

# Collybistin is required for both the formation and maintenance of GABAergic postsynapses in the hippocampus

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## Introduction

Fast synaptic transmission in the nervous system is mediated by ligand-gated ion channels which are densely clustered in the postsynaptic membrane. At inhibitory synapses, the scaffolding protein gephyrin is required for the synaptic localization of glycine receptors and major GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) subtypes (Kneussel and Betz, 2000; Moss and Smart, 2001). Ablation of gephyrin expression by either antisense depletion in cultured neurons or gene knock-out in mice prevents the clustering of glycine receptors (Kirsch et al., 1993; Feng et al., 1998) and  $\alpha$ 2- and  $\gamma$ 2-subunit containing GABA<sub>A</sub>Rs (Essrich et al., 1998; Kneussel et al., 1999) at developing postsynaptic sites. Inversely, inactivation of the GABA<sub>A</sub>R  $\gamma$ 2 subunit gene results in a marked reduction of postsynaptic gephyrin cluster densities (Essrich et al., 1998; Schweizer et al., 2003).

Gephyrin depends on both actin microfilaments and microtubules for synaptic targeting and submembraneous scaffold formation (Allison et al., 2000; Bausen et al., 2006; Kirsch and Betz, 1995; Maas et al., 2006). In addition, it interacts with actin-binding and cytoskeleton regulatory proteins (Bausen et al., 2006; Fuhrmann et al., 2002; Giesemann et al., 2003; Mammoto et al., 1998) including collybistin (Cb), a brain-specific guanine nucleotide exchange factor (GEF) for small Rho-like GTPases (Kins et al., 2000). Cb exists in multiple splice variants

(I–III) (Harvey et al., 2004; Kins et al., 2000), and its human homologue, hPEM-2, has been shown to function as a Cdc42-specific GEF in fibroblasts (Reid et al., 1999). Coexpression of Cb II with gephyrin in human embryonic kidney (HEK) 293 cells alters the subcellular distribution of gephyrin by relocating it from large intracellular aggregates into small clusters at the plasma membrane (Kins et al., 2000). More recently, the crystal structure of the Cdc42–Cb II complex has been determined (Xiang et al., 2006). Biochemical data in this study showed that gephyrin negatively regulates the GEF activity of Cb II, suggesting that gephyrin binding to Cb might help to terminate cellular processes triggered by the Cdc42 signaling cascade which are important during early stages of synapse formation but not required subsequently (Xiang et al., 2006).

In a previous study using Cb-deficient mice, we have found that Cb is an essential determinant of gephyrin and GABA<sub>A</sub>R synaptic clustering in selected regions of the mammalian central nervous system (Papadopoulos et al., 2007). In the hippocampus of adult Cb KO mice, the densities of postsynaptic gephyrin and  $\alpha$ 2- and  $\gamma$ 2-subunit containing GABA<sub>A</sub>R clusters were strongly reduced. This loss of inhibitory postsynaptic proteins was accompanied by a substantial decrease of GABAergic inhibition and significant changes in hippocampal synaptic plasticity. To unravel whether these anatomical and functional deficits reflect a requirement for Cb in synapse formation and/or maintenance *in vivo*, we now used the Cre-loxP system to specifically inactivate the Cb gene in mice at embryonic and postnatal stages. Here, we report that deletion of Cb both during embryonic development and at later

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postnatal stages results in the loss of synaptic gephyrin and GABA<sub>A</sub>R clusters from the dendrites of CA1 pyramidal neurons and the accumulation of gephyrin within cytoplasmic aggregates. This result indicates that Cb is essential for both the initial localization and subsequent stabilization of gephyrin and gephyrin-dependent GABA<sub>A</sub>Rs at specific inhibitory postsynaptic sites.

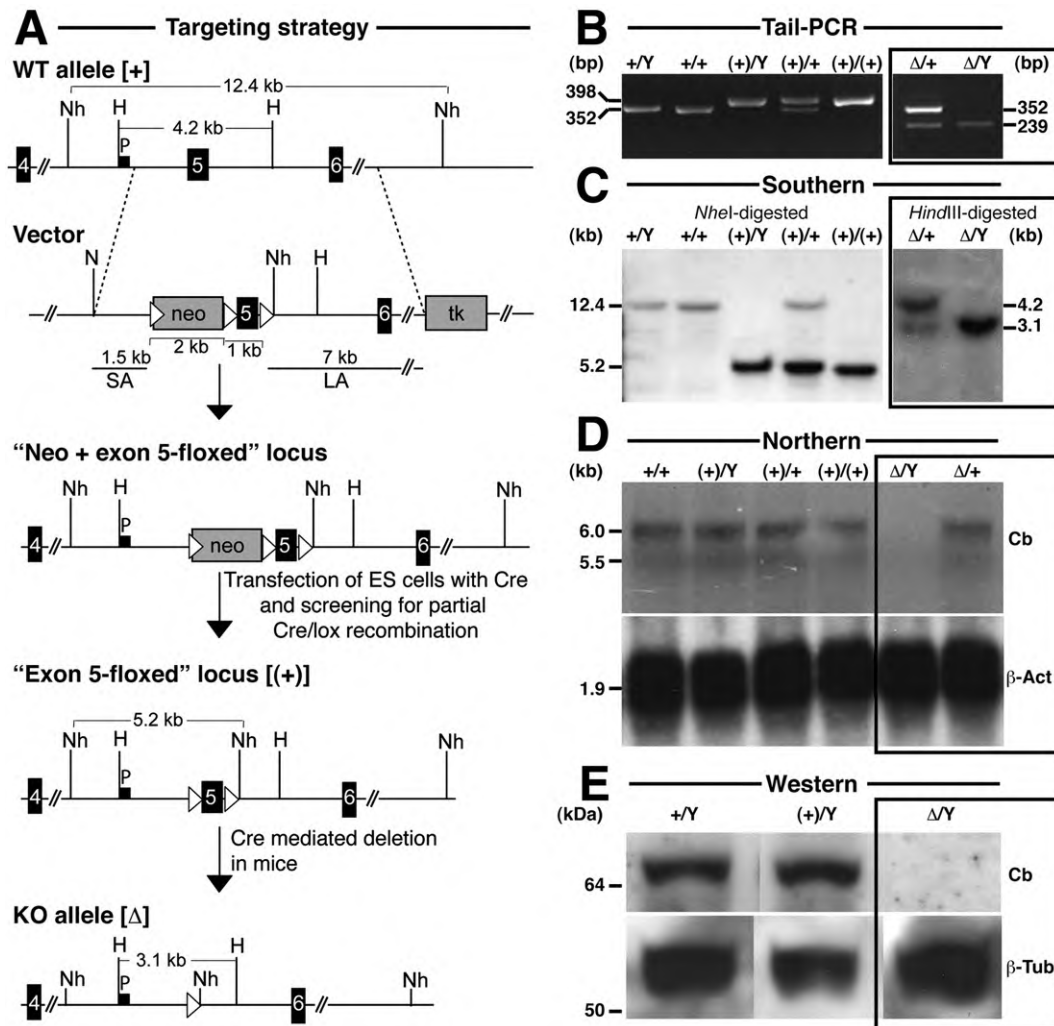
## Results

### Generation of floxed-Cb mice

We have recently demonstrated that inactivation of the Cb gene causes a region-specific loss of postsynaptic gephyrin and GABA<sub>A</sub>R clusters in the adult mouse brain (Papadopoulos et al., 2007). To disrupt the Cb gene in mice in a spatiotemporally defined manner and to clarify its roles during synaptogenesis and at mature synapses, we used the

targeted embryonic stem (ES) cells described previously (Papadopoulos et al., 2007) for generating a mouse line carrying a “floxed” Cb allele. In these ES cells, both a neomycin (neo) resistance cassette and exon 5 of the Cb gene were flanked by loxP sites (Fig. 1A). By transfecting the cells with a Cre-expression vector, we selectively deleted the neomycin (neo) cassette and produced two independent “exon 5-floxed” ES cell clones (data not shown). In these clones, the floxed Cb gene [(+)] differed from the WT locus [+] by the insertion of two loxP sites into the intronic regions upstream and downstream of exon 5 (Fig. 1A). Both ES cell clones were injected into blastocysts to generate chimeric male mice. The resulting animals were then crossed with C57BL/6 female mice to establish germline transmission and to generate (+)/+ females. These mice were then again crossed with C57BL/6 to obtain (+)/Y males. Note that the mouse Cb gene (*Arhgef9*) is located on the X-chromosome.

Mouse genotyping was carried out by PCR (Fig. 1B) and Southern blot analysis (Fig. 1C) of mouse tail DNA. Similar amounts of Cb RNA



**Fig. 1.** Generation of mice with a floxed-Cb gene and Cre-mediated inactivation of the floxed Cb gene. (A) Schematic representation of the targeting strategy showing from top: the WT Cb gene [+], the targeting vector, the exon 5-floxed locus containing the neomycin resistance cassette (neo), the exon 5-floxed locus [(+)] and the KO allele [Δ]. The steps up to homologous recombination in ES cells have been described in Papadopoulos et al. (2007). Exons are represented as black boxes. The neo cassette and the herpes simplex virus thymidine kinase gene (tk) of the targeting vector are indicated as grey boxes, and loxP sites as triangles. P corresponds to the probe used for Southern analysis. SA, short arm; LA, long arm. Relevant restriction sites are indicated: H, HindIII; N, NotI; Nh, NheI. (B) PCR-genotyping of the F1 progeny of representative (+)/+ female mice crossed with either +/Y, (+)/Y or Ell $\alpha$ -Cre (boxed area) C57BL/6-males. The sequences of the three primers used are described under Experimental methods. The longer (398 bp) product is amplified from the (+) locus and includes a loxP site; the 352 bp product corresponds to the + locus, and the short 239 bp product to the inactivated Δ locus resulting from Cre-induced recombination of the (+) locus (see Fig. 3A1). (C) Southern blot analysis of NheI- and HindIII-digested mouse tail DNA from offspring of (+)/+ female mice crossed with either +/Y, (+)/Y (NheI-digestion) or Ell $\alpha$ -Cre (HindIII-digestion in the boxed area) C57BL/6-males. The 12.4 kb and the 5.2 kb bands represent the WT and the floxed allele, respectively. The 4.2 kb and the 3.1 kb bands of tail DNA digested with HindIII represent the WT and the deleted allele, respectively. (D) Upper panel: Northern blot of brain total RNA with a 430-bp cDNA probe encoding exons 1–3 of the mouse Cb gene reveals 5.5 and 6.0 kb Cb transcripts in WT, floxed and heterozygous female mice but not in Δ/Y-offspring of (+)/+ female mice crossed with Ell $\alpha$ -Cre C57BL/6-males (boxed area). Lower panel: control hybridization with a random-primed probe derived from a 714-bp fragment of the mouse β-actin (β-act) cDNA demonstrates that comparable amounts of total RNA were loaded. (E) Western blot analysis of brain homogenates (60 μg protein/lane) using a polyclonal Cb antibody. Note the absence of Cb protein in the Δ/Y sample (boxed area). A β-tubulin (β-Tub)-specific antibody was used to confirm that similar amounts of protein were loaded.

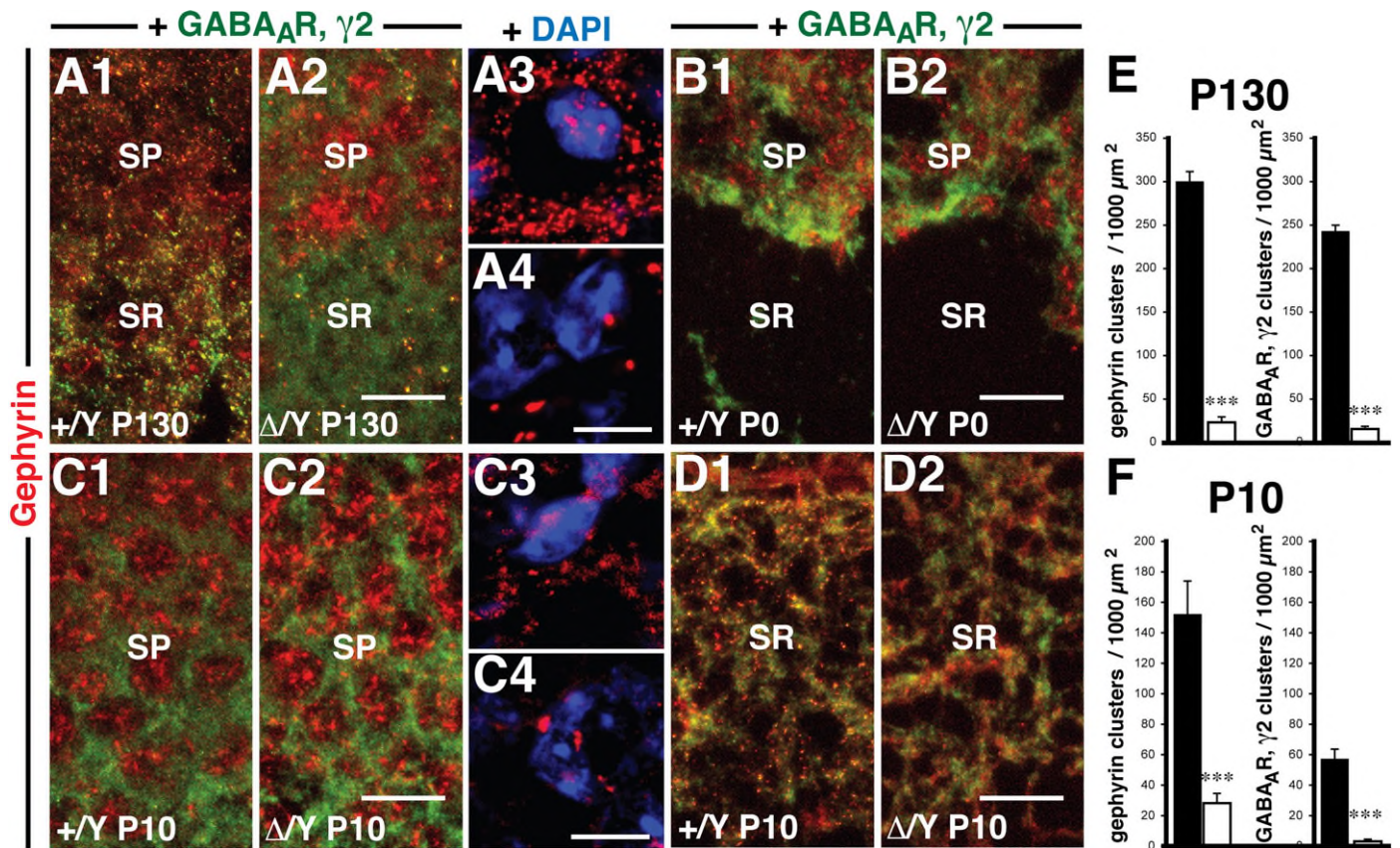
and protein were found in the brains of WT and floxed Cb mice by Northern blot (Fig. 1D) and Western blot (Fig. 1E) analyses, respectively. These data indicate that the incorporation of loxP sites into the intronic regions flanking exon 5 did not impair the expression of the Cb gene. Mice containing only the floxed Cb allele ((+)/(+) or (+)/Y) were phenotypically normal and indistinguishable from WT female (+/+) and male (+/Y) mice in two behavioural tests which previously had revealed highly significant differences between WT and Cb KO mice, i.e. the open field and the elevated plus-maze (see Papadopoulos et al., 2007; data not shown). In addition, the postsynaptic localization of gephyrin and gephyrin-dependent  $\gamma$ 2-subunit containing GABA<sub>A</sub>Rs in brain sections prepared from adult +/Y and (+)/Y mice was similar in both genotypes (not shown). Taken together, these data indicate that the modified Cb gene is fully functional and comparable to the WT gene.

In order to confirm Cre-mediated inactivation of the Cb (+) locus in mice, (+)/+ animals were crossed with a transgenic mouse line carrying the Cre coding sequence under the control of the adenovirus E11 $\alpha$  promoter (E11 $\alpha$ -Cre transgenic mouse line; (Lakso et al., 1996) Mating of floxed mouse lines with E11 $\alpha$ -Cre mice has been shown to result in efficient Cre-mediated recombination at the zygote state of embryonic development (Lakso et al., 1996). When the F1 generation of the Cb (+)/Y E11 $\alpha$ -Cre or the Cb (+)/+ E11 $\alpha$ -Cre matings was crossed

with C57BL/6 mice, the exon 5-deleted Cb locus was efficiently transmitted to the F2 generation (data not shown). Genotyping of the offspring was performed by PCR and Southern blot analysis (Figs. 1B and C, boxed areas). The absence of Cb transcripts and protein in mutant ( $\Delta$ /Y) animals was demonstrated by Northern (Fig. 1D, boxed area) and Western (Fig. 1E, boxed area) blot analysis, respectively.

*Cb is required for the clustering of gephyrin and  $\gamma$ 2-subunit containing GABA<sub>A</sub>Rs during synaptogenesis*

We next analyzed whether Cre-mediated deletion of Cb in E11 $\alpha$ -Cre transgenic mice led to similar reductions of gephyrin and GABA<sub>A</sub>  $\gamma$ 2-subunit immunoreactive clusters in the hippocampus as seen with the conventional Cb KO mice. In agreement with our previous report (Papadopoulos et al., 2007), a strong reduction in the densities of gephyrin and  $\gamma$ 2-subunit clusters was found in the stratum radiatum (SR) of adult (P130)  $\Delta$ /Y mice (Fig. 2A2) as compared to their +/Y littermates (Fig. 2A1). Quantification of the number of gephyrin and  $\gamma$ 2-positive clusters per 1000  $\mu$ m<sup>2</sup> SR area revealed density values of 300 $\pm$ 11 versus 24 $\pm$ 6 clusters/1000  $\mu$ m<sup>2</sup> for gephyrin, and 243 $\pm$ 7 versus 16 $\pm$ 3 clusters/1000  $\mu$ m<sup>2</sup> for the  $\gamma$ 2-subunit, in +/Y and  $\Delta$ /Y sections, respectively (Fig. 2E). In the sections derived from the +/Y mice, numerous mAb 7a immunoreactive puncta were found in the SP



**Fig. 2.** Loss of gephyrin and GABA<sub>A</sub>  $\gamma$ 2 clusters in the hippocampus of  $\Delta$ /Y mice. Brain sections through the hippocampus [CA1, stratum pyramidale (SP) and stratum radiatum (SR)] of +/Y and  $\Delta$ /Y mice were stained with the gephyrin-specific mAb 7a (red) and an antibody specific for the  $\gamma$ 2-subunit of GABA<sub>A</sub>Rs (green) at the postnatal stages indicated and processed for confocal microscopy. (A1–A4) At adult stages (P130), the punctate staining of gephyrin and the GABA<sub>A</sub>  $\gamma$ 2-subunit was strongly reduced in the SR of the CA1 region of  $\Delta$ /Y animals (A2) as compared to +/Y mice (A1). In the SP of sections derived from +/Y mice, gephyrin immunoreactivity was prominent in perisomatic clusters, as indicated by visualization of the nuclei (blue) after incubation in DAPI containing PBS (A3). In contrast, in  $\Delta$ /Y neurons somatic gephyrin immunoreactivity was found in aggregates in the cytoplasm (A4). (B1–B2) At P0, mAb 7a immunoreactivity was concentrated in aggregates which could be observed in the cell body of CA1 pyramidal neurons but not in the SR. The distribution of GABA<sub>A</sub>  $\gamma$ 2-immunofluorescence in the SP was diffuse and did not colocalize with the gephyrin aggregates. At this developmental stage, both gephyrin and  $\gamma$ 2-immunoreactivities were indistinguishable between +/Y (B1) and  $\Delta$ /Y (B2). (C1–D2) At P10 after the onset of synaptogenesis, mAb 7a immunoreactive intracellular aggregates were still prominent in the SP of sections derived from both +/Y (C1) and  $\Delta$ /Y (C2) mice. Some of these aggregates were located close to the nucleus of +/Y (C3) and  $\Delta$ /Y (C4) pyramidal cells, as indicated by DAPI-staining (blue). In the somata of  $\Delta$ /Y pyramidal neurons, however, these aggregates were bigger in size as compared to +/Y. At this developmental stage, a significant reduction of dendritic gephyrin and GABA<sub>A</sub>  $\gamma$ 2 clustering in the SR was observed in sections derived from  $\Delta$ /Y animals (D2) as compared to +/Y (D1). Scale bars, 20  $\mu$ m (A2, B2, C2, D2), 10  $\mu$ m (A4, C4). (E–F) Quantification of gephyrin and GABA<sub>A</sub>  $\gamma$ 2-subunit immunoreactive cluster densities in the SR at different postnatal stages. Each bar corresponds to mean values ( $\pm$ s.e.m.) obtained with sections from 3 individual brains (\*\*\*)  $P < .001$ ; Student's *t*-test.

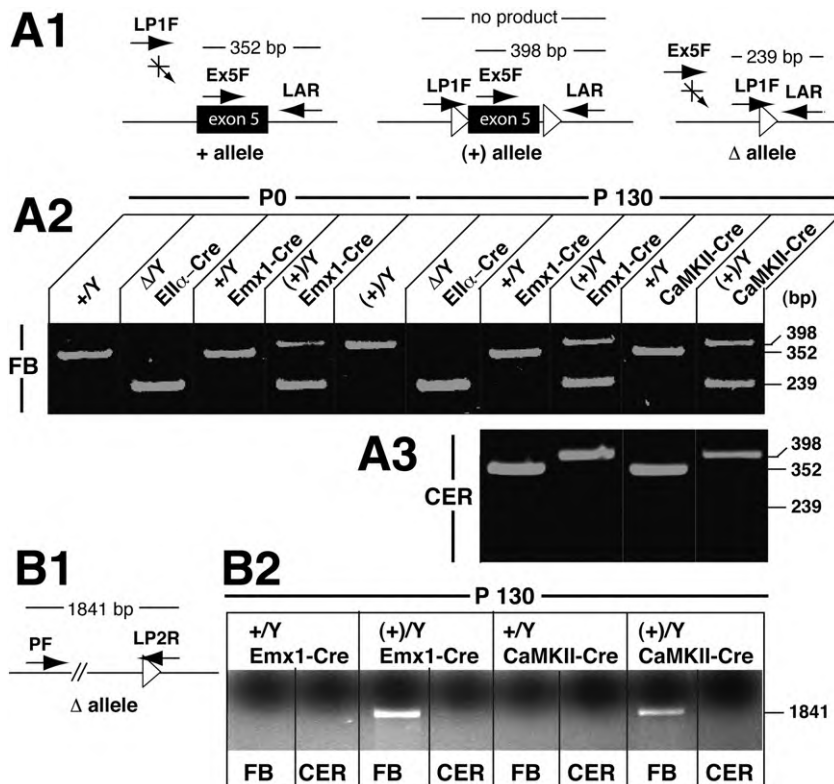
at the cell periphery (Figs. 2A1 and A3); these puncta were synaptically localized, as revealed by their apposition to presynaptic VIAAT immunoreactivity (data not shown; see Papadopoulos et al., 2007). In contrast, in the sections derived from the P130  $\Delta/Y$  animals (Figs. 2A2 and A4) larger gephyrin aggregates were still found in the cytoplasm. This is consistent with our findings reported previously (Papadopoulos et al., 2007).

In order to examine whether Cb is required during the early stages of inhibitory synapse formation in the hippocampus, we analyzed the clustering of gephyrin and the GABA<sub>A</sub>R  $\gamma 2$ -subunit in sections derived from brains of postnatal day 0 (P0) and P10  $\Delta/Y$  animals in comparison to their  $+/Y$  littermates. At P0, i.e. before the onset of hippocampal synaptogenesis, gephyrin and  $\gamma 2$  immunoreactive clusters could be hardly detected in the SR of the CA1 hippocampal area in sections derived from brains of  $+/Y$  and  $\Delta/Y$  mice (Figs. 2B1 and B2). In the stratum pyramidale (SP),  $\gamma 2$ -subunit immunoreactivity was diffusely distributed. Punctate gephyrin immunoreactivity detected by mAb 7a staining was enriched in the SP within the cell bodies of CA1 pyramidal neurons in both genotypes. These puncta were localized in the cytoplasm but also next to or even within the nucleus as indicated by DAPI-staining (not shown). Cytoplasmic and nuclear gephyrin staining at early stages of neuronal maturation has been reported previously (Colin et al., 1996; Sassoe-Pognetto and Wassle, 1997). Gephyrin aggregates in the SP were also observed in sections derived from P10  $+/Y$  and  $\Delta/Y$  mice (Figs. 2C1 and C2). However, the onset of gephyrin translocation into perisomatic clusters resembling those seen in sections derived from adult  $+/Y$  mice could be already detected in the SP of P10  $+/Y$  mice (Fig. 2C3) but not in the SP of their  $\Delta/Y$  littermates (Fig. 2C4). In the SR (CA1 area) of the  $+/Y$  mice, gephyrin

was intensely clustered and colocalized with GABA<sub>A</sub>R  $\gamma 2$ -subunit immunoreactivity at this stage (Fig. 2D1). In contrast, in the SR of P10  $\Delta/Y$  animals the density of puncta immunoreactive for both markers was strongly reduced (Fig. 2D2). Quantification of the numbers of gephyrin and  $\gamma 2$ -positive clusters per 1000  $\mu\text{m}^2$  SR area in brains derived from P10 mice revealed density values of  $152 \pm 22$  versus  $28 \pm 6$  for gephyrin, and of  $57 \pm 6$  versus  $3 \pm 1$  for the  $\gamma 2$ -subunit, in  $+/Y$  and  $\Delta/Y$  samples, respectively. These results indicate that the clustering of gephyrin and  $\gamma 2$ -subunit containing GABA<sub>A</sub>Rs within the dendritic compartment of CA1 pyramidal neurons derived from Cb-deficient mice is impaired already during the major stage of hippocampal synaptogenesis.

#### Strategy for forebrain-specific inactivation of the Cb gene

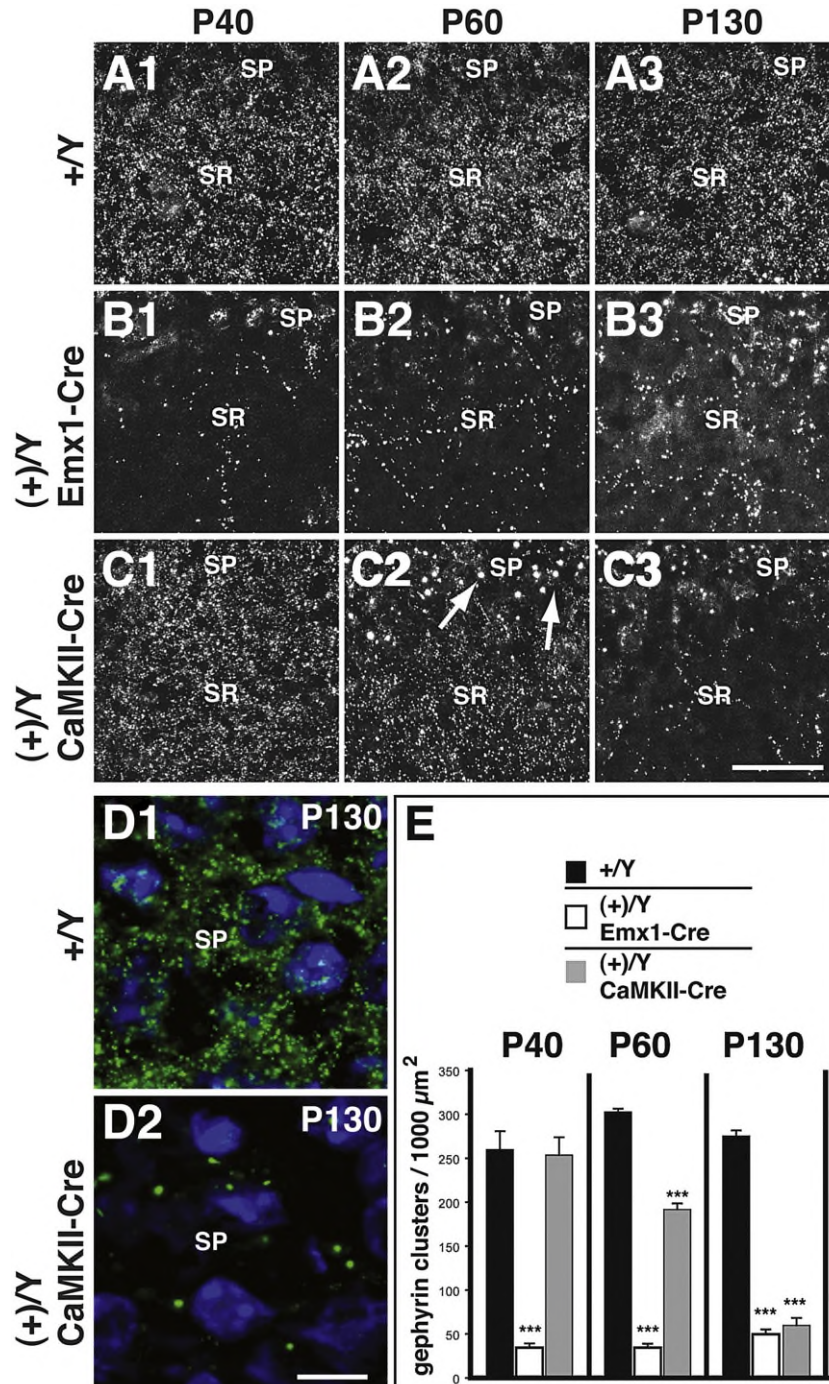
For developmentally controlled and forebrain-specific deletion of Cb in mice, we crossed the  $(+)/+$  mice with two different Cre-expressing mouse lines, an empty spiracles homolog 1 (Emx1)-Cre knock-in mouse and the Ca<sup>2+</sup>/calmodulin-dependent kinase (CaMKII)-Cre2834 transgenic mouse. Recombination directed by the Emx1-Cre gene is detected first at embryonic day 10 (E10) (Guo et al., 2000; Jin et al., 2000) whereas recombination mediated by the CaMKII-Cre2834 transgene starts at P17 (Schweizer et al., 2003). Both Emx1- and CaMKII-promoter driven Cre expression cause effective deletion in the cerebral cortex and hippocampus and result in recombination of floxed target genes in glutamatergic but not in parvalbumin (PAV)-positive GABAergic interneurons (Earnheart et al., 2007; Gorski et al., 2002). Here, we first analyzed the efficiency of CaMKII-Cre2834 mediated recombination by using Z/EG



**Fig. 3.** Cre-mediated deletion of the Cb gene in the forebrain at different developmental stages. (A1) Schematic representation of the 3-primer PCR strategy (see Experimental methods) showing from left: the +, the (+), and the  $\Delta$  alleles. Exon 5 is represented as a black box, and the loxP sites as open triangles. Primer positions are indicated by black arrows. Crossed arrows indicate lack of primer binding to the corresponding allele. (A2) PCR of biopsies using the three primers (Ex5F, LP1F and LAR) revealed Cre-induced recombination of the floxed-Cb locus, as indicated by the presence of the shorter 239 bp product in the forebrains (FB) of newborn (P0) and adult (P130)  $(+)/Y$  Emx1-Cre mice, and of adult  $(+)/Y$  CaMKII-Cre mice. FB-biopsies of  $+/Y$ ,  $\Delta/Y$  Ell $\alpha$ -Cre and  $(+)/Y$  mice were used as controls for the amplification of the 352 bp, 239 bp and 398 bp products, respectively. (A3) Analogous PCR analysis of cerebellar (CER)-biopsies. Note that the shorter 239 bp product is absent, indicating that Cre-mediated recombination was forebrain-specific. (B1) Scheme showing the strategy used for the selective amplification of the 5' region of the  $\Delta$  allele (see Experimental methods). Primer positions are indicated by black arrows and the loxP site by an open triangle. (B2) PCRs of FB and CER biopsies using primers PF and LP2R confirm FB-specificity of Cre-induced recombination in adult  $(+)/Y$  Emx1-Cre and  $(+)/Y$  CaMKII-Cre mice.

Cre reporter mice, in which Cre-induced recombination can be mapped to individual cells based on the expression of GFP (Novak et al., 2000). This confirmed the lack of Cre-mediated recombination at P10 in the hippocampus (data not shown); at P20, GFP-positive neurons were present, with their densities increasing about 2-fold in the CA1 region, and about 4-fold in the granule cell layer of the

dentate gyrus, until P90 (Supplementary Fig. 1, and data not shown). This temporal pattern of Cre-mediated reporter expression resembles that reported previously (Schweizer et al., 2003). Cre-induced recombination in both (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre male mice was verified further by PCR analysis (Figs. 3A1 and B1). The modified Cb gene locus lacking the floxed exon 5 (Cb  $\Delta$ ) could be



**Fig. 4.** Cre-induced reduction of gephyrin clusters in the hippocampus of (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice. (A–C) Hippocampal sections prepared from +/Y, (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice were stained with mAb 7a at the postnatal stages indicated and processed for confocal microscopy. Note the strong reduction of punctuate gephyrin staining in sections of (+)/Y Emx1-Cre mice (B1–B3) at all postnatal stages analyzed as compared to +/Y (A1–A3). At P40, similar densities of gephyrin immunoreactive puncta were observed in sections derived from (+)/Y CaMKII-Cre (C1) and +/Y (A1) mice. By P60, a significant reduction of gephyrin clusters was observed in the SR of (+)/Y CaMKII-Cre mice (C2) as compared to +/Y (A2), which was accompanied with an accumulation of gephyrin aggregates in the SP (arrows in C2). At P130, a strong reduction of gephyrin immunoreactivity was readily detected in sections derived from (+)/Y CaMKII-Cre mice (C3) as compared to their +/Y littermates (A3). (D1–D2) CaMKII-Cre-mediated deletion of the Cb gene in mature CA1 pyramidal neurons led to the accumulation of gephyrin (green) aggregates in the cytoplasm, as indicated by their proximity to nuclei visualized by DAPI (blue) staining (D2). In sections derived from +/Y mice, gephyrin clusters were smaller and perisomatically distributed (D1). Scale bars, 32  $\mu\text{m}$  (A–C), 10  $\mu\text{m}$  (D). (E) Quantification of hippocampal gephyrin cluster densities per 1000  $\mu\text{m}^2$  area in the SR of the different mouse lines at the indicated postnatal stages. Each bar corresponds to mean values ( $\pm$ s.e.m.) obtained with sections from 3 individual brains (\*\*\*) $P < 0.001$ ; Student's *t*-test).

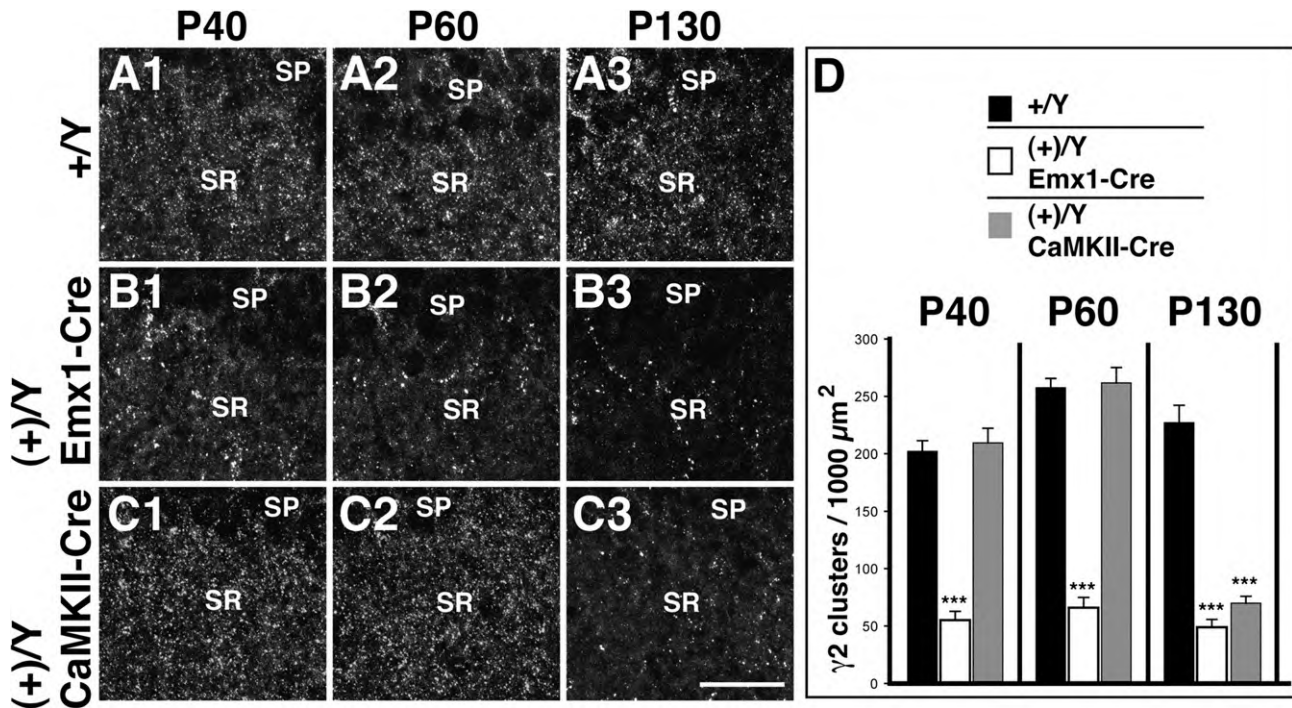
successfully amplified from forebrain (FB) biopsies derived from both adult (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice, as well as from P0 (+)/Y Emx1-Cre mice (Figs. 3A2 and B2). The lack of Cre-mediated recombination in non-forebrain regions was confirmed by PCR on DNA purified from cerebellar (CER) biopsies (Figs. 3A3 and B2). Together these results show that both the Emx1-Cre and CaMKII-Cre2834 matings produced region-specific inactivation of the floxed Cb allele.

*Cb is required for the maintenance of gephyrin and  $\gamma$ 2-subunit containing GABA<sub>A</sub>Rs at mature synapses*

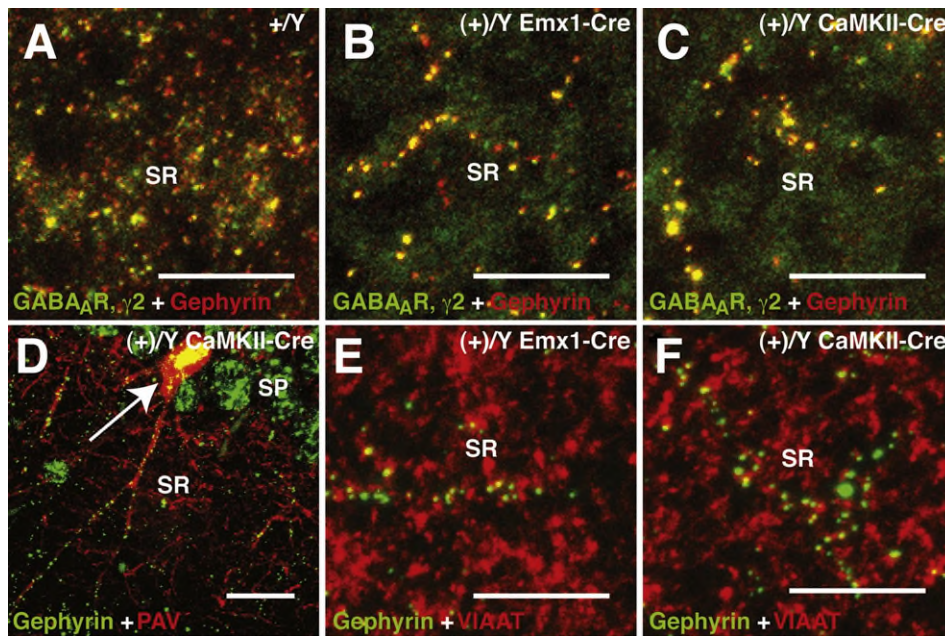
To evaluate whether ablation of Cb from mature synapses affects the clustering of gephyrin in the CA1 area of the hippocampus, we performed gephyrin stainings on brain sections derived from (+)/Y CaMKII-Cre mice at different postnatal stages. In addition, gephyrin immunoreactivity was analyzed in sections derived from (+)/Y Emx1-Cre and +/Y mice as controls. In +/Y sections, a high density of gephyrin clusters was found, with no overt differences between P40, P60 and P130 (Figs. 4A1–A3). In contrast, a strong reduction in gephyrin cluster numbers was observed in the hippocampus of brains derived from (+)/Y Emx1-Cre mice at all postnatal stages analyzed, as expected from Cre-mediated recombination occurring at embryonic stages in this mouse line (Figs. 4B1–B3). At P40, punctate gephyrin immunoreactivities were indistinguishable between sections derived from (+)/Y CaMKII-Cre and +/Y mice (compare Fig. 4A1 with C1). However, at P60 (+)/Y CaMKII-Cre brains showed a significant reduction in gephyrin cluster density in the SR of the CA1 area (Fig. 4C2) that was associated with an accumulation of gephyrin aggregates in the SP (indicated by arrows) as compared to +/Y samples (Fig. 4A2). This loss of gephyrin clusters from the hippocampus of (+)/Y CaMKII-Cre mice was even more pronounced at P130 (Fig. 4C3). In the SP of P130 (+)/Y CaMKII-Cre mice, gephyrin aggregates were concentrated in the cytoplasm of CA1 pyramidal neurons (Fig. 4D2) and resembled the aggregates seen in the

hippocampi of P130  $\Delta$ /Y animals (Fig. 2B4). This differs from the CA1 pyramidal neurons of +/Y mice, in which gephyrin clusters were exclusively perisomatically distributed (Fig. 4D1). Quantification of the number of gephyrin clusters per 1000  $\mu$ m<sup>2</sup> SR area revealed density values at P40 of 260  $\pm$  21, 35  $\pm$  5 and 253  $\pm$  20, at P60 of 302  $\pm$  4, 35  $\pm$  4 and 192  $\pm$  7, and at P130 of 275  $\pm$  6, 50  $\pm$  5 and 60  $\pm$  8, for +/Y, (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice, respectively (Fig. 4E). In the cerebellum (granule cell layer, Purkinje cells, molecular layer), the clustering of gephyrin was unaffected at all postnatal stages analyzed in sections derived from both (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre animals as compared to +/Y sections (data not shown). This confirms the regional specificity of Emx1-Cre and CaMKII-Cre-mediated recombination.

As the ablation of Cb from mature synapses led to a strong reduction of gephyrin clusters in the CA1 area of the hippocampus, we examined whether this was associated with a corresponding reduction of postsynaptic  $\gamma$ 2-subunit containing GABA<sub>A</sub>Rs. In (+)/Y Emx1-Cre mice, we consistently found a strong decrease in the density of GABA<sub>A</sub>R  $\gamma$ 2-subunit immunoreactive puncta in CA1 pyramidal neurons at all postnatal stages analyzed as compared to +/Y animals (Figs. 5A1–B3). In contrast, no difference was observed between +/Y and (+)/Y CaMKII-Cre animals in the density of  $\gamma$ 2-subunit immunoreactive puncta at developmental stages up to P60 (Figs. 5A1–A2 and C1–C2). This differs from the more pronounced reduction of gephyrin cluster density seen at P60 (Fig. 4E). Notably, the gephyrin clusters remaining at P60 all colocalized with punctate GABA<sub>A</sub>R  $\gamma$ 2-subunit immunoreactivity (data not shown). At P130, however, sections derived from (+)/Y CaMKII-Cre animals showed a marked loss of  $\gamma$ 2-subunit containing GABA<sub>A</sub>Rs as compared to +/Y littermates (compare Fig. 5C3 with A3). Quantification of the number of  $\gamma$ 2-subunit positive clusters per 1000  $\mu$ m<sup>2</sup> SR area revealed density values at P40 of 202  $\pm$  9, 55  $\pm$  8 and 209  $\pm$  12, at P60 of 257  $\pm$  8, 66  $\pm$  9 and 262  $\pm$  13, and at P130 of 227  $\pm$  15, 49  $\pm$  7 and 70  $\pm$  6, for +/Y, (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice, respectively (Fig. 4E). In conclusion, ablation of Cb from mature synapses causes a loss of



**Fig. 5.** Gephyrin-dependent reduction of  $\gamma$ 2 GABA<sub>A</sub>R clusters in the hippocampus of (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice. (A–C) Brain sections through the hippocampus of +/Y, (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice were stained for the GABA<sub>A</sub>R  $\gamma$ 2-subunit at the postnatal stages indicated and processed for confocal microscopy. Note the strong reduction of punctate  $\gamma$ 2 staining in sections of (+)/Y Emx1-Cre mice (B1–B3) at all postnatal stages analyzed as compared to +/Y sections (A1–A3). At P40 and P60, similar densities of  $\gamma$ 2-subunit immunoreactive puncta were observed in sections derived from (+)/Y CaMKII-Cre (C1–C2) and +/Y (A1–A2) mice. However, at P130 a strong reduction of punctate  $\gamma$ 2-subunit immunoreactivity was seen in sections derived from (+)/Y CaMKII-Cre mice (C3) as compared to their +/Y littermates (A3). Scale bar, 32  $\mu$ m. (D) Quantification of GABA<sub>A</sub>R  $\gamma$ 2-subunit cluster densities in the SR of the different mouse lines at the indicated stages. Each bar corresponds to mean values ( $\pm$ s.e.m.) obtained with sections from 3 individual brains (\*\*\*) $P$ <0.001; Student's  $t$ -test).



**Fig. 6.** Colocalization of gephyrin and GABA<sub>A</sub>R  $\gamma$ 2-subunit clusters at GABAergic terminals in (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre sections. (A–C) Brain sections through the hippocampus (CA1, SR) of P130 +/Y, (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice were double-stained for gephyrin (red) and the GABA<sub>A</sub>R  $\gamma$ 2-subunit (green) and processed for confocal microscopy. Red and green channels are superimposed to reveal colocalization (yellow). Note the strong reduction of gephyrin and  $\gamma$ 2- immunoreactive puncta in sections prepared from (+)/Y Emx1-Cre (B) and (+)/Y CaMKII-Cre (C) mice as compared to +/Y (A). The remaining gephyrin clusters largely colocalized with  $\gamma$ 2-subunit positive puncta (as indicated by the yellow puncta in B and C). (D) Lack of Cre-mediated recombination, indicated by the unaffected dendritic gephyrin clustering, in a PAV-positive interneuron (highlighted by an arrow) in a section derived from the brain of a (+)/Y CaMKII-Cre mouse. (E–F) SR sections from (+)/Y Emx1-Cre (E) and (+)/Y CaMKII-Cre (F) mice were double-immunostained for gephyrin (green) and VIAAT (red) to reveal GABAergic terminals. Note that the remaining gephyrin clusters mostly colocalize with or are apposed to VIAAT immunoreactive puncta. Scale bars, 20  $\mu$ m.

gephyrin and gephyrin-dependent GABA<sub>A</sub>Rs from inhibitory postsynaptic sites at late postnatal stages.

#### *Emx1-Cre and CaMKII-Cre2834 mediated inactivation of the Cb gene is largely restricted to excitatory neurons*

As mentioned above, Emx1-Cre and CaMKII-Cre2834 transgenes differ in their developmental recombination patterns but should both result in comparable Cb deficits in excitatory forebrain neurons at adulthood (Earnheart et al., 2007; Gorski et al., 2002). Accordingly, the remaining gephyrin and GABA<sub>A</sub>R  $\gamma$ 2-subunit clusters found in the SR of P130 (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice should colocalize at inhibitory postsynaptic sites of PAV-positive GABAergic interneurons. Indeed, despite the strong reduction of immunoreactive puncta in the SR of both (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre as compared to +/Y mice (Fig. 6A), the remaining gephyrin and  $\gamma$ 2-subunit clusters exhibited a high extent of colocalization (Figs. 6B and C). The majority of these colocalized clusters were located on the dendrites of PAV-positive GABAergic interneurons, as demonstrated by double-staining with gephyrin- and PAV-specific antibodies of sections derived from the brain of a (+)/Y CaMKII-Cre animal (Fig. 6D). Furthermore, in the SR of both (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice the remaining gephyrin clusters were closely apposed to punctate immunoreactivity for the presynaptic vesicular inhibitory amino acid transporter (VIAAT) (Figs. 6E and F), consistent with a postsynaptic localization. These results confirm that the loss of postsynaptic gephyrin and GABA<sub>A</sub>R  $\gamma$ 2-subunit clusters seen in the CA1 region of adult (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice is indeed due to a selective inactivation of the Cb gene in principal excitatory neurons.

#### Discussion

In this study, we have used the Cre-loxP strategy to specifically inactivate the Cb gene at different developmental stages. Deletion of Cb at the zygote state of embryonic development in Cb  $\Delta$ /Y EII $\alpha$ -Cre

animals reproduced the anatomical defects in gephyrin and GABA<sub>A</sub>R clustering characteristic of adult conventional Cb KO mice (Papadopoulos et al., 2007). This shows that the floxed Cb allele is efficiently inactivated by the Cre recombinase in vivo. Furthermore, the cell type-specificity and developmental manifestation of the consequences of Cb gene deletion seen here in (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice are consistent with the published spatiotemporal patterns of Cre expression in the Emx1-Cre and the CaMKII-Cre lines (Guo et al., 2000; Jin et al., 2000; Schweizer et al., 2003). This indicates that Cre-mediated inactivation of the Cb (+) allele proceeds reliably at both embryonic and postnatal stages.

A first major conclusion emerging from our anatomical analysis of brains derived from  $\Delta$ /Y EII $\alpha$ -Cre, (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice is that in the hippocampus Cb is required for the postsynaptic clustering of gephyrin during both the initial stages of synaptogenesis and at mature synaptic contacts. At P0, intracellular gephyrin aggregates were consistently found in the somata of CA1 pyramidal neurons derived from both WT and  $\Delta$ /Y EII $\alpha$ -Cre animals. Similar intracellular accumulations of gephyrin have been seen in ventral spinal cord neurons of newborn rats (Colin et al., 1998), after partial denervation of the goldfish Mauthner cell, an identified neuron in the teleost brainstem (Seitanidou et al., 1992), and in CA1 pyramidal neurons of adult Cb-deficient mice (Papadopoulos et al., 2007). At P10, in WT mice the number of these cytoplasmic gephyrin aggregates located in the cell body of CA1 pyramidal neurons was strikingly reduced, and gephyrin immunoreactivity appeared in numerous small perisomatic and dendritic clusters which, in adult animals (P130), represent the major gephyrin-positive structures. These gephyrin clusters correspond to inhibitory postsynapses, as indicated by their apposition to the presynaptic marker VIAAT. Our results clearly show that the developmental redistribution of gephyrin from intracellular aggregates to postsynaptic sites is Cb-dependent, as cytoplasmic gephyrin aggregates prevailed in the cell bodies of CA1 pyramidal neurons derived from P10  $\Delta$ /Y animals and further persisted until adulthood. Furthermore, in both these and the (+)/Y Emx1-Cre mice

the density of postsynaptic gephyrin clusters in the SR was strongly reduced at all postnatal stages examined. Together these findings prove that Cb is crucial for gephyrin scaffold formation during the major phase of inhibitory synapse formation in CA1 pyramidal neurons.

Our results obtained with the (+)/Y CaMKII-Cre mice show that Cb is also required for the maintenance of the postsynaptic gephyrin scaffold at mature synapses. At P130, the density of gephyrin clusters in the SR region of (+)/Y CaMKII-Cre animals, in which Cre expression starts only during the third postnatal week (Schweizer et al., 2003), was reduced to the same extent as in the embryonically inactivating (+)/Y Emx1-Cre mice. In contrast, at P40 no difference in cluster density was found between (+)/Y CaMKII-Cre and WT animals, and even at P60 there was only a modest reduction as compared to (+)/Y Emx1-Cre mice. This indicates that in adult animals postsynaptic gephyrin clusters are lost only at a slow rate in the absence of Cb. Whether this reflects a slow turnover rate of the membrane-apposed gephyrin scaffold or partial compensation by other postnatally expressed GEF proteins, is unknown presently. Denervation and antagonist inhibition experiments at the goldfish Mauthner cell have suggested that, at glycinergic synapses, the postsynaptic clustering of gephyrin and glycine receptors is regulated by trophic signals derived from the contacting presynaptic terminals (Seitanidou et al., 1992). We therefore speculate that the comparatively slow loss of gephyrin clusters observed in the (+)/Y CaMKII-Cre mice reflects the continued presence of GABAergic nerve terminals, which stabilize differentiated postsynaptic structures.

In agreement with our previous study (Papadopoulos et al., 2007), the data presented here confirm that in Cb-deficient mice lacking postsynaptic gephyrin clusters  $\gamma 2$ -subunit containing GABA<sub>A</sub>Rs are not concentrated at inhibitory postsynaptic sites. Both in  $\Delta/Y$  Ell $\alpha$ -Cre and (+)/Y CaMKII-Cre adult mice, the density of  $\gamma 2$ -positive clusters in the SR was dramatically reduced. The precise mechanisms responsible for the reduced number of GABA<sub>A</sub>R clusters found in Cb-deficient neurons are presently unknown but may simply reflect the lack of submembraneous gephyrin scaffolds that trap laterally diffusing GABA<sub>A</sub>Rs at sites of GABAergic nerve terminal contact (Choquet and Triller, 2003). Alternatively, the loss of clustered GABA<sub>A</sub>Rs may be due to an impairment of cytoskeletal functions that require Cb. Consistent with this proposal, the deposition of gephyrin beneath the plasma membrane has been shown to depend on intact microfilaments (Bausen et al., 2006), and gephyrin is known to interact with different proteins controlling actin cytoskeletal dynamics including profilin (Mammoto et al., 1998) and Mena/VASP (Giesemann et al., 2003). Also, previous studies have suggested that gephyrin deposition at postsynaptic sites might involve gephyrin bound to receptor transport vesicles (Hanus et al., 2004; Maas et al., 2006). Accordingly, insertion of gephyrin-coated receptor-rich transport vesicles into the neuronal plasma membrane would provide a receptor pool from which GABA<sub>A</sub>R-gephyrin complexes could be recruited to sites of GABAergic nerve terminal contact (Bogdanov et al., 2006). Cb activated at the plasma membrane by nerve terminal derived signals (Kneussel and Betz, 2000) might help to orient such transport vesicle fusion reactions to regions of high GABAergic synapse density.

Our quantitative analysis of hippocampal slices derived from (+)/Y CaMKII-Cre mice revealed that, upon Cb-gene inactivation during adolescence, dendritic gephyrin clusters disappear more rapidly than the GABA<sub>A</sub>R  $\gamma 2$ -subunit-associated punctate fluorescence. A similar observation has recently been made at perisomatic inhibitory synapses located on cerebellar Purkinje cells (Viltoño et al., 2008). There, the gephyrin clusters formed during the early stage of synaptogenesis are lost between days P7 and P14, whereas the colocalized GABA<sub>A</sub>R clusters persist. Together, these data show that the subsynaptic gephyrin scaffold can be disassembled at inhibitory postsynaptic sites prior to the dissociation of GABA<sub>A</sub>R clusters. Furthermore, as outlined above, our immunohistochemical analysis

of brains derived from  $\Delta/Y$ , (+)/Y Emx1-Cre and (+)/Y CaMKII-Cre mice disclosed cytoplasmic gephyrin aggregates in both WT and Cb-deficient immature neurons as well as in mature neurons that became Cb-deficient only after postnatal inactivation of the Cb gene. This suggests a dynamic exchange of gephyrin between the cytoplasm and synaptically localized clusters, whose disruption causes gephyrin to form aggregates. Together, all the results presented here corroborate that Cb is required throughout life for the formation and maintenance of selected inhibitory synapses. Our data are difficult to reconcile with previous suggestions that gephyrin might inhibit Cb's GEF activity, and thereby terminate reactions of the Cdc42 signaling cascade which are important during early but not later stages of synapse formation (Xiang et al., 2006). Our findings are, however, compatible with Cb regulating the dynamics of the actin cytoskeleton via Cdc42 at both developing and mature postsynaptic sites. Evidence for a role of microfilaments in the regulation of gephyrin clustering and receptor mobility at inhibitory synapses has been obtained with cultured hippocampal and spinal neurons (Kirsch and Betz, 1995; Bausen et al., 2006; Charrier et al., 2006). The underlying mechanisms are, however, not understood. Clearly further work will be required to unravel the sequence of events through which Cb specifies the formation and stabilization of inhibitory postsynapses.

## Experimental methods

### *Generation of floxed-Cb mice and Cre-induced recombination in mice*

For the generation of mice carrying a floxed-Cb gene, the previously described targeted ES cells were employed, in which both a neomycin (neo) resistance cassette and exon 5 of the Cb gene were flanked by loxP sites (Papadopoulos et al., 2007). The cells were transiently transfected with a Cre-encoding plasmid, and surviving clones were screened for partial deletion of the neo selection cassette by PCR and Southern blotting (data not shown). Two of the positive ES cell clones containing the floxed exon 5 were injected into C57BL/6 blastocysts to generate chimeric animals that were backcrossed with female C57BL/6 mice to establish germline transmission. The different Cb loci were confirmed by Southern blotting and PCR using the following primers: an exon-5 specific forward primer (Ex5F: 5'-ACAGATTGAGACT-TTTCTTGACGG-3'), a loxP1-specific forward primer (LP1F: 5'-TGGTTACTAGAAT-TGGATCCATAAC-3'), and the long arm-specific reverse primer (LAR: 5'-AGATGA-AACGCCTACTTCCACTC-3'). Northern and Western blotting were performed as described previously (Papadopoulos et al., 2007).

For Cre-induced recombination in mice, floxed-Cb mice were crossed with the following Cre transgenic mouse lines: (i) a line carrying the Cre transgene under the control of the adenovirus Ell $\alpha$  promoter (Ell $\alpha$ -Cre) (Lakso et al., 1996), (ii) an Emx1-Cre knock-in mouse (Jin et al., 2000), and (iii) the CaMKII-Cre2834 mouse (Schweizer et al., 2003), all of which had been backcrossed into the C57BL/6 background for at least twelve generations. Cre-mediated recombination in the offspring was confirmed by PCR analysis as described above, or by PCR of forebrain (FB) and cerebellum (CER) biopsies, with either the above mentioned 3-primer combination or the following 2-primer combination: forward primer (PF): 5'-GCAGAGATTTTGTCTGGATAAGTC-3' and the loxP2-specific reverse primer (LP2R): 5'-AATGAGCTGCAGGCTAGCATAAC-3'. Cre-positive mice were identified by PCR using the following primers: forward primer 5'-GCCTGCATTACCGTTCGATGCAACGA-3' and reverse primer 5'-GTGGCAGAT-GCGCGGCAACACCATT-3'.

### *Immunostaining and confocal microscopy on cryostat sections*

For double-immunostaining with the gephyrin-specific monoclonal antibody (mAb) 7a and an antibody specific for VIAAT (Synaptic Systems GmbH, Goettingen, Germany), or an antibody specific for PAV



(Swant, Bellinzona, Switzerland), mice were deeply anesthetized and decapitated. The brains were immediately removed and incubated overnight at 4 °C in 50 ml fixative containing 4% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The tissue was then cryoprotected overnight at 4 °C with 30% (w/v) sucrose in phosphate-buffered saline (PBS). Frontal 20 µm sections were prepared from frozen tissue and collected free-floating in PBS. Sections were washed once with PBS, mounted on SuperFrost Plus slides (Menzel GmbH, Braunschweig, Germany) and air-dried. Sections were then postfixed for 10 min with 4% (w/v) paraformaldehyde in PBS at 4 °C, washed twice for 2 min in PBS and once with SC buffer (10 mM sodium citrate, 0.05% (v/v) Tween-20, pH 8.0). Sections were immersed in a pre-heated staining dish containing SC buffer and incubated for 30 min at 95 °C. After allowing the slides to cool at room temperature for 20 min, sections were rinsed twice for 2 min in PBS, permeabilized with 0.3% (w/v) Triton X-100, 4% (v/v) goat serum in PBS, blocked for 3 h with 10% (v/v) goat serum in PBS and incubated overnight at 4 °C with the primary antibodies at appropriate dilutions in PBS/10% goat serum. All other immunostainings with primary and secondary antibodies performed in this study were performed with 14 µm cryostat sections as described (Papadopoulos et al., 2007). Images were collected by confocal microscopy, and immunofluorescent puncta were quantified, as detailed previously (Papadopoulos et al., 2007).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2008.06.006.

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