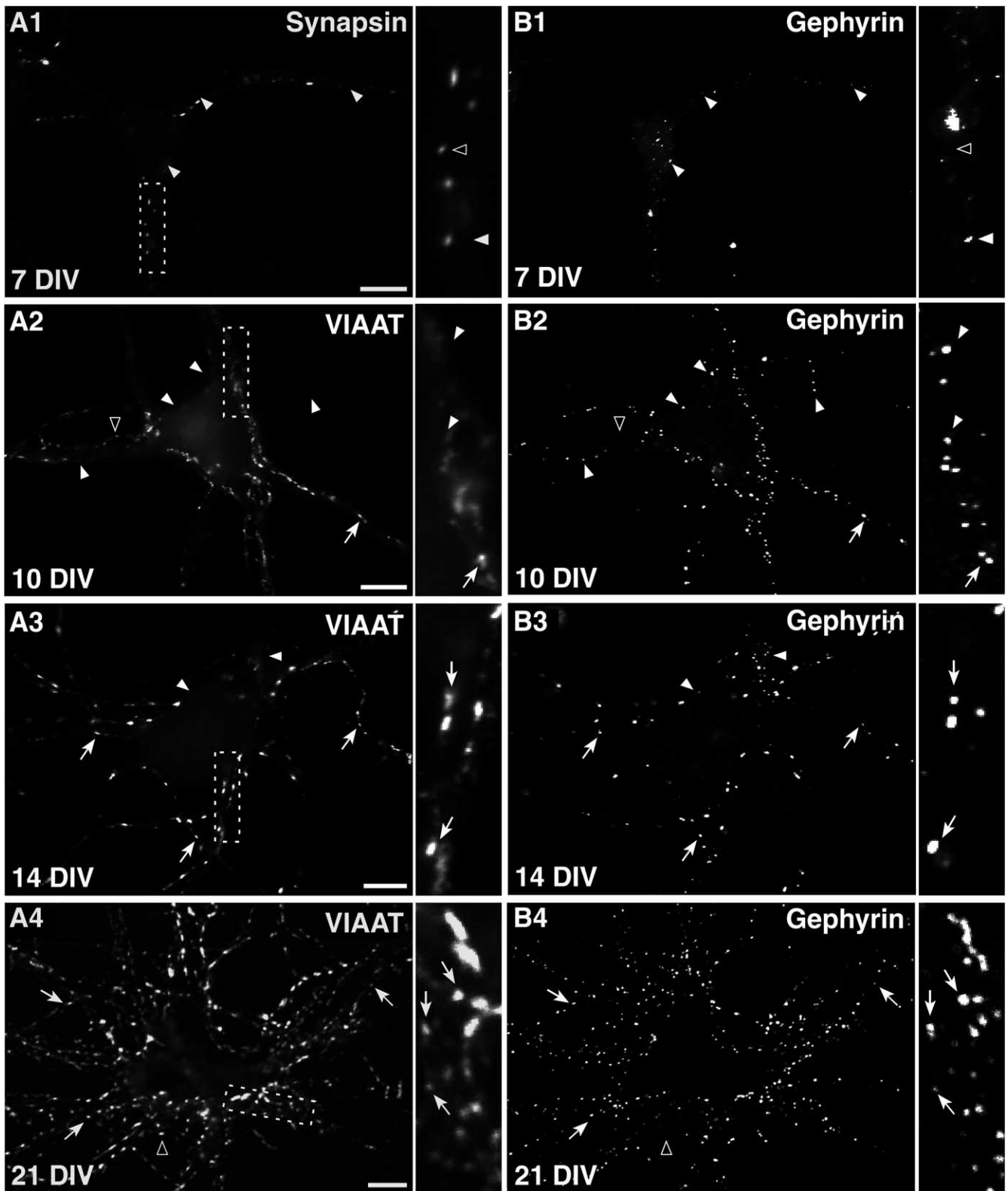


Fig. 5. Gephyrin at synaptic and extrasynaptic sites after 7, 10, 14, and 21 DIV. Double detection of gephyrin (B1–4) with synapsin (A1) or VIAAT (A2–4). Panels on the right are magnifications of the boxed windows. At 7 DIV, few gephyrin-IR clusters with low intensity were detected at extrasynaptic sites (arrowheads). Some synapsin-IR puncta, or at later stage, some VIAAT-IR puncta were not apposed to gephyrin-IR clusters (open arrowheads). The number of gephyrin clusters associated with VIAAT immunoreactivity (arrows) increased with time. Note that in mature culture, even small clusters of gephyrin could be apposed to VIAAT-IR (B4, right arrow). Scale bars, 10 μ m.

Discussion

In this study, we have found that: (1) the GABAergic presynaptic elements were generated 3 to 7 days after the glutamatergic boutons; (2) the GABA_AR subunits became mainly synaptic about 4 days after initial differentiation of the presynaptic elements; and (3) GABA_AR and gephyrin clusters were first expressed as independent extrasynaptic clusters and were associated prior to being detected at synaptic sites.

Inhibitory and excitatory synaptogenesis display a different but coordinated temporal sequence

Excitatory and inhibitory terminals are known to be segregated on different cellular target domains (Benson and Cohen, 1996), but the developmental aspect of this organization has not been analyzed. This is important since in vivo as well as in culture, GABAergic neurotransmission is first excitatory (at 4 to 9 DIV in culture; Ganguly et al., 2001) and then is inhibitory. During the early developmental period, all neurons display a GABA-mediated depolarization due to a high intracellular chloride concentration (Ganguly et al., 2001). Upon maturation, a chloride-extruding system becomes operative, intracellular chloride concentration decreases, and GABA begins to exert its conventional hyperpolarization (refs in Ben-Ari, 2002). We have now observed that in cultured hippocampal neurons, during the early developmental period when GABA is excitatory, punctiform accumulation of GABAergic presynaptic markers could be immunologically detected only in very few neurons (not shown). This early and scarce expression is consistent with a previous observation (Benson and Cohen, 1996) of a few GAD-65-IR boutons contacting neurons 4 days after plating. In contrast, a massive punctiform VIAAT and GAD-65 staining only appears around 10 DIV, at the time when GABA switches from a depolarizing to a hyperpolarizing effect. Since vesicular presynaptic proteins are not expressed before this stage, this suggests that depolarization if any may result from a nonvesicular release of GABA (see also Demarque et al., 2002; Mozhayeva et al., 2002).

Synapsin- and VGLUT1-IR boutons were detected on neurons at 3–7 DIV, that is, 3–4 days before detection of GABAergic terminals. Yet, at the postsynaptic side, the GABA_AR and gephyrin massively colocalize by 14 DIV and are strongly synaptic after about 3 weeks in culture (see also Craig et al., 1996; Levi et al., 2002). It has been shown that during the second week of culture, PSD-95 can associate with nonsynaptic NMDA receptor clusters and that postsynaptic glutamatergic domains display a mature pattern of expression 3 weeks after plating (Rao et al., 1998). This suggests that although early stages for excitatory and inhibitory bouton differentiation are not synchronized, the total duration of synaptogenesis is somehow similar.

Gephyrin and GABA_AR associate at synaptic and extrasynaptic sites

GABA_AR clusters have been reported to extensively colocalize with the tubulin-binding protein gephyrin (Dumoulin et al., 2000; Fischer et al., 2000; Giustetto et al., 1998; Levi et al., 1999; Sassoe-Pognetto et al., 1995, 2000; Triller et al., 1987). However, the synaptic vs extrasynaptic localization of gephyrin-associated GABA_AR had not been investigated during development. We have now directly demonstrated the association of both synaptic and extrasynaptic GABA_AR $\gamma 2$ subunits with gephyrin. This association at extrasynaptic sites was also demonstrated for the GABA_AR $\beta 3$ subunits. Indeed, at 21 DIV, more than 86% of the GABA_AR $\beta 3$ subunit clusters were associated with gephyrin (Fig. 4), whereas 33% of these clusters were extrasynaptic. This implied that, as observed for the GABA_AR $\gamma 2$ subunit, a significant proportion of extrasynaptic GABA_AR $\beta 3$ subunit clusters are associated with gephyrin.

GABA_AR $\gamma 2$ and $\beta 2/3$ subunits behave differently during synaptic maturation. GABA_AR $\gamma 2$ subunits were readily expressed as clusters, whereas the GABA_AR $\beta 3$ subunits were first diffusely distributed. This implies that at this stage, some diffusely distributed $\beta 3$ subunits containing GABA_AR do not contain the $\gamma 2$ subunit. These data agree with the fact that at 21 DIV, 35% of the GABA_AR clusters containing $\beta 2/3$ subunits do not contain $\gamma 2$ subunits (Scotti and Reuter, 2001). At 10 DIV, the clustered GABA_AR $\beta 3$ subunits were more strongly associated with gephyrin (63%) than the GABA_AR $\gamma 2$ subunits (48%), but this difference diminished at 21 DIV (86% for the $\beta 3$ subunits, versus 91% for the $\gamma 2$ subunits). However, it is worth noting that at 21 DIV, gephyrin was more frequently associated with GABA_AR $\gamma 2$ (95.4%) than with the GABA_AR $\beta 3$ subunits (75%). At this stage, whereas a large proportion (>80%) of GABA_AR $\gamma 2$ subunits are detected at synaptic sites, a smaller portion of GABA_AR $\beta 2/3$ subunits were apposed to terminal boutons (66%; see Fig. 4B). In some experiments performed at a late stage in vitro (21 DIV) and using rat or mouse neurons (Kannenberget al., 1999; Kneussel et al., 1999; Levi et al., 2002; Scotti and Reuter, 2001), the proportion of GABA_AR clusters at synapses varied from 30 to 68% and from 35 to 72% for the $\beta 2/3$ and $\gamma 2$ subunits, respectively. Our quantitative results at 21 DIV are in agreement with those of Rao et al. (1998) and Levi et al., (2002). It is worth noting that the GABA_AR $\gamma 2$ subunit antibody (Fritschy and Mohler, 1995) that we have used is particularly efficient for detection (Brunig et al., 2002a); this may explain the higher synapsin–GABA_AR $\gamma 2$ subunit colocalization detected in our study. In addition, we have used the anti-synapsin antibody, which labels both inhibitory and excitatory terminals; therefore the GABA_AR $\gamma 2$ subunit clusters not apposed to synapsin-IR terminal endings are truly extrasynaptic and are not apposed to glutamatergic endings.

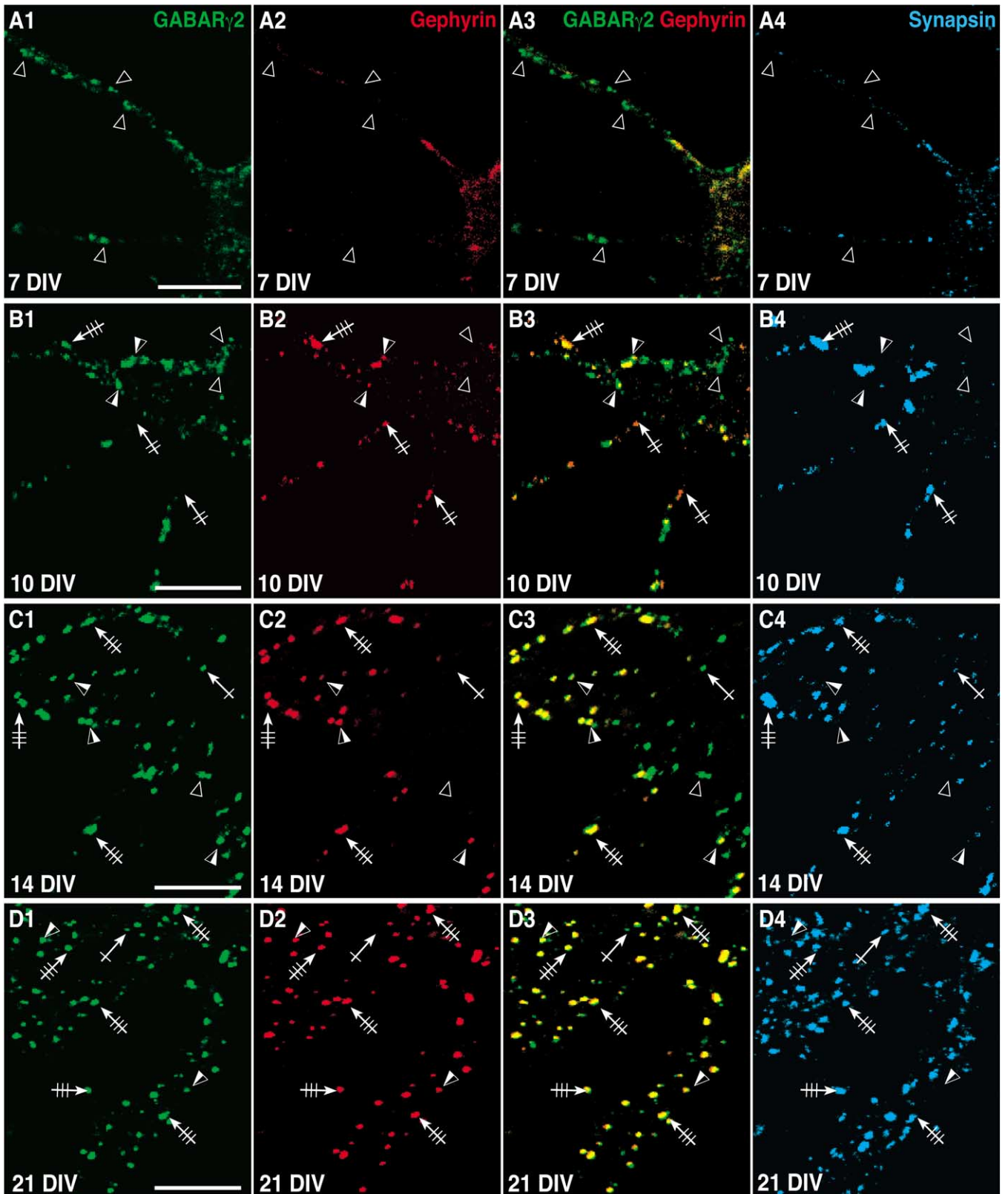


Fig. 6. Triple detection of GABAAR $\gamma 2$ subunit (green), gephyrin (red), and synapsin (blue) at 7 (A) 10 (B), 14 (C), and 21 (D) days in culture. Symbols are: crossed arrows, synapsin–GABAAR $\gamma 2$ subunit apposition; double-crossed arrows, synapsin–gephyrin apposition; triple-crossed arrows, synapsin–gephyrin–GABAAR $\gamma 2$ subunit apposition; open arrowheads, GABAAR $\gamma 2$ subunit clusters alone; open-closed double arrowheads, gephyrin–GABAAR $\gamma 2$ subunit colocalization with no synapsin apposition. Note that at 7 DIV (A), clusters of GABAAR $\gamma 2$ subunits are extrasynaptic and not associated with gephyrin (open arrowheads). At 10 DIV (B), clusters of gephyrin were observed without GABAAR $\gamma 2$ subunits at synaptic sites (double-crossed arrows) or colocalized with GABAAR $\gamma 2$ subunits clusters (double arrowheads) and the proportion of extrasynaptic GABAAR $\gamma 2$ subunit clusters not associated with gephyrin (open arrowheads) decreased. At 14 days (C), most of the GABAAR $\gamma 2$ staining colocalized with gephyrin either at synaptic (triple-crossed arrows) or extrasynaptic sites (double arrowheads). At 21 DIV (D), the proportion of triple-labeled clusters (triple-crossed arrows) increased but extrasynaptic GABAAR $\gamma 2$ –gephyrin complexes (double arrowheads) were still detected. Pictures were acquired on a confocal microscope. Scale bars, 10 μm .

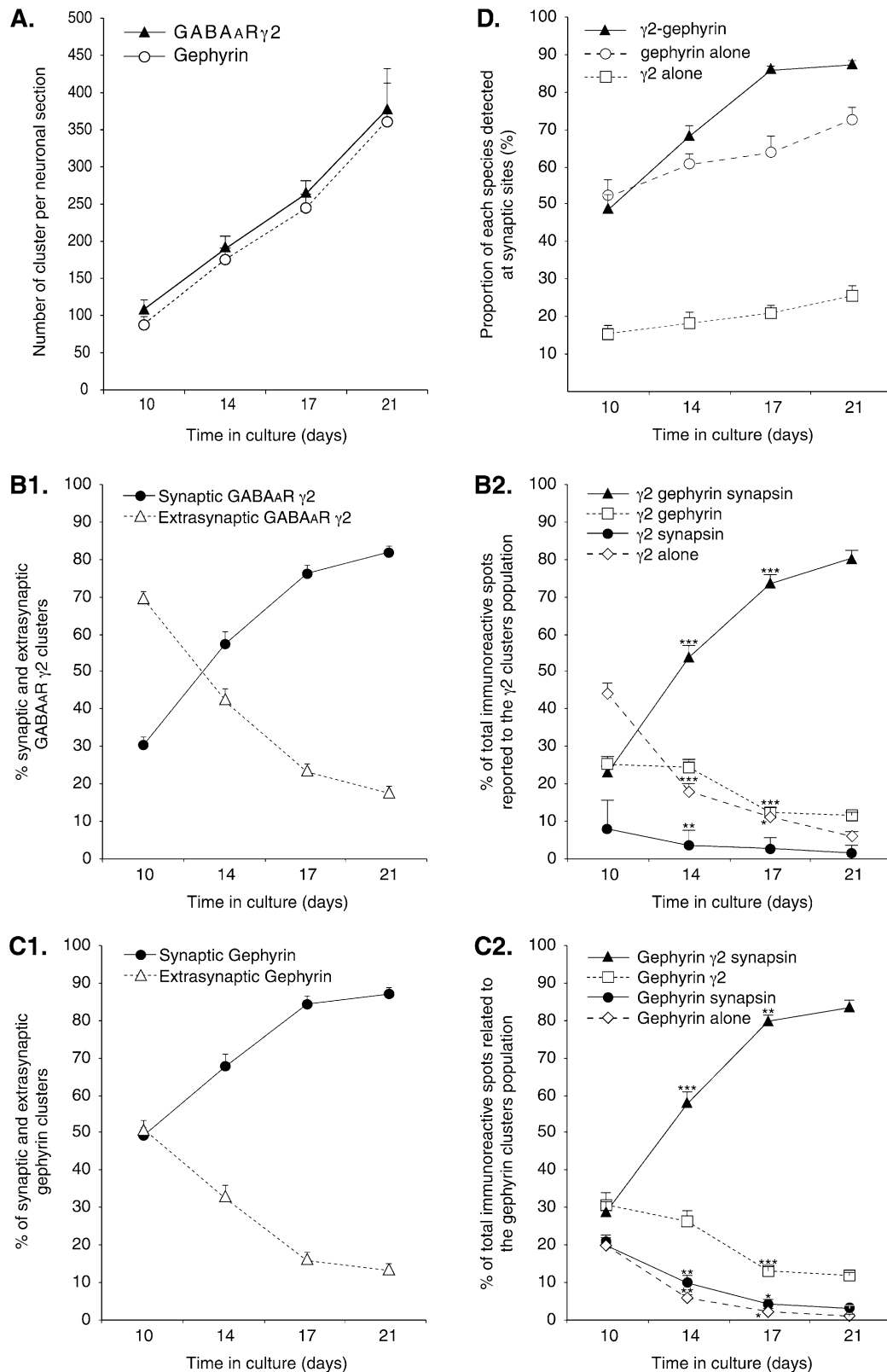


Fig. 7. Quantification of gephyrin, synapsin, and GABAAR $\gamma 2$ colocalization or apposition during hippocampal neuron maturation. (A) Mean number of clusters of GABAAR $\gamma 2$ subunits and gephyrin per confocal neuronal section (16 neurons). (B1) Percentage of synaptic vs extrasynaptic GABAAR $\gamma 2$ clusters during development. The proportion of synaptic GABAAR $\gamma 2$ increased, but even at 21 DIV a significant proportion of GABAAR remained extrasynaptic. (B2) Percentage of GABAAR $\gamma 2$ clusters colocalizing with gephyrin clusters and/or synapsin puncta during neuronal maturation. The absolute number of cluster associations was related to the GABAAR $\gamma 2$ cluster population. Asterisks indicate significant differences between the point and the preceding one (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (C1) Percentage of synaptic vs extrasynaptic gephyrin clusters during development. (C2) Percentage of gephyrin clusters associated with GABAAR $\gamma 2$ clusters or synapsin puncta. The absolute number of cluster associations was related to the gephyrin population. Asterisks indicate significant differences between the point and the preceding one (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (D) Gephyrin-enhanced synaptic

Gephyrin is dispensable for GABA_AR clustering, but is implied in their stabilization

It has been proposed that in cultured motoneurons (Levi et al., 1999) as well as in hippocampal neurons, larger ($0.4\text{--}10\ \mu\text{m}^2$) and smaller ($0.2\ \mu\text{m}^2$) clusters would be synaptic and extrasynaptic, respectively (Craig et al., 1996). In our work, 41.4% ($n = 4352$ clusters) of the gephyrin clusters are $<0.4\ \mu\text{m}^2$ at 21 DIV and 90% of the gephyrin clusters were synaptic. This indicates that even small gephyrin clusters can be synaptic (see Fig. 5B4). An *in vivo* study in the dentate gyrus has suggested that the size of the gephyrin clusters could be related to the nature of the neurons (Simburger et al., 2000). We have not observed in our culture a cell bias in the gephyrin cluster sizes. Actually, it was shown *in vivo* and in mature networks that the size of the gephyrin clusters matched those of the postsynaptic density (Triller et al., 1985, 1987) and that of the adjacent postsynaptic vesicular grid (Sur et al., 1995; Triller et al., 1990). This was also found to be true for inhibitory synapses in rat spinal cord neurons *in vivo* (Alvarez et al., 1997; Colin et al., 1998).

Before 10 DIV, few gephyrin clusters were detected and only in few neurons, whereas GABA_AR $\gamma 2$ subunit clusters were observed in all neurons. This is in apparent contradiction with the observation that in gephyrin knockout (KO) mice, GABA_AR $\gamma 2$ and $\beta 2/3$ subunits are expressed, but that the formation of clusters is impaired (Kneussel et al., 1999, 2001). Actually, GABA_AR $\gamma 2$ subunit clusters have been described in retinal and hippocampal cultures and in spinal cord sections of the gephyrin KO mice (Fischer et al., 2000; Kneussel et al., 2001; Levi et al., 2002), indicating that the GABA_AR $\gamma 2$ clustering might be independent of gephyrin expression. This is supported by our observation that a significant and decreasing proportion (from 50% at 10 DIV to 8% at 21 DIV) of GABA_AR $\gamma 2$ and $\beta 3$ subunits clusters not associated with gephyrin were detected at all stages. Two hypotheses may account for this observation. First, upon maturation, the GABA_AR $\gamma 2$ subunit clusters could be stable and would associate subsequently with the increasing number of gephyrin clusters. However, the parallel evolution of the absolute number of GABA_AR $\gamma 2$ subunits and gephyrin clusters (Fig. 7), as well as the loss of GABA_AR $\gamma 2$ clusters in gephyrin KO mice (Kneussel et al., 1999), argues against such a mechanism. Alternatively, the association of GABA_AR clusters with gephyrin could stabilize the previously clustered receptors by prolonging their half-time and preventing internalization (Kneussel et al., 1999; Rasmussen et al., 2002). This explanation is compat-

ible with the dramatic decrease in the number of gephyrin clusters not containing the GABA_AR $\gamma 2$ subunit observed during maturation as well as the remaining 12% of extrasynaptic GABA_AR $\gamma 2$ -gephyrin complexes found at 17 and 21 DIV. These results are in accordance with those of Essrich et al. (1998), who established that gephyrin staining is profoundly reduced in cortical neurons of GABA_AR $\gamma 2$ -deficient mice.

Recently, several studies have highlighted the selective association of dystrophin glycoprotein complex (DGC) with GABAergic synapses in hippocampal neurons (Brunig et al., 2002b; Knuesel et al., 1999; Levi et al., 2002). However, this complex is not essential for GABAergic synaptogenesis (Levi et al., 2002) and its clustering is unaffected by the absence of GABA_AR $\gamma 2$ subunit (Brunig et al., 2002b) or gephyrin (Brunig et al., 2002b; Levi et al., 2002). It has been proposed that DGC might stabilize the scaffold at inhibitory synapses (Brunig et al., 2002b) and that dystrophin may regulate the synaptic clustering of GABA_AR independently of gephyrin (Knuesel et al., 1999). This putative function of DGC could explain why only few synaptic GABA_AR $\gamma 2$ subunits cluster without gephyrin (≈ 8 to 2% between 10 and 21 DIV).

The existence of extrasynaptic clusters of GABA_AR subunits had previously been reported (Christie et al., 2002; Kannenberg et al., 1999). We have now demonstrated that some of these extrasynaptic clusters are associated with gephyrin. At 21 DIV, 87% of the GABA_AR $\gamma 2$ subunit-gephyrin complexes were synaptic, whereas only 26% of the non-gephyrin-containing GABA_AR $\gamma 2$ clusters were at synapses (Fig. 7D). This is consistent with the notion that gephyrin favors the synaptic anchoring of GABA_AR. If this holds to be true, something is still missing since in 21 DIV neurons 11.6% of the GABA_AR $\gamma 2$ subunit clusters colocalized with gephyrin were extrasynaptic. Actually, extrasynaptic clusters of GABA_AR have been proposed to serve as GABA spillover sensors (Brunig et al., 2002a). They could also be kinetic intermediates before recruitment to postsynaptic domains. As reported in spinal interneurons, newly synthesized glycine receptors diffuse in the plasma membrane as stable clusters (Rosenberg et al., 2001), and the interaction of glycine receptor with gephyrin increases its rate of accumulation at synapses (Meier et al., 2000, 2001). An association of receptors with anchoring proteins prior to their accumulation at synapses has already been reported for the acetylcholine receptor and rapsyn (Marchand et al., 2001). In cultured hippocampal neurons, some nonsynaptic NMDA receptor clusters are associated with Psd-95/Sap90 and GKAP (Rao et al., 1998). Thus, a preas-

localization of GABA_AR $\gamma 2$ clusters. Black triangle, number of synaptic GABA_AR $\gamma 2$ -gephyrin complexes was related to the number of the GABA_AR $\gamma 2$ -gephyrin complex (synaptic + extrasynaptic GABA_AR $\gamma 2$ -gephyrin). Open circle, number of synaptic gephyrin clusters not associated with GABA_AR $\gamma 2$ clusters was related to the population of gephyrin clusters (synaptic and extrasynaptic) that were not associated with GABA_AR $\gamma 2$ clusters. Open square, number of synaptic GABA_AR $\gamma 2$ clusters not associated with gephyrin was related to the total population (synaptic and extrasynaptic) of GABA_AR $\gamma 2$ clusters that were not associated with gephyrin. Respectively 1623, 2848, 3950, and 5661 GABA_AR $\gamma 2$ clusters and 1316, 2636, 3667, and 5430 gephyrin clusters were analyzed on 16 neurons at each stage. Values are means \pm SEM.

sembly of synaptic components while being targeted to the postsynaptic domains might exist as suggested for the pre-synaptic components (Ahmari et al., 2000; Zhai et al., 2001).

Although gephyrin plays a central role in inhibitory synapse biology, our *in vitro* developmental analysis indicates that neither formation of GABA_AR clusters nor their accumulation at synapses depends on a “gephyrin-only” mechanism. Other sets of molecules should be involved in GABA_AR cluster formation, accumulation at synapses, and subsequent stabilization. These three mechanisms may depend upon different molecules and specific regulations. Many molecules interacting with gephyrin (Kneussel and Betz, 2000; Moss and Smart, 2001) have been identified, but their exact functions in clustering and stabilization remain to be determined.

Experimental methods

Cell cultures

Hippocampal cultures were prepared as described (Banker and Cowan, 1977; Goslin and Banker, 1998). Briefly, hippocampi were dissected from 18-day-old, fetal Sprague–Dawley rats. Cells were dissociated by treatment with 0.25% trypsin for 15 min at 37°C, and triturated through a fire-constricted Pasteur pipette in DNase (0.1 mg/ml; Sigma). Neurons were plated onto polyornithine-coated glass coverslips (12-mm diameter) at a density of 5000 cells/cm² in minimal essential medium (MEM; Gibco BRL) supplemented with 10% horse serum, 0.6% glucose, 0.2% sodium bicarbonate, 2 mM glutamine, and 10 IU/ml penicillin–streptomycin. Neurons were grown on an astroglial feeder layer and maintained in serum-free neurobasal medium supplemented with B27 (Life Technologies) for up to 7 weeks at 37°C in a 5% CO₂ humidified incubator.

Antibodies

Synapsin was detected with a rabbit antiserum (0.1 µg/mL; Novus Biological, Littleton, CO) or a mouse monoclonal antibody (1:3000, Synaptic System GmbH). Inhibitory terminal boutons were identified either with a mouse monoclonal antibody against GAD-65 (5 µg/mL, Boehringer–Mannheim) or a rabbit anti-VIAAT (1:200; kindly provided by B. Gasnier, Paris). Glutamatergic terminal boutons were identified by a rabbit anti-VGLUT1 (1:200; kind gift of B. Giros). Mab 7a specific for gephyrin was from Alexis Biochemicals (San Diego, CA, 1 µg/mL). For staining of GABA_AR receptor subunits, we used: (1) a mouse monoclonal anti-GABA_AR β2/3 subunit (mAb bd17 10 µg/mL, Chemicon Int.), (2) a rabbit anti-GABA_AR β3 subunit (1:20; a generous gift from W. Sieghart, Vienna; Todd et al., 1996) and (3) a guinea pig anti-GABA_AR γ2 (1:2000; generously given by J.M. Fritschy, Zurich; Fritschy and

Mohler, 1995). Double detection experiments were performed with mAb (mouse) and pAb (rabbit or guinea pig) antibodies. Except for the polyclonal anti-GABA_AR, mAb and pAb were combined with Cy3-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, dilution 1:500). The pAb anti-GABA_AR β3 subunit was revealed by a biotinylated goat anti-rabbit IgG, and the pAb anti-GABA_AR γ2 subunit by a biotinylated goat anti-guinea pig IgG, followed by streptavidin–FITC.

Immunocytochemistry

For all detection, neurons were assayed every 3 days from 3 to 21 DIV. Except for GABA_AR detection (see below), neurons were fixed with 4% paraformaldehyde–4% (w/v) sucrose in PBS for 20 min at room temperature, quenched in 50 mM NH₄Cl in PBS for 20 min, permeabilized with 0.1% (w/v) Triton, 0.1% (w/v) gelatin in PBS for 4 min, and then blocked in 0.25% (w/v) gelatin in PBS for 30 min. They were then incubated with primary antibody in 0.1% (w/v) gelatin in PBS for 1 h at room temperature and subsequently incubated with secondary fluorescent antibodies for 45 min at room temperature. For the GABA_AR γ2 staining, living cells were incubated for 35 min at 37°C with anti-GABA_AR γ2 antibody, fixed for 10 min in methanol at –20°C, blocked in 0.25% (w/v) gelatin in PBS for 30 min, and then incubated with other antibodies. This labeling and fixation protocol has been shown to prevent antigen capping by the secondary antibodies and clustering artifacts (Brunig et al., 2002a). For the GABA_AR β3 immunostaining, living cells were fixed for 10 min in graded ethanol, blocked in 0.25% (w/v) gelatin in PBS for 30 min, and then incubated with antibodies.

Standard epifluorescence microscopy

Fluorescence images were acquired on a Leica DMRD microscope (objective 63X/1.32 or 40X/1.25) using FITC, Cy3, and DAPI-specific sets of filters and a high-resolution camera (MicroMax Princeton Instruments) driven by the Metamorph Image Analysis System. Image files were merged for colocalization using Adobe Photoshop 5.0.

Confocal microscopy and quantifications

Specimens were observed with a 63X oil immersion objective, followed by a 1.6× digital zoom magnification. Images were acquired on a Leica SP2 confocal microscope by sequential scanning of the emission lines. FITC was detected by using the 488-nm line of an argon laser for excitation; Cy3 and Cy5 were respectively excited by the 543-nm line of a green neon laser and the 650-nm line of a helium neon laser. Typically, stacks of five to seven sections (1024 × 1024 pixels), with an interval of 0.32 µm, were scanned three times, to optimize the signal/noise ratio.

Quantifications were done on the section showing the highest staining on dendrites. FITC, Cy3, and Cy5 channel images were assembled in Adobe Photoshop 5.0. To avoid the bias introduced by electronic thresholding, the spots were counted manually, centering the soma in the middle of the picture. From every time point and combination of labeling, 15 to 20 sections from 3 to 4 different cultures were counted. Statistical significance was evaluated using Statview (Abacus concept). The level of significance (Mann–Whitney) is indicated by one ($P < 0.05$), two ($P < 0.01$), or three ($P < 0.001$) symbols.

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