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Quantitative localisation of synaptic and extrasynaptic $GABA_A$ receptor subunits on hippocampal pyramidal cells by freeze-fracture replica immunolabelling

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Abstract

Hippocampal CA1 pyramidal cells, which receive γ-aminobutyric acid (GABA)ergic input from at least 18 types of presynaptic neuron, express 14 subunits of the pentameric $GABA_A$ receptor. The relative contribution of any subunit to synaptic and extrasynaptic receptors influences the dynamics of GABA and drug actions. Synaptic receptors mediate phasic GABA-evoked conductance and extrasynaptic receptors contribute to a tonic conductance. We used freeze-fracture replica-immunogold labelling, a sensitive quantitative immunocytochemical method, to detect synaptic and extrasynaptic pools of the alpha1, alpha2 and beta3 subunits. Antibodies to the cytoplasmic loop of the subunits showed immunogold particles concentrated on distinct clusters of intramembrane particles (IMPs) on the cytoplasmic face of the plasma membrane on the somata, dendrites and axon initial segments, with an abrupt decrease in labelling at the edge of the IMP cluster. Neuroligin-2, a GABAergic synapse-specific adhesion molecule, co-labels all beta3 subunit-rich IMP clusters, therefore we considered them synapses. Double-labelling for two subunits showed that virtually all somatic synapses contain the alpha1, alpha2 and beta3 subunits. The extrasynaptic plasma membrane of the somata, dendrites and dendritic spines showed low-density immunolabelling. Synaptic labelling densities on somata for the alpha1, alpha2 and beta3 subunits were 78–132, 94 and 79 times higher than on the extrasynaptic membranes, respectively. As GABAergic synapses occupy 0.72% of the soma surface, the fraction of synaptic labelling was 33–48 (alpha1), 40 (alpha2) and 36 (beta3)% of the total somatic surface immunolabelling. Assuming similar antibody access to all receptors, about 60% of these subunits are in extrasynaptic receptors.

Introduction

The activity of pyramidal cells in the cerebral cortex is governed by several differentially timed γ-aminobutyric acid (GABA)ergic inputs, acting via $GABA_A$ and $GABA_B$ receptors. In the CA1 area of the hippocampus, at least 18 distinct presynaptic GABAergic neurons release GABA to pyramidal cells (Klausberger & Somogyi, 2008). For example, the somata of pyramidal cells receive GABAergic input from three distinct basket cell populations expressing either parvalbumin, or cholecystokinin and vasoactive intestinal peptide (VIP), or cholecystokinin and vesicular glutamate transporter type 3 (VGLUT3), and they also show distinct firing behaviour during network oscillations (Klausberger *et al.*, 2005). Such differences suggest different roles in the network supported by specialised molecular signalling machineries (Foldy *et al.*, 2007).

The postsynaptic receptors activated by these GABAergic neurons may also be expressed in an input-dependent manner. Pyramidal cells express at least 14 different subunits of the $GABA_A$ receptor contributing to multiple pentameric, hetero-oligomeric receptor species

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(Persohn *et al.*, 1992; Wisden *et al.*, 1992; Sperk *et al.*, 1997; Ogurusu *et al.*, 1999). Most $GABA_A$ receptors include two alpha (alpha1–6), two beta (beta1–3), and either a gamma (gamma1–3), delta, epsilon or theta subunit (Sieghart & Sperk, 2002). The same alpha and/or beta subunit can have one or two copies in a receptor, increasing diversity, each receptor having a possible differential surface expression. Indeed, Thomson and colleagues have demonstrated differences in the pharmacology of unitary GABAergic inputs to CA1 pyramidal cells from identified interneurons (Pawelzik *et al.*, 1999; Thomson *et al.*, 2000; Ali & Thomson, 2008). Fast spiking basket cells activated receptors indicative of the alpha1 subunit, whereas regular spiking basket cells acted via receptors indicative of alpha2/3 subunits. Thus, pyramidal cells may target distinct receptor species to particular synaptic inputs. This was supported by the demonstration that the alpha1 subunit was enriched in synapses received from parvalbumin-immunopositive terminals (Klausberger *et al.*, 2002), whereas the alpha2 subunit was enriched in synapses received from parvalbumin-negative GABAergic boutons, presumably originating from cholecystokinin-expressing basket cells (Nyiri *et al.*, 2001). However, due to the lack of suitable antibodies, the relationship between synapses positive for the alpha1 or alpha2 subunit has not been evaluated directly. Therefore, in the present study, having raised the appropriate antibodies, we have used freeze-fracture replica immunogold labelling (FRIL), a sensitive immunocytochemical method (Fujimoto, 1995; Rash & Yasumura, 1999), to investigate the presence of both the alpha1 and alpha2 subunits in single synapses on the somata of pyramidal cells. The advantage of FRIL is that the plasma membrane distribution of proteins can be revealed *en face*, in two-dimensional membrane sheets *in situ*, at a lateral resolution of about 20 nm.

Correlated FRIL and electrophysiological recordings showed a single gold particle corresponding to each functional α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor (Tanaka *et al.*, 2005). Such sensitivity makes it possible to detect $GABA_A$ receptors expressed at low density, for example at extrasynaptic sites (Somogyi *et al.*, 1989), which are responsible for a tonic current evoked by GABA in the extracellular spaces (Semyanov *et al.*, 2004; Farrant & Nusser, 2005; Glykys & Mody, 2007; Belelli *et al.*, 2009). These extrasynaptic receptors may be of the same subunit composition as synaptic receptors, which cycle in and out of the synapse by lateral diffusion (Bannai *et al.*, 2009). Some extrasynaptic receptors may be of different subunit composition from synaptic receptors, as shown by electron microscopic immunolabelling (Nusser *et al.*, 1998; Wei *et al.*, 2003), and by the pharmacological characteristics of the tonic vs synaptic currents (Nusser & Mody, 2002; Caraiscos *et al.*, 2004a; Belelli *et al.*, 2005; Glykys *et al.*, 2007). The gamma2 subunit is necessary for synaptic localisation (Essrich *et al.*, 1998), and the replacement of the gamma by the delta subunit leads to the exclusion of the receptor from the synapse (Nusser *et al.*, 1998). The relative participation of the other subunits in synaptic and extrasynaptic receptors is unclear, partly due to positional effects within the pentamer (Minier & Sigel, 2004) and the inclusion of two different alpha and/or beta subunits (Tretter *et al.*, 1997). To test the relative contribution of the alpha1, alpha2 and beta3 $GABA_A$ receptor subunits to synaptic and extrasynaptic receptors, we have quantified their distribution using FRIL. Abstracts have been published (Somogyi *et al.*, 2004; Kasugai *et al.*, 2006).

Materials and methods

All procedures involving experimental animals used in this study in the UK were carried out under a Home Office project license in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated procedures. The experiments on animals in the laboratory at Okazaki, Japan, were conducted in accordance with the guidelines of the National Institute of Physiological Science's Animal Care and Use Committee. A total of 21 adult rats (specified below), four guinea pigs, five wild-type and five gene-deleted mice were used (see below).

Sodium dodecyl sulphate (SDS)-digested freeze-fracture replica preparation

We followed the procedure developed by Fujimoto (1995), with recent modifications (Tanaka *et al.*, 2005). Thirteen adult male Wistar rats were anaesthetised with Pentobarbital (50 mg/kg, i.p.), and perfused first for 3 min with 25 mM phosphate-buffered saline (PBS), then for 12 min with a fixative composed of 2% paraformaldehyde, ∼0.2% w/v picric acid in 0.1 M sodium phosphate buffer (PB, pH 7.2–7.4), followed by PB for 3 min. The brains were dissected and sectioned coronally with a Lineaslicer PRO7 (Dosaka EM, Kyoto, Japan) at 120-μm thickness. The hippocampal CA1 area was dissected in PBS and cryoprotected with 30% glycerol in 0.1 M PB overnight at 4 °C. The sections were frozen quickly in a high-pressure freezing machine (HPM 010; Bal-Tec, Balzers, Liechtenstein). The frozen slices were then fractured at −120 °C and coated with carbon (5 nm), shadowed by platinum (2 nm) at 60°, and then coated with carbon (15 nm) again in a BAF 060 machine (Bal-Tec). After thawing, the tissue was removed from the replica by autoclaving for 15 min at 105 °C in a Tris buffer (15 mM, pH 8.3) containing 2.5% SDS and 20% sucrose. The specimens were removed from the autoclave after the temperature in the chamber had returned to room temperature in about 5 h, and were processed further or left in the same solution at room temperature until further processing.

Production of primary antibodies

Polyclonal antibodies were developed in guinea pig (beta3) and rat (alpha1) using glutathione-S-transferase (GST)-fusion proteins produced in *Escherichia coli* cells (BL21, Agilent Technologies, Santa Clara, CA, USA). The plasmid constructs that were used contained cDNA for GST fused with a portion of the intracellular loop corresponding to residues 328–382 of the alpha1 or residues 345–408 of the beta3 subunits of the $GABA_A$ receptor. The rat and mouse sequences at these positions are identical. For immunisation, these fusion proteins were purified by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, the gels were washed briefly in several changes of deionised water and then transferred to 0.3 M $CuCl_2$. The gels were shaken gently for 3 min, and the broad protein bands that appeared in the gel against a blue background were excised. The gel fragments containing the purified fusion protein were emulsified with Freund's adjuvant (Nakalai tesque, Kyoto, Japan). Eight rats (Sprague–Dawley, female, 6 weeks old at first immunisation) and four guinea pigs (adult, female) were immunised by subcutaneous injections at several sites in the back with a total of 50–100 μg of the beta3 subunit fusion protein, or 20–50 μg of the alpha1 fusion protein, respectively. They received three sequential sets of injections at 2-week intervals. The first injection contained complete Freund's adjuvant, the two later injections were done with incomplete Freund's adjuvant. The antisera were collected 2 weeks after the last injection by exsanguination of the animals following anaesthesia with Pentobarbital.

The anti-alpha1 (328–382), anti-alpha2 (322–357), anti-alpha5 (337–388), anti-beta2 (351–405), anti-beta3 (345–408) and

anti-gamma2 (319–366) antibodies were generated by immunising rabbits with a maltose-binding protein (MBP)-alpha1 (328–382)-7His, MBP-alpha2 (322–357)-7His, MBP-alpha5 (337–388)-7His, MBP-beta2 (351–405)-7His, MBP-beta3 (345–408)-7His or MBP-gamma2 (319–366)-7His fusion protein. Then the antibodies were purified by using the corresponding GST fusion protein coupled to affigel 10 (Bio-Rad, Hercules, CA, USA), as described earlier (Slany *et al.*, 1995; Tretter *et al.*, 1997; Jechlinger *et al.*, 1998; Poltl *et al.*, 2003; Table 1). The anti-alpha5 (337–388) antibody was described previously (Poltl *et al.*, 2003; Ogris *et al.*, 2006) as anti-alpha5 (337–380).

Tests for the specificity of antibodies

Proteins separated by SDS–PAGE (12%) from a crude membrane fraction of whole rat brains (Sprague–Dawley, male) were used to prepare Western blots, as described earlier (Notomi & Shigemoto, 2004). Both the rat antiserum to the alpha1 subunit and the guinea pig antiserum to the beta3 subunit were used at a dilution of 1 : 500. These antisera also recognised GST, as seen in Western blots of SDS–PAGE-separated protein samples of lysed *E. coli* used for production of the fusion proteins and tested with rabbit antibodies to GST (Sigma-Aldrich, St Louis, MO, USA; Table 1). The purified rabbit anti-alpha1 (328–382) and anti-beta3 (345–408) antibodies were used at a dilution of 1 : 1000. In addition, the purified rabbit anti-alpha2 (322–357) antibody was used here in a Western blot of membrane proteins from wild-type mice and alpha2 subunit gene-deleted mice, as described earlier for other antibodies (Ogris *et al.*, 2006).

Transient transfection of $GABA_A$ receptor subunits in cultured cells

The cDNAs for the complete polypeptide sequence of the rat alpha1, alpha2 and alpha5, beta1, beta2, beta3 and gamma2 subunits of the $GABA_A$ receptor were cloned into a pCl plasmid vector, which uses the cytomegalovirus immediate-early enhancer/promoter region for strong constitutive expression. Human embryonic kidney cells (HEK-293) were subcultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (GIBCO, Invitrogen, Paisley, UK), glutamine (0.29 g/L), sodium bicarbonate (2.2 g/L), penicillin (100 000 U/L) and streptomycin (10 mg/L) in an atmosphere of 5% CO_2/95% air at 37°C. The HEK-293 cells (0.5×10^6 cells per well) were grown for approximately 24 h in six-well plates on glass coverslips coated with poly-L-lysine. Transient transfection of the HEK-293 cells by the plasmids was carried out using FuGENE6 (Roche Applied Science, Indianapolis, USA) following the manufacturer's recommended protocol. For a single transfection, 1 μg of plasmid and 3 μL of FuGENE6 were separately mixed in a volume of 50 μL OptiMEM (GIBCO). For double and triple transfections, the amount of DNA for each subunit was reduced, and the final ratio of FuGENE6 solution to plasmid was maintained at 1 μg DNA to 3 mL FuGENE6. After 5 min the plasmid was mixed with the diluted FuGENE6 and kept for 20 min at room temperature. Transfection was initiated by adding the entire 100 μL of each transfection mixture containing DNA to each well. The cells were grown for 48 h before fixation.

The cells were fixed with 4% paraformaldehyde and ~0.2% w/v picric acid dissolved in 0.1 M PB (pH 7.4) for 30 min at room temperature. The fixed cells were washed three times in 0.1 M PB for 5 min. Non-specific antibody binding was blocked with 20% normal horse serum (VECTOR, Burlingame, CA, USA) in 0.3% Triton X-100 and Tris[hydroxymethyl]amino-methane-buffered saline (TBS-T) for 1 h. Single antibodies, or mixtures of up to three primary antibodies, raised in different host species, were applied in 1% normal horse serum in TBS-T for 2 h at room temperature (Table 2). After washing, the cells were incubated with secondary antibodies. For simultaneous detection of different primary antibodies, Alexa Fluor-488 goat anti-rabbit IgG (1 : 1000; Invitrogen, CA, USA), Cy3-coupled anti-guinea pig (1 : 400; Jackson ImmunoResearch Europe, Cambridgeshire, UK) and Cy5-coupled anti-rabbit, anti-guinea pig or anti-rat (1 : 400; Jackson ImmunoResearch Europe, Suffolk, UK) subtype- and species-specific secondary antibodies were used. Secondary antibodies were diluted with TBS-T, and reacted for 1 h at room temperature. After washing twice with TBS-T and once with TBS, the cells on glass coverslips were covered with Vectashield anti-fading medium (Vector, Burlingame, CA, USA). Epifluorescence images were captured with a Digital charge-coupled device (CCD) camera on a LEITZ DM RB microscope equipped with the following filter blocks A4, L5, Y3 and Y5, which ensured that only one fluorochrome was recorded in each channel. Image processing was carried out with the Openlab 4.0.4 software (Improvision, PerkinElmer Comp, Coventry, UK).

$GABA_A$ receptor subunit-deficient mice

A further test of the specificity of the antibodies was carried out on SDS-digested freeze-fracture replica produced from two mice lacking the $GABA_A$ receptor alpha1 subunit (Sur *et al.*, 2001), and two wild-type littermates (kindly provided by Drs D. Belleli and J. Lambert at the Neurosciences Institute, University of Dundee, and originated from Dr Thomas Rosahl, Merck Sharp & Dohme, UK). Brains from two mice each lacking the alpha2 subunit (Boehm *et al.*, 2004) or the alpha5 subunit (used as positive controls for unaffected subunits), and wild-type littermates were kindly provided by Dr Thomas Rosahl (Merck Sharp & Dohme, UK). Two mouse brains lacking the beta3 subunits were obtained also from Dr G. Homanics, Dept Anesthesiology, Univ. Pittsburgh, School Med, PA, USA (Homanics *et al.*, 1997).

Specificity of antibodies

Western blots of membrane preparations from rat brains showed a protein band at the expected molecular mass for the antibodies tested. Results from immunoblots were published previously for the affinity-purified rabbit antibodies to the alpha1 (Tretter *et al.*, 1997; Ogris *et al.*, 2006), alpha5 (Ogris *et al.*, 2006), beta2 (Jechlinger *et al.*, 1998), beta3 (Slany *et al.*, 1995; Poltl *et al.*, 2003) and gamma2 (Tretter *et al.*, 1997) subunits, which in addition have been shown to precipitate recombinant receptors consisting of these subunits and did not have cross-reactivity with other subunits. The Western blot of rat brain membranes with the rat antiserum to the alpha1 subunit showed a major band at 51 kDa, as did the rabbit antibody in the same experiment. The antiserum raised in guinea pig and the purified rabbit antibodies to the beta3 subunit showed two strong bands at 53 and 56 kDa, as described previously (Todd *et al.*, 1996), and some weaker bands. The Western blot of a wild-type mouse brain with the purified antibody alpha2 (322–357) showed a major protein band at approximately 53 kDa, and this was absent in the blot of brain membrane proteins from an alpha2 gene-disrupted mouse**.**

In addition, two immunohistochemical methods were used to assess the specificity of the antibodies on fixed cells for this study. Firstly, HEK cells transiently transfected with plasmids encoding cDNAs for different $GABA_A$ receptor subunits were tested by immunofluorescence labelling (Table 2). The antibodies for the alpha1 (rat, rabbit), alpha2 (rabbit) and beta3 (guinea pig, rabbit) subunits reliably labelled

TABLE 1. Identity, source and characterisation of antibodies

Molecule	Code# in original lab. or supplier	Host animal	Developer	Epitope, amino acid residues	Protein concentration	Optimal dil. FRIL	Characterisation tests: IH, subunits expressed, HEK cells*	Characterisation tests: FRIL, gene deleted & wild-type mice	Characterisation tests: Western blot	Reference, production, characterisation
Neuroligin-2	NL2B-KLH (bleed#1, rabbit CU904)	Rabbit	B. Chih N. Limthong P. Scheiffele	750–767	Purified ab., not known	400×	Not known	No	Brain tested**	Budreck & Scheiffele (2007)
$GABA_A$-R α1	GABAa α1, GST-Rt2	Rat	Y. Kasugai R. Shigemoto	328–382	Serum	50×	Tested	Tested	Brain tested	This paper
$GABA_A$-R α1	α1(328–382), R11/12	Rabbit	W. Sieghart	328–382	1.2 mg/mL	500×	Tested	Tested	Wt and gene-deleted mouse**	Tretter *et al.* (1997); Ogris *et al.* (2006)
$GABA_A$-R α2	α2(322–357), R18/19	Rabbit	W. Sieghart	322–357	991 μg/mL	900×	Tested	Tested	Wt and gene-deleted mouse	Poltl *et al.* (2003); this paper
$GABA_A$-R α5	α5(337–388) R13/12	Rabbit	W. Sieghart	337–388	410 μg/mL	50×	Tested	Tested	Brain tested**	Poltl *et al.* (2003); Ogris *et al.* (2006)
$GABA_A$-R ß2	ß2(351–405) R22/10	Rabbit	W. Sieghart	351–405	468 μg/mL	200×	Tested	No	Purified receptor tested**	Jechlinger *et al.* (1998)
$GABA_A$-R ß3	$GABA_A$ ß3-Gp4	Guinea pig	Y. Kasugai R. Shigemoto	345–408	Serum	50×	Tested	Tested	Brain tested	This paper
$GABA_A$-R ß3	ß3(345–408) R14/22	Rabbit	W. Sieghart	345–408	325 μg/mL	500×	Tested	Tested	Purified receptor tested**; brain tested	Slany *et al.* (1995); Poltl *et al.* (2003); this paper
$GABA_A$-R γ2	γ2(319–366) R12/20	Rabbit	W. Sieghart	319–366	264 μg/mL	200×	Tested	No	Receptor in HEK cells tested**	Tretter *et al.* (1997)
$Na_v1.6$	K87A/10.2 (7/20/01)	Mouse monoclonal	J. S. Trimmer	1904–1976 (C-t), rat Nav1.6	6.1 mg/mL	500×	Not known	No	Brain tested**	Rasband *et al.* (2003)
PSD-95/SAP90	CAT No 05-427 NeuroMab clone K28/43	Mouse monoclonal	Upstate Biotech., now NeuroMab	77–299 human PSD-95	1 mg/mL	500×	Not known	No	Wt and gene-deleted mouse**	Beique *et al.* (2006)
Glutathione-S-transferase (GST)	CAT No G7781	Rabbit	Sigma-Aldrich	Whole protein	10 mg/mL	5000×	Not known	No	Recombinant GST	Calon *et al.* (2006)

*see Table 2; **Data presented in cited publication. GABA, γ-aminobutyric acid; HEK, human embryonic kidney cells; IH, immunohistochemistry; FRIL, freeze-fracture replica immunolabelling; Wt, wild-type.

TABLE 2. Immunofluorescence labelling tests of antibody specificity on transiently transfected HEK cells expressing subunits of the $GABA_A$ receptor

Transfected $GABA_A$ receptor subunit	Primary antibodies (dilutions), applied mixed	Secondary antibodies (dilutions), applied mixed	Labelling
$\alpha1$	Rat anti-$\alpha1$ (1 : 100)	Anti-rat Cy3 (1 : 400)	++
	Rabbit anti-$\beta3$ (1 : 500)	Anti-rabbit Alexa Fluor-488 (1 : 1000)	–
$\alpha1$	Rabbit anti-$\alpha1$ (1 : 500)	Anti-rabbit Alexa Fluor-488 (1 : 1000)	+++
	Guinea pig anti-$\beta3$ (1 : 100)	Anti-guinea pig Cy3 (1 : 400)	–
$\alpha2$	Rabbit anti-$\alpha2$ (1 : 500)	Anti-rabbit Alexa Fluor-488 (1 : 1000)	+++
$\alpha2$	Guinea pig anti-$\beta3$ (1 : 100)	Anti-guinea pig Cy3 (1 : 400)	–
	Rabbit anti-$\alpha1$ (1 : 500)	Anti-rabbit Alexa Fluor-488 (1 : 1000)	–
$\alpha2$	Rat anti-$\alpha1$ (1 : 100)	Anti-rat Cy3 (1 : 400)	–
	Rabbit anti-$\beta3$ (1 : 500)	Anti-rabbit Alexa Fluor-488 (1 : 1000)	–
$\alpha5$	Rabbit anti-$\alpha5$ (1 : 200)	Anti-rabbit Alexa Fluor-488 (1 : 1000)	+++
	Rat anti-$\alpha1$ (1 : 100)	Anti-rat Cy3 (1 : 400)	–
$\alpha5$	Rabbit anti-$\alpha1$ (1 : 500)	Anti-rabbit Alexa Fluor-488 (1 : 1000)	–
	Guinea pig anti-$\beta3$ (1 : 100)	Anti-guinea pig Cy3 (1 : 400)	–
$\beta3$	Guinea pig anti-$\beta3$ (1 : 100)	Anti-guinea pig Cy3 (1 : 400)	+++
	Rabbit anti-$\alpha1$ (1 : 500)	Anti-rabbit Alexa Fluor-488 (1 : 1000)	–
$\beta3$	Rat anti-$\alpha1$ (1 : 100)	Anti-rat Cy3 (1 : 400)	–
	Rabbit anti-$\beta2$ (1 : 500)	Anti-rabbit Alexa Fluor-488 (1 : 1000)	–
$\beta2$	Rabbit anti-$\beta2$ (1 : 500)	Anti-rabbit Alexa Fluor-488 (1 : 1000)	+++
	Rat anti-$\alpha1$ (1 : 100)	Anti-rat Cy3 (1 : 400)	–
$\alpha1 + \beta3 + \gamma2$	Rat anti-$\alpha1$ (1 : 100)	Anti-rat Alexa Fluor-488 (1 : 1000)	++
	Guinea pig anti-$\beta3$ (1 : 100)	Anti-guinea pig Cy5 (1 : 400)	++
	Rabbit anti-$\gamma2$ (1 : 300)	Anti-rabbit Cy3 (1 : 400)	++

GABA, γ-aminobutyric acid.

those cells transfected with the corresponding plasmids, and no non-specific cross-labelling was detected for any of the other tested subunits (Table 2). A second test of the specificity of the antibodies to the alpha1 (rat, rabbit), alpha2 (rabbit) and beta3 (guinea pig, rabbit) subunits was FRIL on hippocampi from wild-type and gene-deleted mice. In wild-type mice, we observed qualitatively similar immunogold distribution to that described in the rat below, but this was absent in the gene-deleted mice (not shown). We conclude that immunogold labelling on the protoplasmic-face (P-face) of cells in FRIL represents recognition of the respective subunits.

Replica immunolabelling

We followed recent modifications (Tanaka *et al.*, 2005; Masugi-Tokita & Shigemoto, 2007; Tarusawa *et al.*, 2009) of the technique developed by Fujimoto (1995). The replicas treated with the SDS solution were washed thrice in a washing buffer (WB) containing 0.05% bovine serum albumin (BSA; Nakalai tesque, Kyoto, Japan), 0.1% Tween 20 and 0.05% sodium azide dissolved in TBS. Non-specific binding was blocked by incubating the replicas for 2 h at room temperature in a blocking solution consisting of 5% BSA in WB. Replicas were then incubated overnight at 4 °C on a shaker while submerged in the primary antibodies, diluted in blocking solution. The source, testing of specificity and concentration of primary antibodies used are shown in Table 1. The following day, after three washes in the WB, the replicas were incubated with secondary antibodies conjugated to gold particles of either 5, 10 or 15 nm (British Bio-cell International, Cardiff, UK) for 2 h at 37 °C. For double-labelling, the various antibodies were applied sequentially as follows – the first primary antibody and then 5-nm gold-conjugated secondary antibody, followed by the second primary antibody and 10-nm gold-conjugated secondary antibody. The replicas were washed twice in WB and then in distilled water twice for 3 min. They were collected on 100-mesh copper grids and examined with an electron microscope (JEOL1010 or Philips CM100). Some images were taken on films and the negatives printed for measurement using a digitising pad. Most of the images were recorded using a Gatan UltraScan 1000 CCD camera (Gatan UK, Abingdon, UK) and measured directly on a computer screen. For the quantitative analysis of synaptic and extrasynaptic labelling, original images that included one or more synapses and covered an area of at least 1.4×1.4 μm, at 2048×2048 pixels of resolution, were used.

Measurement of synaptic area and quantification of the density of immunogold particles

The antibodies used in the study reliably showed labelling concentrated on the cytoplasmic face (P-face) of the plasma membrane, whereas the extracellular half of the plasma membrane (exoplasmic, E-face) had a very low level of labelling. Some of the P-face labelling was highly concentrated over small membrane patches rich in intramembrane particles (IMPs), which were also labelled for neuroligin-2 and considered to be synaptic junctions. All synapses on an analysed cell body that were present on the membrane close to perpendicular to the electron beam were collected. The specimens were not tilted, therefore synapses on areas of membrane with significant slope provide an area smaller than their real lateral extent, and were not included. The synaptic area was delineated manually using ImageJ (NIH, USA) or iTEM software (Olympus Soft Imaging Solutions GmbH, Munster, Germany) following the contour of the high IMP concentration, by two investigators independently for training sessions. If the difference in the measurement was larger than 20%, the area was re-measured by three investigators until an agreed area was arrived at. Gold particles within a synapse were counted to obtain the synaptic density, which is expressed as number of gold particles per square micrometre. Extrasynaptic immunolabelling density measurements were taken from the same images and cells from which synaptic measurements were obtained. Background labelling was measured on E-face membrane areas of the same cells from which P-face measurements were taken, or if they did not have E-face membrane areas, more often, from neighbouring cells. From

each cell at least three images were taken, the E-face area was measured and the gold particles counted at high magnification.

The significance level for statistical tests was set at 0.05. Correlations were analysed by two-tailed Pearson correlation test. Results are expressed as mean ± SD.

Results

Distribution of $GABA_A$ receptor subunit immunogold labelling in rat CA1 area

Identification of replicated membranes, synapses and selection of antibodies

The somata of pyramidal cells are known to receive a high density of GABAergic synapses from several types of basket cell (Ramon y Cajal, 1893; Andersen *et al.*, 1963; Buhl *et al.*, 1994; Pawelzik *et al.*, 2002) and provide a largely homogeneous population of cell bodies for testing the molecular composition of synapses. Interneurons represent only a few percent of the neuronal population in the pyramidal layer (Aika *et al.*, 1994). The cell bodies of CA1 pyramidal cells are easily identifiable in freeze-fractured replicas of the hippocampus as a band of large elliptical or triangular structures located in the stratum pyramidale (Fig. 1A). In order to evaluate the antibodies and the membrane partitioning of receptors we studied these fractured somatic membranes. Preliminary screening of antibodies to the extracellular N- or C-terminals of subunits, which are accessible on the extracellular half (E-face) of the plasma membrane, and to the intracellular loop between transmembrane domains 3 and 4, which is accessible to antibodies on the cytoplasmic half (protoplasmic, P-face) of the fractured membrane, showed that a much higher level of labelling is obtained on the P-face than on the E-face (data not shown). These membrane domains can be identified by features such as the arrangements of IMPs (Rash & Yasumura, 1999). The E- and P-faces can be differentiated by the much higher density of IMPs in the P-face and the concavity of the structures. Nevertheless, there was significant specific labelling also on the E-face with antibodies to the C- or N-terminal of subunits, which are located on the extracellular surface of the membrane. Although the pattern of E-face labelling matched that of the P-face, it was not analysed, because the immunogold particle clusters that may represent synapses were not associated with any obvious structural feature (see below).

Using antibodies to the intracellular loop of the alpha1 (raised in rat or rabbit), alpha2 (rabbit) and beta3 (raised in rabbit or guinea pig) subunits, two distinct labelling patterns were observed on the P-face of somatic membrane replicas. Small (~0.02–0.05 μm^2) areas had high immunogold density coinciding with a concentration of IMPs of a characteristic size and shape. The high immunogold density abruptly dropped at the edge of the IMP cluster (Fig. 1A, inset). Of all the IMPs in the P-face, those in the $GABA_A$ receptor subunit-dense areas were relatively uniform and of medium height and diameter as compared with other IMPs. Adjacent to these immunolabelled areas, larger diameter and higher IMPs were often present in small clumps, but were not immunolabelled. There were also unlabelled patches of high-density clusters of larger size IMPs distinct from the immunolabelled patches. The rest of the plasma membrane had scattered immunogold particles of varying density, depending on the antibody, and these particles could not be allocated to particular IMPs, although an association could not be excluded.

The size of the IMP clusters labelled for $GABA_A$ receptor subunits was similar to that of GABAergic synaptic areas reported from serial thin sections reconstructed on CA1 pyramidal cells (Nyiri *et al.*, 2001). To further probe if the labelled IMP clusters are indeed synapses, we tested whether they also contained neuroligin-2 ($n = 3$, animals), a protein present apparently only in GABAergic and glycinergic synapses on the cell surface (Varoqueaux *et al.*, 2004). In two animals, FRIL for neuroligin-2 (10-nm gold) resulted in the labelling of IMP-dense patches only on the P-face (Fig. 1B), which were similar in size (animal 1, $n = 62$, 0.053 ± 0.023 μm^2; animal 2, $n = 34$, 0.047 ± 0.017 μm^2) and shape to patches obtained with antibodies to $GABA_A$ receptor subunits (see below). In a third animal we used 5-nm particles for the beta3 subunit and 10-nm particles for neuroligin-2 immunolabelling, and found that every IMP cluster labelled for the beta3 subunit (guinea pig antibody, $n = 26$, 0.046 ± 0.019 μm^2) was also labelled for neuroligin-2 (Fig. 1C and D). We concluded that P-face membrane patches densely labelled for $GABA_A$ receptor subunits are synaptic junctions. The size of neuroligin-2-labelled IMP-rich areas were not different in the three animals (Kruskal-Wallis test, $P = 0.370$), therefore they were pooled, resulting in a mean synaptic IMP cluster area of 0.050 ± 0.021 μm^2 (range, 0.015–0.121 μm^2).

Distribution of synaptic and non-synaptic $GABA_A$ receptor labelling on dendrites, spines and the axon initial segment

In general, it is not easy to establish the identity of the cell type relating to a particular freeze-fracture replicated membrane in the neuropil. However, due to the regular laminar organisation of the hippocampus and the characteristic shapes of pyramidal cells, some structures can be reliably identified. The main apical dendrites of pyramidal cells can be recognised on the basis of their radial origin from the cell body and/or their large diameter and perpendicular orientation to the layers. None of the GABAergic interneuron types have such large dendritic diameters. Synapses on main apical dendrites, as identified by receptor subunit-rich IMP clusters, had the same shape and IMP content (Fig. 2) as those synapses found on the oblique dendrites, which in fortuitous cases could be followed out from the apical dendrites (Fig. 3). The extrasynaptic labelling density did not appear to change along the apical and oblique dendrites at a distance of up to 150 μm from the soma. The GABAergic dendritic innervation of pyramidal cells is diverse, and in the region that we studied is supplied by at least 13 distinct interneuron types (Buhl *et al.*, 1994; Pawelzik *et al.*, 1999, 2002; Thomson *et al.*, 2000; Klausberger & Somogyi, 2008). These synapses may be heterogeneous in terms of their subunit composition and it is uncertain if they all contain the beta3 subunit. Dendritic spines were identified by their size, shape and occasionally by the thin neck connecting them to the parent dendrite (Fig. 2), or by shape and co-labelling for PSD-95, a glutamatergic synaptic scaffolding protein (Fig. 2). Receptor subunit-rich IMP clusters were not encountered on dendritic spines, but the spines were diffusely labelled by extrasynaptic immunoparticles (Fig. 2).

We also investigated if the FRIL technique is suitable for the characterisation of $GABA_A$ receptor subunit composition of the axon initial segment of pyramidal cells, which receives GABAergic innervation exclusively from parvalbumin-positive axo-axonic cells (Somogyi *et al.*, 1983). These synapses are particularly rich in the alpha2 subunit (Nusser *et al.*, 1996), and are known to contain also the alpha1, beta2/3 and gamma2 subunits (Somogyi *et al.*, 1996). We identified axon initial segments on the basis of selective labelling for the $Na_v1.6$ alpha subunit of the sodium channel, as also reported recently (Lorincz & Nusser, 2010), and tested for $GABA_A$ receptor subunits with 5-nm particles. Because the membrane of the axon initial segment represents a very small fraction of all the membranes in strata pyramidale and oriens, axon initial segments were very rare, and we found only seven with $GABA_A$ receptor subunit-rich IMP clusters,

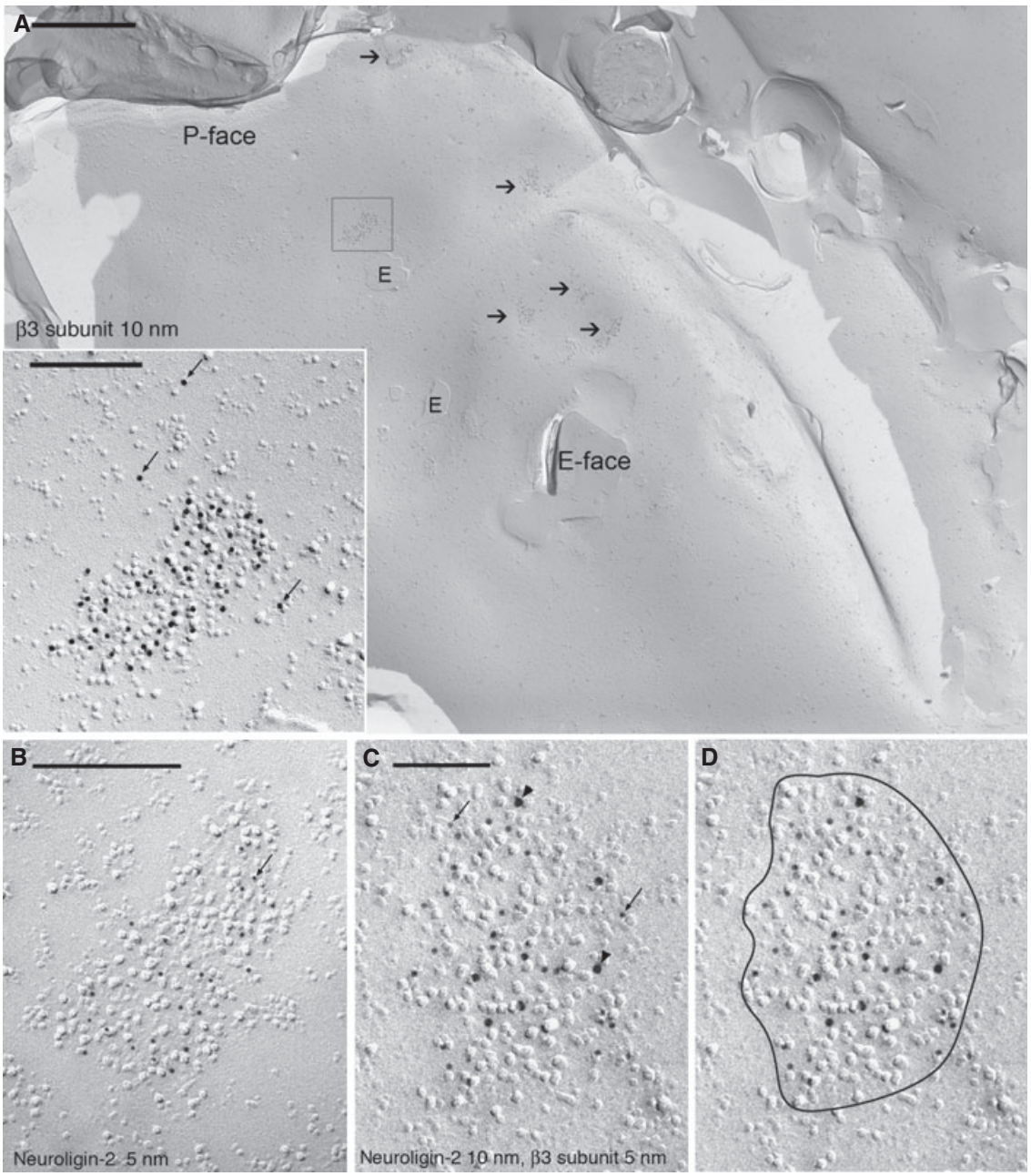

FIG. 1. GABAergic synapses on the surface of CA1 pyramidal cell somata revealed by SDS-digested FRIL. (A) Low-magnification view of a replica of the soma surface of a pyramidal cell labelled with an antibody to the intracellular loop of the beta3 subunit of the $GABA_A$ receptor and 10-nm immunogold particles conjugated to secondary antibodies. The cytoplasmic face of the plasma membrane (protoplasmic; P-face) was replicated and the gold particles are concentrated on IMP clusters (arrows, framed area shown as inset), and also scattered at low density outside these clusters (arrows in inset). A medium sized area of exoplasmic (E)-face membrane replica and some smaller areas (E) of the membranes of neighbouring cells are attached to the P-face replica of the soma. (B) Immunolabelling for neuroligin-2 (5 nm gold, e.g. arrow). This is a protein specifically enriched in GABAergic synapses on the cell surface in the hippocampus; it is highly concentrated over an island of IMPs on the P-face of the replicated plasma membrane of another cell. (C) High-magnification image of the replicated plasma membrane of a pyramidal cell showing the co-localisation of neuroligin-2 (10 nm immunogold, arrowheads) and the beta3 subunit of $GABA_A$ receptors (5 nm immunogold, arrows) concentrated over an IMP cluster. (D) Delineation of the IMP cluster for measuring the synaptic area. Scale bars: 1 μm (A); 0.2 μm (inset A, B); 0.1 μm (C, D).

three labelled for the alpha2 subunit (Fig. 4) and four labelled for the beta3 subunit. All these synapses were trans-fractured, i.e. the plane of membrane split jumped from the axon initial segment membrane to the presynaptic terminal membrane across the synaptic cleft, revealing the E-face of the latter (Fig. 4). Thus, only partial synapses were seen on axon initial segments having IMP organisation qualitatively similar to that of other GABAergic synapses on neurons. The density of extrasynaptic labelling on the axon initial segments could not be assessed quantitatively due to the small membrane areas and the low number of extrasynaptic particles.

Quantification of synaptic and extrasynaptic $GABA_A$ receptor subunit labelling on pyramidal cell somata

One of our aims was to quantify immunolabelling of synapses and of the extrasynaptic membrane, and this depends on labelling efficacy.

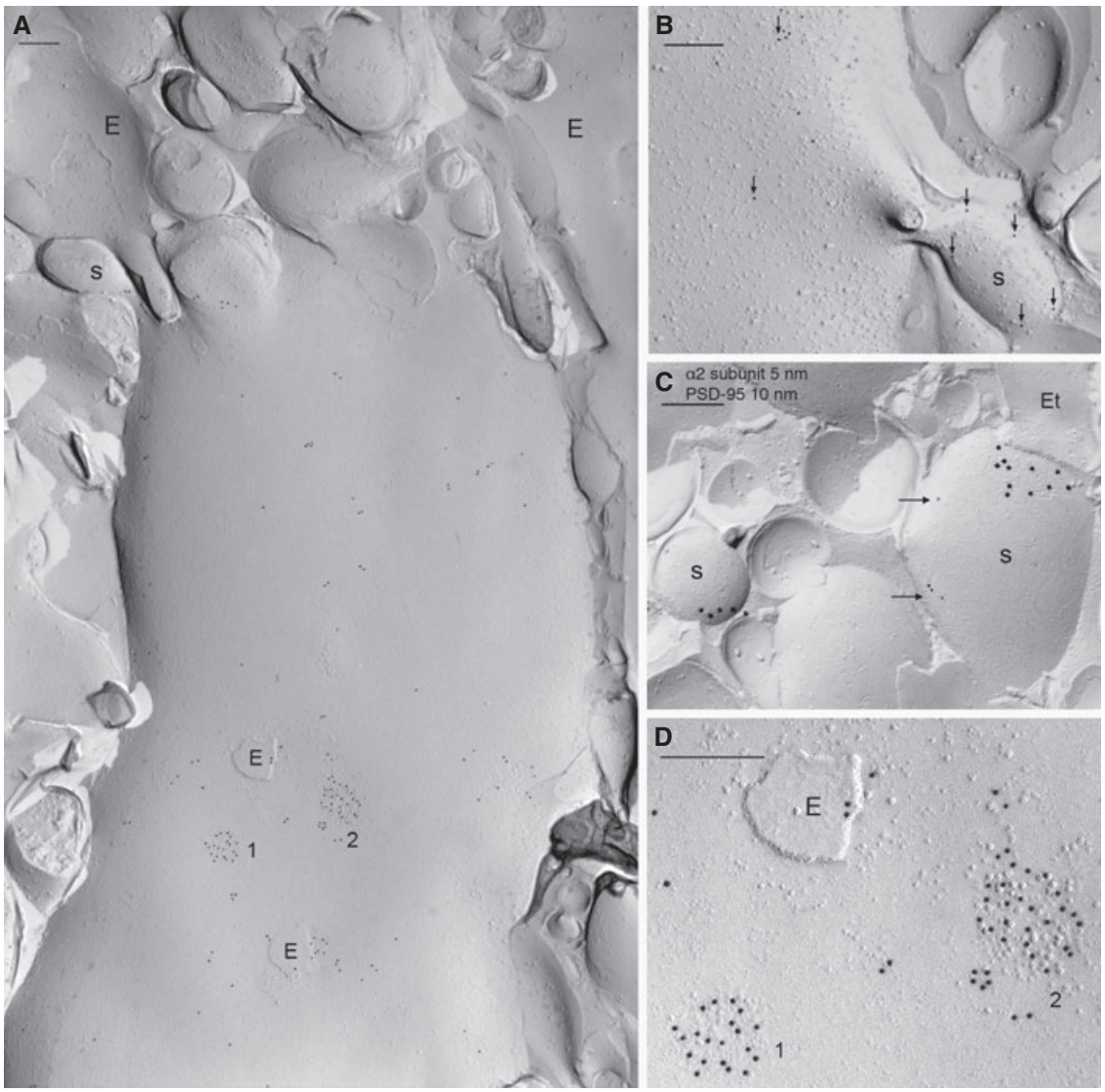

FIG. 2. Synaptic and extrasynaptic $GABA_A$ receptor subunit labelling on pyramidal cell apical dendrites and dendritic spines. (A) An apical dendrite in stratum radiatum shows beta3 subunit labelling in two synapses (1, 2) and scattered extrasynaptic labelling that includes the neck of a dendritic spine (s). The synapses are shown at higher magnification in (D). The amount of extrasynaptic labelling exceeds synaptic labelling in this area. Two small E-face plasma membrane patches (E) of adjacent cells remain attached. Note the lack of labelling on the E-face plasma membranes (top) and in other structures. (B) Extrasynaptic labelling for the beta3 subunit (e.g. arrows) on an apical dendrite and in an emerging spine (s, arrows). At the neck of the spine, the broken neck of another spine is seen. (C) Two dendritic spines (s) identified by their shape and PSD-95 immunolabelling in glutamatergic synapses. The larger one on the right shows extrasynaptic alpha2 subunit labelling (arrows). The glutamatergic synapse is partially covered by the E-face of the presynaptic nerve terminal (Et). (D) Higher-magnification view of the two synapses shown in (A). Scale bars: 0.2 μm (A–D).

We tested several of the antibodies using the same primary antibody dilution, and either 5-nm or 10-nm gold particles coated with secondary antibodies on separate replicas in the same experiment. The 5-nm particles resulted in 1.5–2 times higher synaptic immunogold densities than the 10-nm particles (data not shown). Therefore, we proceeded to analyse the densities of $GABA_A$ receptor labelling using 5-nm particles.

Synaptic areas and $GABA_A$ receptor subunits labelling densities

The distribution of $GABA_A$ receptor subunits was tested on pyramidal cell somata by the application of antibodies specific to the intracellular loop of the alpha1 (rat antiserum), alpha2 (rabbit antibody) and beta3 (guinea pig antiserum) subunits. A consistent labelling pattern was evident in all four rats in batch 1 (Fig. 5; Table 3). A total of 67 cells were measured, an average of 5.5 cells per animal per antibody (range 4–8). A synapse was considered immunopositive if it contained at least three gold particles, and the total area of each immunogold-labelled IMP cluster was measured, i.e. including the area of IMPs that had no overlying gold particles. All labelled synapses were recorded on the fractured part of the soma of each cell. The size of the smallest neuroligin-2-immunopositive IMP cluster was 0.0153 μm^2, and this value was set as a lower limit for further analysis, which resulted in the exclusion of only a few labelled IMP clusters. The values of synaptic areas obtained for rats 1–4 labelled for the receptor subunits were (*n*, range) – 0.0502 ± 0.0261 μm^2 (146, 0.0156–0.1536); 0.0513 ± 0.0254 μm^2 (200, 0.0159–0.1719); 0.0472 ± 0.0252 μm^2 (275, 0.0160–0.2710); 0.0535 ± 0.0230 μm^2 (110, 0.0156–0.1407). There

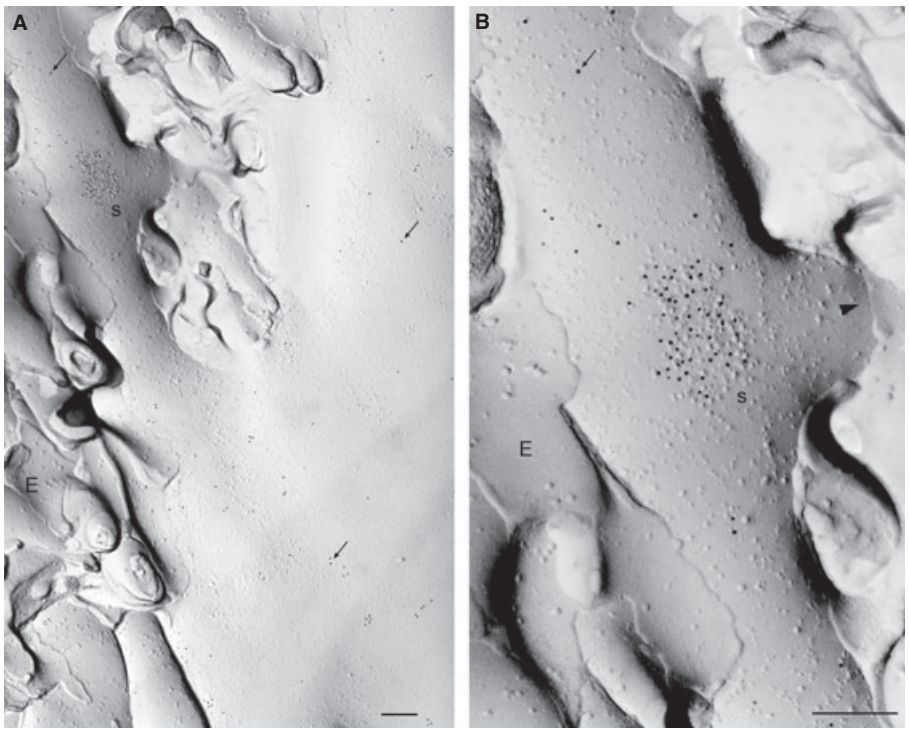

FIG. 3. Synaptic and extrasynaptic labelling for the beta3 subunit of the $GABA_A$ receptor on a main apical dendrite and a small oblique dendrite in stratum radiatum. (A) The oblique dendrite branches from the apical dendrite to the left and receives a synapse (s) identified by the high density of IMPs and immunogold particles with a sharp border. Both the main apical dendrite and the oblique dendrite have scattered immunolabelling (e.g. arrows) of extrasynaptic subunits. Note that there is no synapse in this membrane area on the main apical dendrite. There is no labelling on E-face (E) membranes. (B) High-magnification view showing the synaptic and extrasynaptic labelling. Note that the synapse is at a site where the oblique dendrite branches to the right (arrowhead). Scale bars: 0.2 μm (A, B).

was no significant difference between the synaptic areas labelled for $GABA_A$ receptor subunits (n = 4 rats) or neuroligin-2 (n = 3 rats; Kruskal–Wallis test, P = 0.0758). The pooled mean synaptic area obtained from single receptor subunit immunolabelling was 0.0498 ± 0.0252 (n = 731). Pooled synaptic area size was not distributed normally (Kolmogorov–Smirnov, Z = 2.351, P = 0.00003), and showed a skewed distribution towards larger values (Fig. 6).

Synaptic and extrasynaptic immunolabelling densities were corrected in each animal and for each reaction, individually, by subtracting the labelling density measured on the somatic E-face membrane areas (see below) in the same replicas. The average synaptic labelling densities (mean ± sd, gold particles per μm^2) of four rats for the alpha1 subunit were 219 ± 30 (range of animal mean, 192–261; range of synaptic densities, 38–505), for the alpha2 subunit 576 ± 64 (range of animals mean, 488–642; range of synaptic densities, 40–1582) and for the beta3 subunit 646 ± 109 (range of animals mean, 553–789; range of synaptic densities, 63–1688; Fig. 6B). The distribution of values representing the number of immunoparticles per synapse pooled across animals for each subunit was not normally distributed (Kolmogorov–Smirnov, two-tailed, alpha1, n = 249, Z = 2.198, P = 0.00013; alpha2, n = 257, Z = 2.365, P = 0.00003; beta3, n = 225, Z = 1.893, P = 0.00154), and showed a skewed distribution towards larger values (Fig. 6C, E and G).

There was a strong positive correlation between synapse size and number of immunoparticles (Pearson correlation test, two-tailed) in 11 of the 12 cases (four animals, three subunits each). The mean correlation coefficients were 0.544 ± 0.120 (n = 4, 0.014 ≥ P ≥ 8.05E-11), 0.725 ± 0.019 (n = 3, 2.47E-09 ≥ P ≥ 3.51E-19) and 0.731 ± 0.072 (n = 4, 2.01E-06 ≥ P ≥ 4.32E-14) for the alpha1, alpha2 and beta3 subunits, respectively. Rat 4 showed no correlation for the alpha 2 subunit (Pearson, r = 0.219, P = 0.192). In most cases, there was no or only very weak correlation between synapse size and the density of immunoparticles. The distribution of synapses according to labelling density was normal for the alpha2 (Kolmogorov–Smirnov, two-tailed, n = 257, Z = 0.754, P = 0.621), beta3 subunits (n = 225, Z = 0.583; P = 0.885), and if the two largest (values ≥ 500) outliers were omitted also for the alpha1 subunit (n = 247, Z = 1.331, P = 0.058). Accordingly, a main contributor to the skewed synaptic labelling strength distribution (particles per synapse) is the skewed synapse size distribution, as is also suggested by the correlation of synaptic area and particle number. On individual pyramidal cells, the density of immunogold particles per synapse could vary over a range of up to one order of magnitude for the same subunit (Fig. 7).

Extrasynaptic immunolabelling density and background labelling measurements

Extrasynaptic immunolabelling was measured on the same micrographs taken for recording synapses. The synaptic areas and any attached E-face membrane fragment areas were measured and subtracted from the total somatic P-face membrane to arrive at the extrasynaptic membrane area. In the four animals, the average extrasynaptic particle density (corrected for background, gold particles/μm^2) was 4.5 ± 3.9 (range 1.8–10.2) for the alpha1 subunit, 6.3 ± 1.7 (range 4.3–7.9) for the alpha2 subunit and 9.0 ± 4.4 (range 6.5–15.7) for the beta3 subunit.

The above data critically depend on the level of background labelling. Ideally this would be measured on the P-face membranes of cells that do not express the subunit in the same specimens, but such

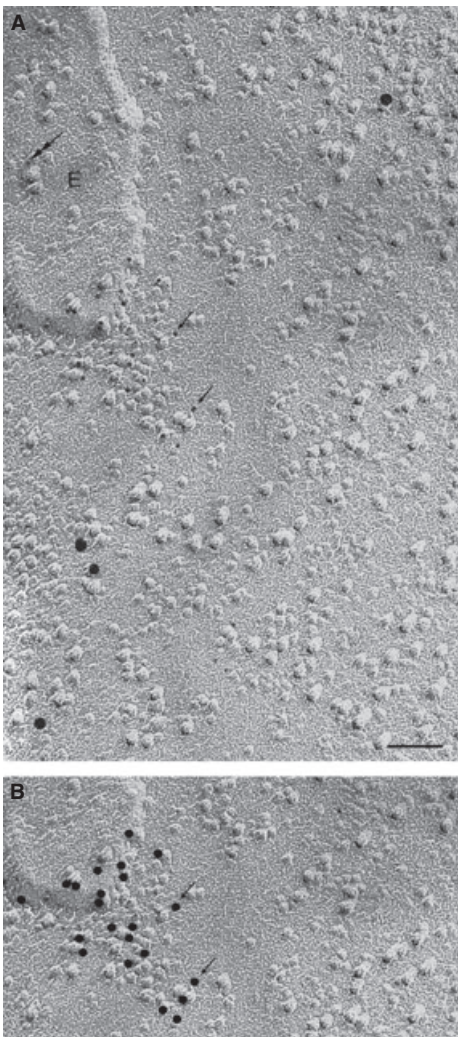

FIG. 4. Localisation of the alpha2 subunit of the $GABA_A$ receptor in a synapse on an axon initial segment. (A) The radially oriented axon initial segment is identified by immunolabelling for the $Na_v1.6$ sodium channel alpha subunit (15-nm gold particles), which is highly expressed in the initial segment membrane of CA1 pyramidal cells. The alpha2 subunit is localised by small immunogold particles (nominally 5 nm), which are concentrated over a patch of IMPs, identifying it as a synapse, partially covered by the E-face (E) of the presynaptic terminal. There is also one low electron density particle on the E-face (double arrow). (B) To assist interpretation of the small gold particles, which are partially electron lucent and had low contrast, each was covered with a black marker dot. Note that at the boundary of the shift of the fracture plane from the membrane of the axon initial segment to the membrane of the presynaptic bouton, immunoparticles overlap the E-face, which is most likely due to a small overlap of undigested or trapped P-face membrane under the E-face membrane half, and is not the location of alpha2 subunit-containing receptors in the presynaptic terminal. Any intracellular protein domains in the E-face membrane half are covered with the carbon/platinum layer and are not accessible to antibodies. Scale bar: 0.1 μm (A).

membranes cannot be identified. We used the labelling on the E-face membranes as these are also rich in proteins. For example, AMPA- and *N*-methyl-D-aspartate (NMDA)-type glutamate receptors are highly retained in the extracellular half of the plasma membrane replica (Tanaka *et al.*, 2005; Tarusawa *et al.*, 2009). E-face gold density was measured on large areas of individual cell bodies, and three–five random images were captured for each cell, corresponding to 14.2 ± 7.7 μm^2 (n = 47) of somatic E-face membrane per cell. Any attached P-face membrane fragment areas were measured and subtracted from the cell surface. On average, four cells per animal per antibody were measured (range 2–5). In the four animals, the average E-face particle density (mean ± SD, n = 4, gold particles/μm^2) was 0.99 ± 1.08 (range 0.36–2.60) for the alpha1 subunit, 0.71 ± 0.06 (range 0.65–0.80) for the alpha2 subunit and 0.91 ± 0.77 (range 0.49–2.06) for the beta3 subunit. These measurements show that, on average, the background labelling was 0.49% (range 0.14–1.35) of synaptic labelling for the alpha1 subunit, 0.13% (range 0.10–0.16) for the alpha2 subunit and 0.15% (range 0.07–0.36) for the beta3 subunit. For the background-corrected extrasynaptic labelling the proportion of background was inevitably higher. On average, the background labelling was 20.0% (range 12.7–25.6) of extrasynaptic labelling for the alpha1 subunit, 12.2% (range 8.8–18.8) for the alpha2 subunit and 12.0% (range 3.4–28.5) for the beta3 subunit.

From the density values corrected for background, a ratio of synaptic to extrasynaptic density was calculated for each animal and each antibody. The ratios were averaged across animals. On average, the ratio for the alpha1 subunit was 77.9 (Table 3), for the alpha2 subunit was 94.4 and for the beta3 subunit it was 78.6.

Somatic surface synaptic membrane and immunolabelling fraction

In order to calculate the total amount of subunit labelling in the synaptic and extrasynaptic membranes, we measured the plasma membrane area occupied by synaptic junctions on the somata in three additional rats (three cells in each). We used beta3 subunit labelling with an affinity-purified rabbit antibody as a GABAergic synaptic marker, and 10-nm gold particles, which are easily recognised (Fig. 1A). The three rats were not different (Kruskal–Wallis, P = 0.252) and were pooled, resulting in a mean proportion of somatic surface occupied by GABAergic synapses of 0.72 ± 0.11%. Using the mean synaptic and extrasynaptic densities for each subunit in each of the four animals, and taking into account the average synaptic fraction of soma surface, the proportions of synaptic and extrasynaptic labelling were calculated and averaged across animals. The proportion of the total labelling that was in synapses was 33 ± 17% for the alpha1 subunit, 40 ± 5% for the alpha2 subunit and 36 ± 7% for the beta3 subunit.

Estimation of alpha1 subunit distribution with two different antibodies

The above results were obtained with polyclonal antibodies, which are likely to contain several antibody species with different properties. Furthermore, even the same antibody species may lead to different labelling densities depending on how densely the epitopes are located in the membrane or in a single receptor channel. To test the effect of antibodies on synaptic vs extrasynaptic labelling, we compared the labelling for the alpha1 subunit obtained with the rat antiserum, which produced the lowest synaptic labelling signal (see above), to that obtained with affinity-purified rabbit antibodies. Because no tissue was available from the four animals (Batch 1, Table 3) analysed above, we used tissue from three further rats (Batch 2, Table 3) processed together under the same conditions and reacted together by the same person with both the rat and rabbit antibodies to the alpha1 subunit. The comparison is presented in Table 3. The non-parametric Mann–Whitney test was used for comparing variables.

The size of synapses was not different between the two batches of rats measured with either the rat antiserum (Z = −1.414, P = 0.229) or rabbit antibody (Z = −2.121, P = 0.057) to the alpha1 subunit. There

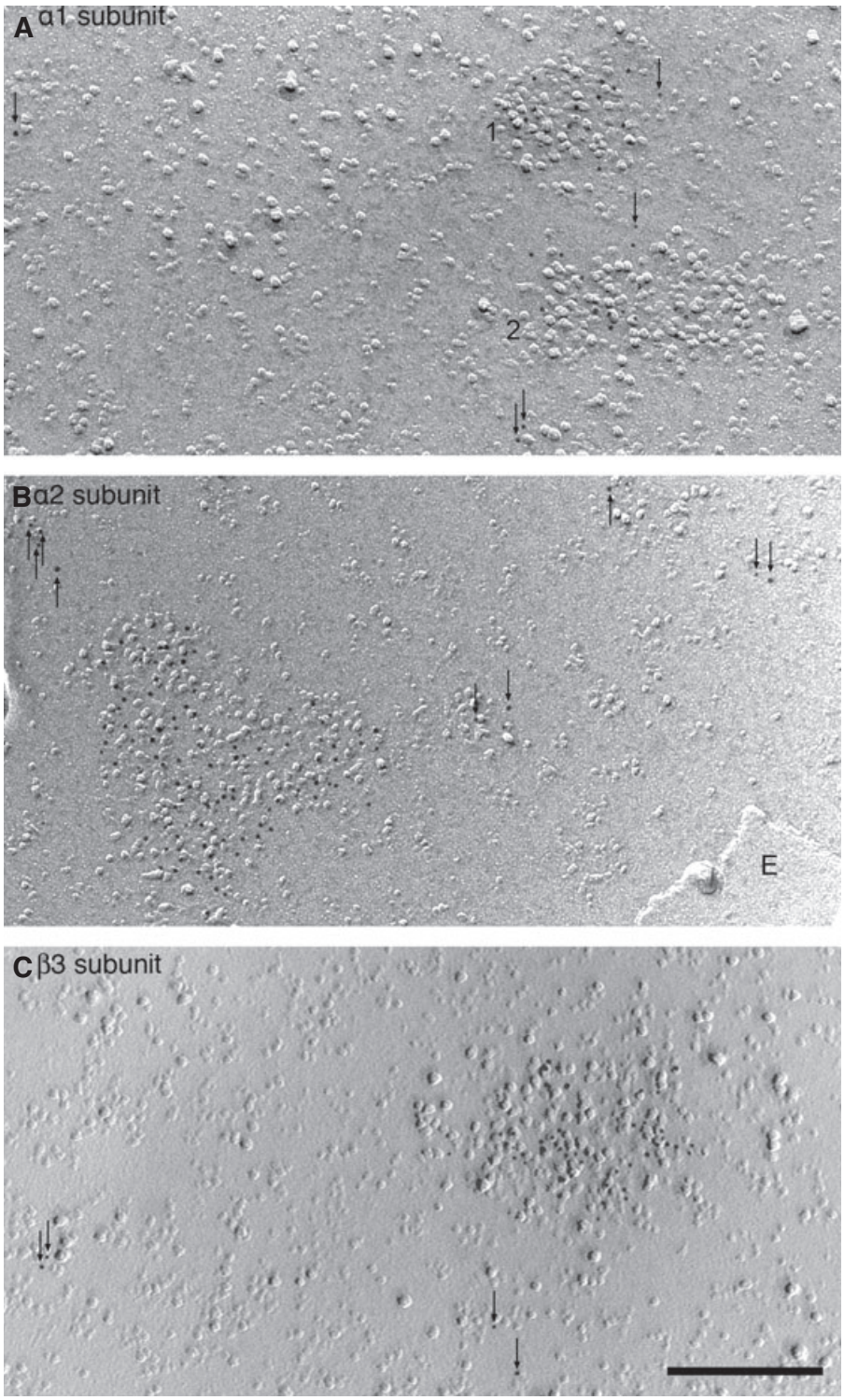

FIG. 5. Synaptic and extrasynaptic localisation of $GABA_A$ receptor subunits on CA1 pyramidal cell somata. (A–C) Labelling for the alpha1, alpha2 and beta3 subunits, respectively (5-nm immunogold particles) is highly concentrated on clusters of IMPs on the P-face of the replicated plasma membrane with low-density scattered immunoparticles in the extrasynaptic regions. Note the high density of synaptic labelling in patches within the IMP clusters. Two synapses (1, 2) are labelled for the alpha1 subunit. (B) The extrasynaptic (E) face of the plasma membrane of a neighbouring cell is in the lower right corner. Scale bar: 0.2 μm (A–C).

was a strong positive correlation between the number of immunoparticles and synapse size (Pearson correlation test, two-tailed) in all three animals, confirming results obtained with the rat antiserum in Batch 1. For the rat antiserum, the mean correlation coefficient was 0.746 ± 0.029 ($n = 3$, $4.54E\text{-}09 \geq P \geq 4.06E\text{-}17$). The distribution of labelling density values was normal (Kolmogorov–Smirnov, two-tailed, $n = 181$, $Z = 0.817$, $P = 0.516$), and there was no correlation between synapse size and the density of immunoparticles (Pearson correlation test, two-tailed, $P > 0.65$) in two animals, whereas the third animal (rat 2) showed a weak negative correlation ($r = -0.290$, $P = 0.046$). For the rabbit antibody, there was also a strong positive correlation between the number of immunoparticles and synapse size (Pearson correlation test, two-tailed) in all three animals. The mean correlation coefficient was 0.842 ± 0.064 ($n = 3$, $2.51E\text{-}11 \geq P \geq 5.2E\text{-}21$). The distribution of labelling density values was normal (Kolmogorov–Smirnov, two-tailed, $n = 158$, $Z = 0.797$, $P = 0.55$), and there was no correlation between synapse size and the density of immunoparticles (Pearson correlation test, two-tailed,

TABLE 3. Comparison of immunolabelling parameters for the alpha1 subunit using two different antisera, and the antiserum raised in rat on two different batches of rats

			Synapses			Extrasynaptic		Synaptic/extrasynaptic			E-face (background)		
			Density* (no/μm^2)		No. gold/ synapse	Density* (no/μm^2)		Ratio			Density (no./μm^2)	% Density of	
Experi-mental animals	Antisera raised in	*n*	Mean ± SD	Range	Mean ± SD	Mean ± SD	Range	Mean ± SD	Range	% Synaptic labelling on soma	Mean ± SD	Synaptic labelling	Extrasynaptic labelling
Rats													
Batch 1	Rat	4	219 ± 30	192–261	11.5 ± 1.7	4.5 ± 3.9	1.8–10.2	78 ± 55	19–148	33 ± 17	0.99 ± 1.08	0.49 ± 0.58	20.0 ± 5.4
Batch 2	Rat	3	237 ± 23	211–251	14.2 ± 1.0	3.3 ± 1.8	1.9–5.2	85 ± 34	47–114	37 ± 10	0.420 ± 0.220	0.175 ± 0.083	15.51 ± 9.54
	Rabbit		443 ± 52	383–473	28.1 ± 1.3	3.5 ± 0.7	2.7–4.0	132 ± 39	102–176	48 ± 7	0.095 ± 0.068	0.022 ± 0.014	2.84 ± 1.68
Cells													
Batch 2	Rat	17	234 ± 35	171–303	14.0 ± 2.8	3.1 ± 1.7	1.0–5.6	99 ± 53	40–229	39 ± 12			
	Rabbit	15	443 ± 84	332–638	28.1 ± 7.2	3.5 ± 1.3	1.7–6.3	141 ± 50	62–256	49 ± 9			
Synapses													
Batch 1	Rat	249	226 ± 98	38–505	11.9 ± 6.6								
Batch 2	Rat	181	232 ± 74	55–489	13.8 ± 6.7								
	Rabbit	158	446 ± 137	130–833	28.5 ± 16.0								

*Immunogold densities were corrected by subtracting the background [exoplasmic (E)-face density in the same specimen] from each synaptic and extrasynaptic density value.

$P > 0.20$) in two animals, whereas the third animal (rat 2) showed a weak negative correlation ($r = -0.269$, $P = 0.043$).

Next, we tested if the rat antiserum produced similar or different results on the two batches, the four rats reacted individually, and the three rats reacted together. The number of immunogold particles per synapse ($Z = -1.768$, $P = 0.114$), the synaptic labelling density ($Z = -0.707$, $P = 0.629$), the extrasynaptic labelling density ($Z = 0$, $P = 1$) and the E-face labelling ($Z = 0$, $P = 1$) showed no difference between the two sets of rats.

Testing the same parameters using the rabbit antibody on specimens from batch 2 rats ($n = 3$) in comparison with rat antiserum showed no difference in the number of immunogold particles per synapse ($Z = -1.964$, $P = 0.100$), synaptic density ($Z = -1.964$, $P = 0.100$), extrasynaptic density ($Z = -0.218$, $P = 1.000$) or E-face labelling ($Z = -1.528$, $P = 0.200$). This was probably due to the low number of animals tested and the high level of variability between animals. For example, the mean number of gold particles per synapse was twice as high, on average, with the rabbit antibody than with the rat antiserum, and the density of synaptic labelling was 87% higher. Therefore, we also compared the parameters between the populations of pyramidal cells sampled from rats in batch 2 ($n = 17$, rat antiserum; $n = 15$, rabbit antibody; Table 3). In this comparison the use of the rabbit antibody resulted in a significantly higher number of gold particles per synapse ($Z = -4.589$, $P = 1.10E-07$) and synaptic labelling density ($Z = -4.815$, $P = 3.50E-09$). The extrasynaptic labelling densities were not different with the two antibodies ($Z = -1.038$, $P = 0.313$), but due to the higher synaptic labelling with the rabbit antibody the ratios of synaptic to extrasynaptic density per cell values were higher ($Z = -2.398$, $P = 0.016$) with the rabbit antibody. A significantly higher synaptic particle number ($Z = -10.52$, $P = 7.00E-26$) and synaptic immunolabelling density ($Z = -13.44$, $P = 3.50E-41$) was also obtained with the rabbit ($n = 158$ synapses) as compared with the rat antiserum ($n = 181$ synapses) when all synapses were pooled across animals. Accordingly, we concluded that the rabbit antibody provides higher labelling efficacy as compared with the rat antiserum in batch 2 rats.

Because the rat antiserum provides similar labelling in the two batches of rats, we pooled these data, and compared the resulting labelling parameters to those obtained with the rabbit antibody on batch 2 rats, for which both antibodies were tested. The number of gold particles per synapse ($Z = -2.393$, $P = 0.017$), the density of synaptic labelling ($Z = -2.393$, $P = 0.017$) and the E-face labelling ($Z = -2.165$, $P = 0.033$) were different for the two antibodies. The density of extrasynaptic labelling ($Z = -0.342$, $P = 0.833$) and the ratio of synaptic to extrasynaptic labelling ($Z = -1.709$, $P = 0.117$) were not different with the two antibodies. The ratio of synaptic to extrasynaptic labelling density (background corrected) was, on average, 131.6 ± 38.8 ($n = 3$) with the rabbit antibody, and that obtained with the rat antiserum was 80.8 ± 43.8 ($n = 7$).

Using the mean synaptic and extrasynaptic densities obtained with the rat antiserum for each of the seven animals, and with the rabbit antibody for each of the animals in batch 2, and taking into account the average fraction of synaptic soma surface obtained earlier (0.72%), the proportion of synaptic alpha1 subunit labelling 37 ± 10% ($n = 3$) obtained with the rat antiserum was not different ($Z = -1.528$, $P = 0.200$) from that obtained with the rabbit antibody 48 ± 7% ($n = 3$). Neither value was significantly different from that obtained with the rat antiserum in the first four rats (33 ± 17; Mann–Whitney, rat antiserum, $Z = -0.354$, $P = 0.857$; rabbit antibody, $Z = -1.414$, $P = 0.229$). In conclusion, 33–48% of the alpha1 subunit labelling on the soma surface was in synaptic junctions.

Co-localisation of two $GABA_A$ receptor subunits in individual synapses

Technical considerations

Previous studies suggested that $GABA_A$ receptors that contain different alpha subunits might be targeted to partially separate sets of synapses on CA1 pyramidal cells (Nusser *et al.*, 1996; Thomson *et al.*, 2000; Nyiri *et al.*, 2001; Klausberger *et al.*, 2002; Ali & Thomson, 2008). To assess the co-existence of multiple $GABA_A$ receptor subunits in individual synapses, we carried out double-labelling using antibodies raised in different host species and immunogold particles of different sizes (5 and 10 nm). Robust evidence for pair-wise co-existence of all three subunit combinations was observed in individual synapses on pyramidal cell somata (Fig. 8; Table 4). Simultaneous application of two primary antibodies to two different subunits reduces labelling intensities, due to steric hindrance if both subunits are present in the same channel. This can lead to false negative results for one or both subunits. Therefore, a

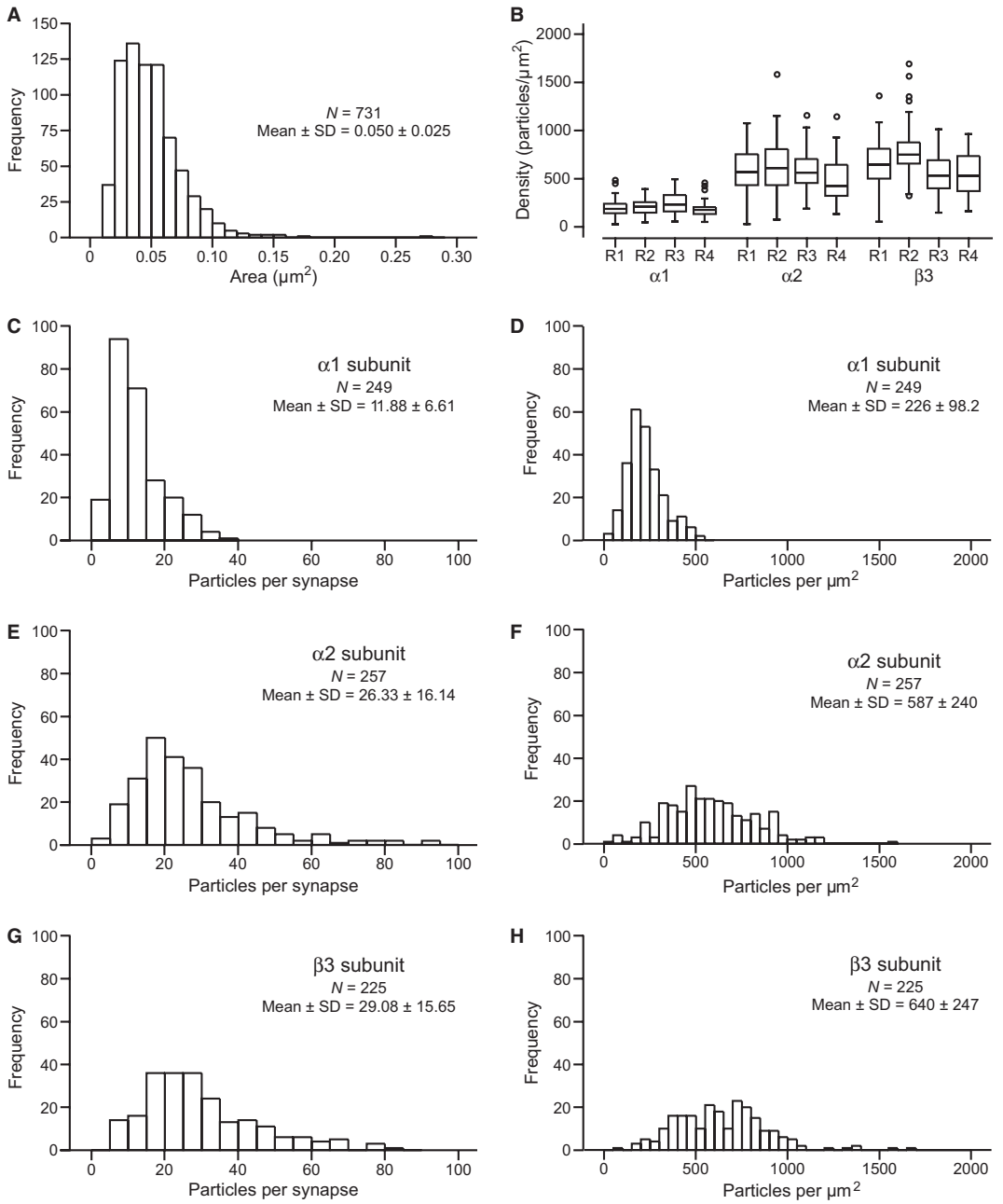

FIG. 6. Distribution of synaptic area values and $GABA_A$ receptor subunit immunolabelling. (A) Synaptic area size is not normally distributed, it is skewed towards larger values. (B) Average synaptic immunolabelling and variability in each of four rats shows consistent labelling. Horizontal lines show median, box indicates 50 percentile, whiskers show upper and lower 25%, and circles show outliers. (C, E, G) The distribution of immunolabelling strength (no. of immunogold particles per synapse) is skewed towards larger values for all subunits. (D, F, H) In contrast to the distributions in (C, E and G), the synaptic density value distributions are normal (see text), indicating uniform labelling density. Note that labelling intensity differences between antibodies do not represent differences in subunit abundance, because the degree of labelling is not comparable between antibodies due to the individual properties of each antiserum.

sequential application of both primary and secondary antibodies was adopted. The first primary antibody (followed by the first secondary antibody) was predicted to represent the maximal population of labelled synapses, particularly when detected with 5-nm particles. Labelling intensity differences between antibodies cannot be taken as differences in subunit abundance, due to the individual properties of each antiserum. Due to the above factors, we did not calculate the ratios of labelling densities in individual synapses and we present the frequency of co-labelled synapses in two of the four rats in batch 1 (Table 4). First, we detected all synapses labelled with at least one 10-nm particle (applied second) and screened them for the presence of labelling by at least three 5-nm particles (applied first). The co-labelling threshold was set lower for the secondarily applied antibody because of the above-mentioned factors that reduce the probability of labelling. In order to test the effect of the reduced labelling probability by the second application of an antibody on the proportion of co-labelled synapses, we reversed the sequence of the two antibodies.

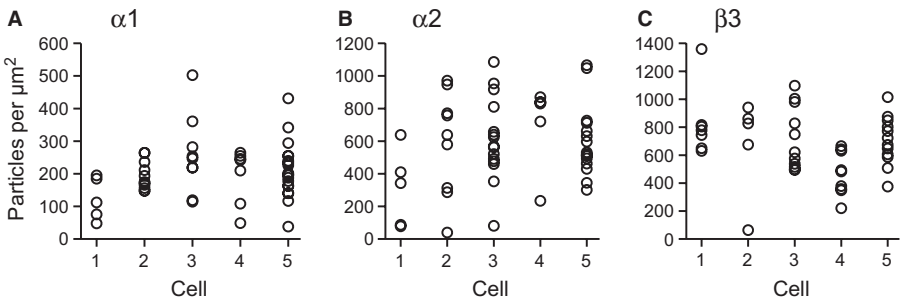

FIG. 7. Variability of synaptic $GABA_A$ receptor labelling for the alpha1, alpha2 and beta3 subunits on individual CA1 pyramidal cell somata. (A–C) All visible synapses were analysed on the somata of five pyramidal cells for each subunit. The cells for a given subunit were not different in synaptic particle density (pooled mean ± SD, particles/μm^2; alpha1, 205 ± 88; alpha2 587 ± 261; beta3 675 ± 240). Synaptic immunolabelling density varied in a range of up to one order of magnitude amongst synapses for a given antibody. Note that labelling intensity differences between antibodies do not represent differences in subunit abundance, because the degree of labelling is not comparable between antibodies due to the individual properties of each antiserum.

Pairwise testing of individual synapses for the alpha1, apha2 and beta3 subunits

A total of 525 synapses (144–203 per antibody combination) were analysed (Table 4). The results were not different between the two animals (Chi square test, DF = 1, 0.000 < Chi-square < 0.268, Fisher's exact $1 > P > 0.67$) for any of the six combinations of the three subunits. Nevertheless, all but one of the proportion values representing the coexistence of two subunits were slightly higher for rat 2, indicating more favourable subunit recognition by all antibodies, probably due to technical factors. The mean number of gold particles per synapse for the first applied antibody was 16.8 ± 8.4 (n = 12 reactions, six in each of two animals, 5-nm gold, including immunonegative synapses) and for the secondarily applied antibody it was 7.8 ± 5.6 (10-nm gold). As expected, all but one of the alpha1 or alpha2 subunit-positive synapses (n = 165) were also positive for the beta3 subunit in the two animals. The vast majority (95%) of synapses tested (n = 144) were also labelled for both the alpha1 and alpha2 subunits, showing the presence of receptors including one or both of these subunits in most basket cell synapses. The degree of synaptic labelling in doubly-labelled synapses as measured by the number of immunoparticles per synapse was highly variable, for both the alpha1 subunit (5 nm, n = 83, range 3–28, median 6; 10 nm, n = 59, range 1–9, median 2) and the alpha2 subunit (5 nm, n = 54, range 3–44, median 22; 10 nm, n = 85, range 3–29, median 10). There was either no or only very weak positive correlation between the number of immunoparticles labelling these two subunits in individual synapses.

Discussion

The results show that GABAergic synapses can be recognised in freeze-fracture replicas in the cytoplasmic, P-face membrane halves as distinct IMP clusters. On somata of CA1 pyramidal cells, the total amount of alpha1, alpha2 and beta3 subunit expression in the synapses is similar to that in the extrasynaptic membrane. Synaptic receptor clusters end with a sharp decrease in labelling density, only occupy 0.72% of the soma surface and have 50–130 times higher immunolabelling densities than the extrasynaptic membrane. Double-labelling experiments with combinations of antibodies to these three subunits indicate that virtually all somatic synapses contain the alpha1, alpha2 and beta3 subunits at varying densities. Synaptic labelling density for a given subunit may vary up to an order of magnitude amongst synapses.

Technical considerations

The labelling efficacy of membrane molecules in immunohistochemistry in general as well as in FRIL depends on many factors that may influence our results. The current method, which evolved after many difficult approaches had been tried to identify proteins in freeze-fracture replicas (Rash & Yasumura, 1999), provides a two-dimensional landscape of the plasma membrane. The breakthrough came with the introduction of SDS by Fujimoto (1995, 1997) to wash the tissue components from the replica film, leaving only molecules, such as membrane-spanning proteins and lipids (Fujimoto *et al.*, 1996), that are directly in contact and trapped by the initial carbon coating deposited over the middle of the split membrane. Subsequently, it was shown that by carefully adjusting the washing conditions, proteins associated with the membrane-spanning molecules, but not having intramembrane domains themselves, can be also retained and immunolabelled on the cytoplasmic P-face membrane leaflet (Hagiwara *et al.*, 2005). This and other unpublished experiments indicate that the SDS washing conditions, such as temperature and duration, may influence the retention of the membrane protein including the $GABA_A$ receptor.

Immunolabelling may also be affected by factors that do not uniformly apply to all parts of the plasma membrane. Fixation may reduce the recognition of the epitopes differentially depending on the local protein environment. A reduction of immunoreactivity by fixation has been reported for $GABA_A$ receptors with antibodies recognising extracellular epitopes (Fritschy *et al.*, 1998b).

We have detected immunoreactivity in both the E-face and the P-face of the split plasma membranes with different antibodies. On the P-face replica the IMPs represent the domains of the membrane proteins that span the outer leaflet of the lipid bilayer and include the extracellular domain as well. It is thought that the splitting of the membrane does not lead to the breaking of covalent bonds, and individual molecules are pulled to either one or the other membrane leaflet (Fisher, 1989). The partitioning of a molecule between the E-face and the P-face may depend on its subcellular location and could be influenced by the local macromolecular environment, particularly by interactions with cytoskeletal and other proteins. Therefore, further studies are needed with face-matched double replicas (Hagiwara *et al.*, 2005; Masugi-Tokita & Shigemoto, 2007) in which the E-face and the P-face leaflets of the same membrane area are analysed with two different antibodies to either extracellular epitopes or intracellular epitopes, respectively, of the same subunit, in order to establish if the partitioning may be biased in different subcellular compartments.

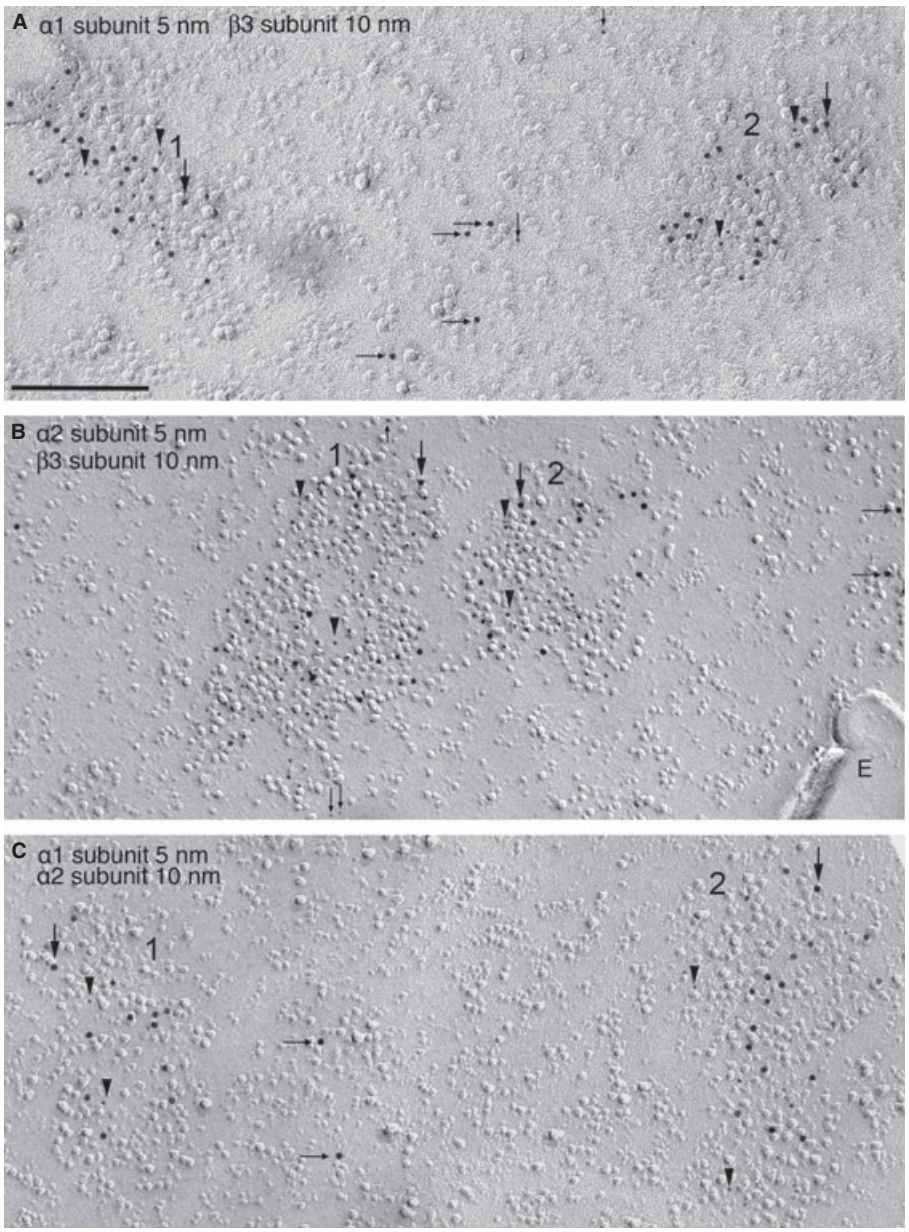

FIG. 8. Co-localisation of alpha1, alpha2 and beta3 subunits in individual synapses on CA1 pyramidal cell somata. Sequential applications of two antibodies with different size immunoparticles for the two subunits in each replica. The antibody labelled with 5-nm particles was applied first. Extrasynaptic subunit labelling is marked by vertical arrows for the 5-nm particles and horizontal arrows for the 10-nm particles. (A) Two synapses (1, 2) are labelled for both alpha1 (5 nm, e.g. arrowheads) and beta3 (10 nm, large vertical arrows) subunits. (B) Two synapses (1, 2) are labelled for both the alpha2 (5 nm, e.g. arrowheads) and beta3 (10 nm, large vertical arrows) subunits. (C) Two synapses (1, 2) are labelled for both the alpha1 (5 nm, e.g. arrowheads) and alpha2 (10 nm, large vertical arrows) subunits. The ratio of labelling for the two subunits is different in the two synapses (synapse 1, alpha1/alpha2, 2/12 particles; synapse 2, alpha1/alpha2, 14/16 particles). Note that labelling intensity differences between antibodies do not represent differences in subunit abundance, because the degree of labelling is not comparable between antibodies due to the individual properties of each antiserum. The application of a primary antibody applied second in the sequence and the use of larger particles both reduce labelling efficacy. Scale bar: 0.2 μm (A–C).

The density of $GABA_A$ receptor channels has been estimated from combined immunogold labelling and recording quantal synaptic responses (miniature inhibitory postsynaptic currents; mIPSCs) *in vitro* to be about 1250 receptors per μm^2 in cerebellar stellate cell synapses (Nusser *et al.*, 1997), where a uniform receptor density distribution was found. In our measurements, the density showed a high variability for a given subunit with coefficients of variation ranging from 0.25 to 0.49 for synapses pooled across animals, and showing a Gaussian distribution. The variability in density may indicate that in the high-density location due to steric hindrance the true subunit abundance is under-reported by our method at the applied antibody concentrations. Using FRIL on unfixed tissue, ~1000 immunoparticles (~5 nm diameter) per μm^2 were found, on average, for the AMPA-type glutamate receptor in developing climbing fibre to Purkinje cell synapses, which matched well the expected number of functional channels (Tanaka *et al.*, 2005). In some synapses, up to 1460 particles per μm^2 were recorded with antibodies that recognised all four different receptor subunit species. Because four subunits are present in an AMPA receptor, this indicates that, on average, only one of them per receptor may be reported by the immunogold particle.

TABLE 4. Frequency of co-localisation of different $GABA_A$ receptor subunits in individual synapses

Antibodies to subunit		Proportion [% (total no. tested)] of double-labelled synapses of the synapses labelled by antibody applied second	
First applied	Second applied	Rat 1	Rat 2
$\alpha1$	$\beta3$	81 (58)	95 (49)
$\beta3$	$\alpha1$	96 (23)	100 (48)
$\alpha2$	$\beta3$	85 (59)	98 (50)
$\beta3$	$\alpha2$	100 (44)	100 (50)
$\alpha1$	$\alpha2$	98 (65)	93 (20)
$\alpha2$	$\alpha1$	87 (30)	98 (29)

A similar under-reporting may also occur for the $GABA_A$ receptors, although the two alpha and two beta subunits are not directly next to each other in the pentameric channel (Minier & Sigel, 2004), and the cytoplasmic loops that are recognised may be more accessible than the extracellular epitopes detected on AMPA receptors. A similarly high density of immunolabelling has been achieved also in fixed tissue in adult retino-geniculate glutamatergic synapses (Tarusawa *et al.*, 2009), and even higher densities, up to 3400 particles per μm^2, were reported in the spinal cord (Antal *et al.*, 2008). The highest labelling density of synaptic receptors in our study was also about 1000–1500 particles per μm^2, lower than for the AMPA-type glutamate receptors cited above, indicating that steric hindrance between antibodies on neighbouring receptors did not reduce labelling.

It is not yet possible to compare labelling densities and subunit abundance across different antibodies, although such comparisons have been reported (Caruncho *et al.*, 1995). Two immunised animals may produce antibodies of widely differing labelling efficacy even to the same immunogen. To relate labelling density to subunit and $GABA_A$ receptor number, independent measures of calibration are required as has been done in the GABAergic synapses on stellate cells (Nusser *et al.*, 1997) or AMPA-type glutamate receptors (Tanaka *et al.*, 2005; Tarusawa *et al.*, 2009). Our comparison of antibodies raised in different species to the alpha1 subunit showed that a doubling of synaptic labelling density can be achieved in samples from the same animal. However, due to the small surface fraction of synaptic areas on the soma, the proportion of synaptic labelling for the subunit in synaptic vs extrasynaptic membrane changed only by 15%. Surprisingly, the extrasynaptic labelling density was not different between the two antibodies. One possible explanation is that in the case of the rat antiserum the extrasynaptic labelling density may contain some non-specific labelling for which the E-face measurement is not a full control. In future studies, the recognition of such P-face membranes, which contain no $GABA_A$ receptors, will provide a better control for non-specific labelling. The other possibility is that labelling with the purified rabbit antibody that provided a higher synaptic labelling density was amplified by the presence of anti-idiotypic antibodies, which may amplify labelling in an antibody density-dependent manner. The immunisation of the rabbit was carried out over a prolonged period, and we cannot exclude the presence of anti-idiotypic antibodies.

The relationship between subunit abundance and receptor abundance also requires further analysis. There are two alpha and two beta subunits, which may not be identical molecular species, in most $GABA_A$ receptors (Sieghart & Sperk, 2002). For example, pyramidal cells may express receptors with two alpha1 subunits or an alpha1 and an alpha2 subunit preferentially in different synapses, or selectively in the synaptic or extrasynaptic membrane. When two identical subunit species are present in a channel, the binding of an antibody to one of them may occlude the binding of a further antibody to the second subunit. However, in a receptor containing two different alpha or beta subunits, every subunit of a particular species may be labelled. The above difference could influence labelling density in particular molecular compartments.

Notwithstanding the above uncertainties, FRIL provides a highly sensitive method to compare $GABA_A$ receptor distribution in membrane microdomains such as synapses. The particular advantages and limitations have been discussed recently (Hagiwara *et al.*, 2005; Masugi-Tokita & Shigemoto, 2007). Here we exploited the advantages of the method that shows the proteins in two-dimensional membrane sheets, which can be sampled uniformly.

Synaptic receptors and phasic inhibition

The presence of alpha1, alpha2 and beta3 subunit immunoreactivity in somatic synaptic junctions of CA1 pyramidal neurons is in line with results of previous immunocytochemical (Nusser *et al.*, 1996; Somogyi *et al.*, 1996; Crestani *et al.*, 1999) and pharmacological (Pawelzik *et al.*, 1999; Thomson *et al.*, 2000) studies of the adult hippocampus in rat and mouse (Crestani *et al.*, 1999; Prenosil *et al.*, 2006).

As virtually all somatic synapses defined by IMP clusters contained beta3 subunit immunoreactivity, it is likely that synapses innervated by both cholecystokinin- and parvalbumin-expressing basket cells contained this subunit, but our results shed no light on the subunit partners in single-receptor pentamers. Even in double-labelled synapses the lateral resolution of about 20 nm does not allow any two immunogold particles to be assigned to a single receptor. In CA1 pyramidal neurons of beta3 gene-deleted mice, a selective loss of slow $GABA_A$ receptor-mediated synaptic responses has been reported (Hentschke *et al.*, 2009), and these are probably of dendritic origin in contrast to the fast IPSCs, many of which are mediated by somatic synapses. The fast IPSCs had only slightly faster decays in beta3 gene-deleted pyramidal cells (Hentschke *et al.*, 2009), indicating that either in the mouse the beta3 subunit makes little contribution to somatic synapses, or other subunit(s) compensated for the loss.

The presence of both alpha1 and alpha2 subunits in virtually all somatic synapses in our study of the rat may appear at odds with conclusions obtained in mice (Prenosil *et al.*, 2006). Making alpha2-containing receptors diazepam insensitive was reported to abolish selectively the diazepam enhancement of synaptic currents in pyramidal cells evoked by extracellular stimulation in the soma layer. Synaptic currents in mice in which alpha1 subunit-containing receptors were rendered diazepam insensitive responded with enhancement to the same stimulus (Prenosil *et al.*, 2006). Although the subcellular source of the currents cannot be identified with extracellular stimulation, these results would predict a predominance of the alpha2 subunits in somatic synapses in the mouse. Apart from species differences between rat and mouse, another explanation may be the different position of the alpha1 and alpha2 subunits in the same single-receptor pentamer; the alpha2, but not the alpha1, subunit being next to the gamma2 subunit and thereby contributing to the benzodiazepine-binding pocket (see below). Although the subunit composition of receptors containing alpha1 or alpha2 subunits have not been defined in hippocampal pyramidal cells, receptors containing both alpha1 and alpha2 subunits are almost as abundant in the mouse forebrain as those containing two alpha2 subunits (Benke *et al.*, 2004).

Is there an input-specific sorting of synaptic $GABA_A$ receptors?

Previous studies of somatic synapses suggested an enrichment of alpha1 or alpha2 subunit-containing $GABA_A$ receptors in synapses established, respectively by parvalbumin- or presumed cholecystokinin-expressing basket cells. First, zolpidem, a benzodiazepine receptor agonist, which shows some selectivity for alpha1 subunit-containing receptors, enhanced IPSPs evoked by fast-spiking (presumed parvalbumin-expressing) basket cells more than IPSPs evoked by regular firing (presumed cholecystokinin-expressing) basket cells (Thomson *et al.*, 2000). Second, a higher level of alpha2 subunit immunoreactivity was found in synapses made by parvalbumin-negative boutons (Nyiri *et al.*, 2001), which were suggested to originate from cholecystokinin-expressing basket cells, than in synapses made by parvalbumin-positive boutons. In a complementary study (Klausberger *et al.*, 2002), the synapses made by parvalbumin-positive boutons had higher alpha1 subunit immunoreactivity than those made by parvalbumin-negative boutons. These quantitative studies used the postembedding immunolabelling technique that has lower sensitivity than the FRIL method.

Immunoreactivity for both alpha1 and alpha2 subunits in virtually all synapses on somata found here appears to contradict these previous conclusions. However, we have not been able to establish the relative abundance of these two subunits in individual synapses, because the recognition of two molecules on the same membrane replica face requires the use of two different sizes of gold particles, the larger one having a lower sensitivity. In addition, in order to produce results comparable to the single antibody labelling used in quantification, the two antibodies need to be applied sequentially leading to a reduced density of labelling for the antibody applied second. Therefore, a potential negative correlation between the levels of the alpha1 and alpha2 subunits, which might follow from the results obtained with the postembedding immunogold method, remains an open question. Because both the E-face and the P-face replicas of the two membrane halves of a single synapse can be immunoreacted with different antibodies (Hagiwara *et al.*, 2005; Shinohara *et al.*, 2008), future studies could address the relative abundance of different receptor subunits in individual synapses using antibodies recognising extracellular epitopes in one subunit on the E-face replica and intracellular epitopes in another subunit on the P-face replica.

Tonic $GABA_A$ receptor-mediated inhibition and extrasynaptic receptor subunits

Following the immunohistochemical demonstration of extrasynaptic $GABA_A$ receptors (Somogyi *et al.*, 1989; Soltesz *et al.*, 1990; Nusser *et al.*, 1995) and the pioneering electrophysiological observations on hippocampal granule cells (Otis *et al.*, 1991), a tonic $GABA_A$ receptor-mediated current was discovered in cerebellar granule cells (Kaneda *et al.*, 1995; Brickley *et al.*, 1996; Wall & Usowicz, 1997). These cells have a particularly high level of alpha6 and delta subunit-containing extrasynaptic receptors (Nusser *et al.*, 1996, 1998). Subsequent recordings in tissue slices *in vitro* suggested that $GABA_A$ receptors also mediate a tonic current in hippocampal granule cells (Nusser & Mody, 2002), CA1 pyramidal cells (Bai *et al.*, 2001; Caraiscos *et al.*, 2004a), thalamic relay cells (Porcello *et al.*, 2003; Belelli *et al.*, 2005; Cope *et al.*, 2005) and hippocampal interneurons (Semyanov *et al.*, 2003; Glykys *et al.*, 2007; Mann & Mody, 2010), among others. Because the delta subunit has not been found in synapses (Nusser *et al.*, 1998; Wei *et al.*, 2003), its involvement in the tonic currents supports its extrasynaptic origin. Low concentrations of the naturally occurring neurosteroid 3α,21-dihydroxy-5α-pregnan-20-one (allotetrahydrodeoxycorticosterone) selectively enhance the tonic current in mouse dentate granule cells, but not in CA1 pyramidal cells, in a delta subunit-dependent manner (Stell *et al.*, 2003). In the mouse CA1 area the tonic current is accounted for by alpha5 subunit-containing receptors, and in the absence of the alpha5 subunit also by delta subunit-containing receptors (Caraiscos *et al.*, 2004a; Glykys & Mody, 2006). There may be species differences between the mouse and rat. It was proposed (Scimemi *et al.*, 2005) that in rat CA1 pyramidal neurons the tonic current is mostly mediated by alpha4/delta subunit-containing receptors at low concentrations of GABA, but not by alpha5 subunit-containing receptors, whereas at higher GABA concentration alpha5 subunit-containing receptors, which probably do not include the delta subunit, carry much of the tonic current *in vitro*. In the mouse, deleting genes coding for the alpha5 subunit (Caraiscos *et al.*, 2004a; Glykys & Mody, 2006) significantly reduces the tonic current in hippocampal pyramidal cells, and other neurons (Glykys *et al.*, 2008). Although receptors containing the alpha5 subunit are thought to be predominantly in the extrasynaptic membrane, synaptic localisation (Fritschy *et al.*, 1998a; Christie & deBlas, 2002) and the involvement in synaptic responses (Ali & Thomson, 2008; Zarnowska *et al.*, 2008) of the alpha5 subunit have also been reported. Pharmacological studies using a low dose of the benzodiazepine inverse agonist, L-655,708, selective for alpha5 subunit-containing receptors, also showed the involvement of this subunit in mediating tonic current (Caraiscos *et al.*, 2004a; Scimemi *et al.*, 2005; Glykys *et al.*, 2008). In mice, deficient in both the delta and alpha5 subunits, virtually all of the tonic current was abolished in hippocampal pyramidal and dentate granule cells *in vitro* (Glykys *et al.*, 2008). However, the above results do not exclude the presence of additional subunits, including the alpha1 and alpha2 subunits, in the receptors mediating the tonic current under normal conditions, but show that in the absence of the delta and/or alpha5 and/or alpha4 subunits, other subunit-containing receptors do not compensate for their absence in mediating a tonic current. Neither the delta nor the alpha5 subunit (when next to the gamma subunit)-containing receptors would be sensitive to zolpidem, which enhances the tonic current in rat CA1 pyramidal cells (Liang *et al.*, 2004). Therefore, the extrasynaptic receptor composition may be different in the rat.

As 50–67% of the alpha1 and alpha2 subunit immunoreactivity is outside synaptic junctions, these subunits may be part of receptors contributing to tonic inhibitory currents with particular pharmacological sensitivity (Belelli *et al.*, 2009). One of the two alpha subunits is positioned between two beta subunits and is not part of the benzodiazepine-binding site. The other alpha subunit is situated between a beta and the gamma2 subunit, and contributes to the benzodiazepine-binding pocket at the interface with the latter (Baumann *et al.*, 2003; Minier & Sigel, 2004; Baur *et al.*, 2006). Although the precise subunit composition of native $GABA_A$ receptors on CA1 pyramidal cells is unknown, some may contain two different alpha subunits.

A large variety of immunoprecipitated receptors have been reported that contained more than one alpha subunit species (Duggan *et al.*, 1991; McKernan *et al.*, 1991; Pollard *et al.*, 1993; Jechlinger *et al.*, 1998; Sieghart & Sperk, 2002; Poltl *et al.*, 2003; Benke *et al.*, 2004). In particular, antibodies specific for the alpha4 subunit, which is often associated with the tonic current, also precipitated alpha1/2/3, but not alpha5 subunits (Benke *et al.*, 1997; Bencsits *et al.*, 1999) from rat forebrain. Accordingly, the alpha1 and alpha2 subunits detected in the extrasynaptic membrane in our study could be part of high-affinity alpha4 subunit-containing receptors, which exhibit a pharmacological profile characteristic of alpha4 subunit-containing receptors. The existence of a specific class of alpha1 subunit-containing, potentially extrasynaptic, receptors has also been suggested on the basis of ligand-

binding autoradiographic experiments involving several lines of subunit-deficient mice, including delta+alpha4 double knockout mice (Halonen *et al.*, 2009). The alpha1 subunit has also been shown to participate in mediating tonic current in partnership with the delta subunit in presumed extrasynaptic receptors on some GABAergic interneurons in the dentate gyrus of the mouse (Glykys *et al.*, 2007). These neurons, unlike CA1 pyramidal cells, do not appear to express a significant level of other alpha subunits. Accordingly, it cannot be excluded that some of the alpha1 subunits that we found in the extrasynaptic membrane of pyramidal cells in the rat participate in the same alpha1/delta subunit combination reported by Glykys *et al.* (2007).

The beta3 subunit in extrasynaptic receptors

Both *in situ* hybridisation and immunohistochemical data suggest that the beta3 subunit is a major contributor to $GABA_A$ receptors in CA1 pyramidal cells (Persohn *et al.*, 1992; Wisden *et al.*, 1992; Pirker *et al.*, 2000), but its role in extrasynaptic receptors has not been evaluated. In whole-brain extracts, the beta3 subunit is thought to assemble more with the alpha2 subunit, and the beta2 subunit more with the alpha1 subunit (Benke *et al.*, 1994). The alpha1 and alpha2 subunits in the extrasynaptic membrane may be associated with different beta subunits, but the abundance of the beta2 subunit in the extrasynaptic membrane remains to be evaluated. In dentate granule cells, the beta2 subunit may contribute more to the tonic current suggested to be mediated by extrasynaptic receptors, than the beta3 subunit, which appears to be more involved in synaptic responses (Herd *et al.*, 2008). Our finding that about 64% of the beta3 subunit immunoreactivity is in the extrasynaptic membrane shows that it is a major contributor to extrasynaptic receptors, but whether it is present in receptors responsible for the tonic current in pyramidal cells remains to be determined.

The two alpha subunits and the beta3 subunit may be present in extrasynaptic receptors composed of only alpha and beta subunits, which have been shown on cultured hippocampal neurons (Mortensen & Smart, 2006). But the enhancement of the tonic current by both diazepam and zolpidem in CA1 pyramidal cells in adult hippocampal slices suggests that at least some of the receptors also contain a gamma subunit and next to it an alpha subunit, which is not an alpha5 subunit (Liang *et al.*, 2004), as the alpha5/gamma interface is insensitive to zolpidem.

Receptor trafficking and extrasynaptic receptors

Some of the receptors detected in the extrasynaptic membrane may have properties identical to synaptic receptors, presumably containing a gamma2 subunit, but forming a pool of receptor transiently outside the synapse, as the receptors move in and out of synapses (Bannai *et al.*, 2009). Receptors destined for synapses may be incorporated outside the synaptic junction and move by lateral diffusion. Indeed, single tagged receptors have been shown to move from synapse to synapse within the extrasynaptic plasma membrane *in vitro* (Bannai *et al.*, 2009). The synaptic receptors are thought to have lower affinity for GABA than extrasynaptic delta subunit-containing receptors, therefore at physiological extrasynaptic GABA concentrations it is uncertain whether receptors with subunit composition identical to synaptic receptors contribute to the tonic current when outside the synapse. Nevertheless, the fact that some hippocampal GABAergic neurons, such as the parvalbumin-expressing fast-spiking cells, have very high level of the alpha1 subunits in their extrasynaptic plasma membrane (Baude *et al.*, 2007) suggests that such receptors contribute to the action of GABA in the extrasynaptic space. A different type of interneuron showing a large tonic current with a likely receptor subunit composition including the alpha1 and delta subunits showed a high density of extrasynaptic immunolabelling (Glykys *et al.*, 2007). These neurons very likely correspond to the neurogliaform cells (Olah *et al.*, 2009).

Roles of synaptic and extrasynaptic $GABA_A$ receptors

Synaptic and extrasynaptic $GABA_A$ receptors have been shown to be differentially regulated in physiological and pathological conditions (Mody & Pearce, 2004; Farrant & Nusser, 2005; Belelli *et al.*, 2009; Skilbeck *et al.*, 2010). Synaptic $GABA_A$ receptors mediate phasic inhibition that influences which postsynaptic neurons participate in information processing and also governs the spike timing of the postsynaptic neurons (Klausberger & Somogyi, 2008). Extrasynaptic receptors, in addition to supplying synapses through receptor trafficking, may also contribute to setting the gain of the input–output function and/or the threshold of firing (Mitchell & Silver, 2003; Semyanov *et al.*, 2004). The strong outward rectification of extrasynaptic $GABA_A$ receptors on CA1 pyramidal cells predicts that they carry most charge near firing threshold and therefore regulate firing threshold rather than gain *in vitro* in juvenile rats (Pavlov *et al.*, 2009). The method that we applied to quantify $GABA_A$ receptors containing selected subunits or their combinations offers a strategy to assess physiological and pathological changes in receptor pools in different parts of neurons and in distinct membrane compartments.

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Abbreviations

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BSA, bovine serum albumin; CCD, charge-coupled device; E-face, exoplasmic-face; FRIL, freeze-fracture replica immunolabelling; GABA, γ-aminobutyric acid; GST, glutathione-S-transferase; HEK, human embryonic kidney cells; IMP, intramembrane particle; MBP, maltose-binding protein; mIPSC, miniature inhibitory postsynaptic potential; PB, phosphate buffer; PBS, phosphate-buffered saline; P-face, protoplasmic-face; SDS, sodium dodecyl sulphate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; TBS, Tris[hydroxymethyl]aminomethane-buffered saline; TBS-T, TBS-Triton; VGLUT3, vesicular glutamate transporter type 3; WB, washing buffer.

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NEUROSYSTEMS

COMMENTARY
$GABA_A$ receptor diversity revealed in freeze-fracture replica (Commentary on Kasugai *et al.*)

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GABAergic inputs from at least 18 types of inhibitory interneurons regulate and coordinate the activity of pyramidal cells in the hippocampal area CA1 (Klausberger *et al.*, 2005), which in turn express at least 14 subunits of the $GABA_A$ receptor ($GABA_AR$) with varying affinity to GABA and other ligands (Persohn *et al.*, 1992; Wisden *et al.*, 1992; Sperk *et al.*, 1997; Ogurusu *et al.*, 1999). Thus, the subunit composition determines the local response of the $GABA_AR$ to synaptically released GABA. Elucidating the subunit composition of synaptic and extrasynaptic $GABA_AR$ is also crucial in understanding the phasic vs. tonic postsynaptic responses evoked by GABA.

In their elegant study, Kasugai *et al.* (2010) performed a series of double-labeling experiments to demonstrate for the first time that virtually all somatic inhibitory synapses in the rat hippocampal CA1 pyramidal cell contained α1, α2, and β3 subunits of $GABA_AR$. The authors developed a new antibody against the α1 subunit to be used for a sensitive immunocytochemical method, freeze-fracture replica-immunogold labeling, that allows for quantitative analyses of transmembrane protein distribution (Fujimoto, 1995; Masugi-Tokita & Shigemoto, 2007). Their finding is consistent with previous post-embedding immunogold studies (Nusser *et al.*, 1996; Somogyi *et al.*, 1996). The presence of the three subunits in all somatic synapses does not imply that all synaptic $GABA_ARs$ consist of these subunits. However, this finding raises an interesting question regarding the relative proportion of the subunits at these synapses, because previous studies showed that α1 and α2 subunits preferentially mediate inputs from fast-spiking and regular-spiking basket cells, respectively (Pawelzik *et al.*, 1999; Thomson *et al.*, 2000; Nyiri *et al.*, 2001; Klausberger *et al.*, 2002).

Kasugai *et al.* (2010) also conducted single-labeling experiments to examine carefully the density of α1, α2 and β3 subunits in the synaptic and extrasynaptic plasma membrane of the pyramidal cells. Thirty to 50% of total labeling was found in synapses with 50–70% being extrasynaptic, suggesting that these subunits are well distributed between synaptic and extrasynaptic membrane. This is perhaps not too surprising as $GABA_ARs$ diffuse laterally in the plasma membrane (Bannai *et al.*, 2009). However, in light of previous studies suggesting that tonic inhibition is mediated by extrasynaptic $GABA_ARs$ containing the α4, α5, α6 and/or δ subunits (Belelli *et al.*, 2009), one might wonder how α1- or α2-containing extrasynaptic receptors are different from the synaptic ones in their subunit composition, targeting and functions.

Elucidating the native subunit composition of $GABA_ARs$ at identified synapses and extrasynaptic membrane will require a combination of research tools, including subunit-specific antibodies against different epitopes (intracellular vs. extracellular), transgenic animals (with specific subunits deleted or mutated in specific neuronal populations) and subunit-specific allosteric modulators. Studies on the interactions between specific subunit and scaffolding proteins would also provide insight into the mechanisms that target and regulate $GABA_ARs$. The data from this study provide a solid anatomical substrate to understand the functions of α1, α2 and β3 subunits in synaptic and extrasynaptic plasma membrane. They begin to elucidate how a diverse population of $GABA_ARs$ and inhibitory interneurons may shape cortical network activity in health and disease.

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