Synaptic GABA_A receptors are directly recruited from their extrasynaptic counterparts

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GABA_A receptors mediate the majority of fast synaptic inhibition in the brain. The accumulation of these ligand-gated ion channels at synaptic sites is a prerequisite for neuronal inhibition, but the molecular mechanisms underlying this phenomenon remain obscure. To further understand these processes, we have examined the cellular origins of synaptic GABA_A receptors. To do so, we have created fluorescent GABA_A receptors that are capable of binding α -bungarotoxin (Bgt), facilitating the visualization of receptor endocytosis, exocytosis and delivery to synaptic sites. Imaging with Bgt in hippocampal neurons revealed that GABA_A receptor endocytosis occurred exclusively at extrasynaptic sites, consistent with the preferential colocalization of extrasynaptic receptors with the AP2 adaptin. Receptor insertion into the plasma membrane was also predominantly extrasynaptic, and pulse-chase analysis revealed that these newly inserted receptors were then able to access directly synaptic sites. Therefore, our results demonstrate that synaptic GABA_A receptors are directly recruited from their extrasynaptic counterparts. Moreover, they illustrate a dynamic mechanism for neurons to modulate GABA_A receptor number at inhibitory synapses by controlling the stability of extrasynaptic receptors.

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Introduction

GABA_A receptors are the major mediators of fast synaptic inhibition in the brain and the sites of action for both benzodiazepines and barbiturates. These receptors are pentameric, Cl[−] selective ligand-gated ion channels that can be

assembled from seven subunit classes: α 1–6, β 1–3, γ 1–3, δ , ϵ and π providing the structural basis for extensive heterogeneity of GABA_A receptor structure (Rudolph *et al*, 2001; Sieghart and Sperk, 2002). However, a combination of molecular, biochemical and genetic approaches suggests that in the brain the majority of benzodiazepine receptor subtypes are composed of α , β and γ 2 subunits (Rudolph *et al*, 2001; Sieghart and Sperk, 2002). γ 2-containing receptors are highly enriched at synaptic sites in neurons, a process that is critical for efficient inhibitory neurotransmission and appropriate animal behavior (Essrich *et al*, 1998; Crestani *et al*, 1999; Kittler and Moss, 2003; Luscher and Keller, 2004; Alldred *et al*, 2005).

The mechanisms by which neurons facilitate the accumulation of GABA_A receptors at synaptic sites remain poorly defined, but it is evident that synaptic GABA_A receptors exhibit reduced rates of lateral mobility compared to their synaptic counterparts (Jacob *et al*, 2005; Thomas *et al*, 2005), consistent with studies on both glutamate and glycine receptors (Meier *et al*, 2001; Borgdorff and Choquet, 2002; Dahan *et al*, 2003). For GABA_A receptors, this confinement is at least partially facilitated by the postsynaptic scaffold protein gephyrin (Kneussel and Betz, 2000; Keller *et al*, 2004), which has recently been shown to decrease the diffusion rates of GABA_A receptors (Jacob *et al*, 2005), promoting their accumulation at synapses (Essrich *et al*, 1998; Kneussel *et al*, 1999). In addition to lateral movements, GABA_A receptors also undergo significant rates of constitutive endocytosis and recycling, which can directly modulate the efficacy of synaptic inhibition (Kittler *et al*, 2000a, 2005; Chen *et al*, 2005).

To further understand how neurons construct inhibitory synapses, we have developed tools to visualize GABA_A receptor endo- and exocytosis in addition to their accumulation at synaptic sites by creating receptor subunits modified with N-terminal pHluorin reporters that are also capable of binding α -bungarotoxin (Bgt) with high affinity. Using labelled Bgt derivatives, we then reveal that the principal sites of both GABA_A receptor endo- and exocytosis are extrasynaptic in hippocampal neurons. In addition, our study demonstrates that newly inserted extrasynaptic receptors are capable of directly accessing synaptic sites. Our results thus reveal that synaptic GABA_A receptors are derived directly from their extrasynaptic counterparts, and constitute a dynamic mechanism for neurons to rapidly modulate receptor number at inhibitory synapses by controlling the availability and stability of extrasynaptic receptors.

Results

Characterizing the properties of GABA_A receptors engineered with the BBS and ecliptic pHluorin

Studies on the dynamics of cell-surface GABA_A receptor populations have been compromised by the lack of suitable high-affinity probes. To measure GABA_A receptor endo- and exocytosis directly, we have engineered the receptor β 3

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subunit with the bungarotoxin binding site (BBS) peptide WRYESSLEPYPD. The BBS is derived from the α subunit of the muscle nicotinic acetylcholine receptor and has been established to bind Bgt with an affinity of approximately 3 nM (Scherf *et al*, 2001; Katchalski-Katzir *et al*, 2003). The BBS together with a pHluorin reporter separated by a 13-amino-acid alanine/proline linker was added between amino acids 4 and 5 of the mature β 3 subunit (Figure 1A). The resulting construct, $\text{pHBBS}\beta$ 3, was then expressed in HEK-293 cells and 48 h after transfection SDS-soluble lysates were subjected to immunoblotting with anti- β 3 antibody. This revealed the presence of a major band of 87 kDa only in

Figure 1 Functional expression of GABA_A receptors incorporating $\text{pHBBS}\beta$ 3 subunits. (A) The structure of the GABA_A receptor $\text{pHBBS}\beta$ 3 subunits. pHluorin and BBS were added between amino acids 4 and 5 of the mature β 3 subunits and were separated by a 13-amino-acid alanine-proline linker. Tm domains 1–4 are shown in gray. (B) Analyzing expression of the $\text{pHBBS}\beta$ 3 subunit by immunoblotting. Control (lane 1) or HEK-293 cells expressing GABA_A receptor α 1 $\text{pHBBS}\beta$ 3 and γ 2 subunit cDNAs (lane 2) were immunoblotted with antibodies against the receptor β 3 subunit. The migration of molecular mass standards is indicated. (C, D) Agonist sensitivity of GABA_A receptors incorporating $\text{pHBBS}\beta$ 3 subunits. HEK-293 cells were transfected with the respective murine cDNAs in a 1:1:1 ratio. At 48 h after transfection, I_{GABA} was recorded under voltage clamp at -50 mV. Responses from individual cells are shown in panel C. Currents at individual agonist concentrations were then normalized to the maximal response observed and fitted with the Hill equation. $I/I_{\text{max}} = 1/(1 + (\text{EC}_{50}/A)^n)$ are shown in panel D. EC_{50} values of 9.89 ± 2.30 , 2.38 ± 0.4 and 6.12 ± 1.94 were seen for GABA_A receptors composed of α 1 β 3 γ 2 ($\square$), α 1 $\text{pHBBS}\beta$ 3 γ 2 ($\circ$) and α 1 $\text{pHBBS}\beta$ 3 γ 2 ($\blacktriangle$), respectively (mean $\pm$ s.e.m.; $n = 6-8$). (E, F) Benzodiazepine modulation of GABA_A receptors incorporating $\text{pHBBS}\beta$ 3 subunits. HEK-293 cells expressing GABA_A receptors were voltage clamped at -50 mV. The magnitude of I_{GABA} at EC_{20} agonist concentration was then measured in the absence and presence of 100 nM flurazepam as illustrated in panel E. Normalized conductances in the absence of flurazepam were given a value of 1. I_{GABA} was potentiated by 2.15 ± 0.18 (α 1 β 3 γ 2), 2.26 ± 0.21 (α 1 $\text{pHBBS}\beta$ 3 γ 2) and 2.09 ± 0.25 (α 1 $\text{pHBBS}\beta$ 3 γ 2) respectively (mean $\pm$ s.e.m.; $n = 9-15$) as shown in panel F.

cells expressing $\text{pHBBS}\beta$ 3, in agreement with the predicted molecular mass of this subunit (Figure 1B).

We further assessed the ability of $\text{pHBBS}\beta$ 3 subunits to assemble with receptor α 1 and γ 2 subunits to form functional heteromeric α 1 β 3 γ 2 receptors using patch clamp recording (Kittler *et al*, 2000b; Bedford *et al*, 2001; Jacob *et al*, 2005). Fast application of GABA produced inward currents (I_{GABA}) for cells expressing receptors composed of α 1 β 3 γ 2, α 1 $\text{pHBBS}\beta$ 3 γ 2 or α 1 $\text{pHBBS}\beta$ 3 γ 2 subunits (Figure 1C). Using dose-response analysis, it was evident that these differing receptors exhibited similar EC_{50} values for GABA of 9.89 ± 2.30 , 2.38 ± 0.4 and 6.12 ± 1.94 μM (mean $\pm$ s.e.m.; $n = 6-8$) respectively in addition to maximal currents, strongly suggesting similar receptor densities on the plasma membrane for these varying subunit combinations (Figure 1D). To measure the incorporation of the γ 2 subunit into functional receptors, we analyzed modulation by benzodiazepines, a property critically dependent upon incorporation of this subunit into heteromeric $\alpha\beta\gamma$ 2 receptors (Rudolph *et al*, 2001; Sieghart and Sperk, 2002). Flurazepam at a concentration of 100 nM produced similar robust enhancements of I_{GABA} at EC_{20} GABA concentrations of approximately 220% for receptors composed of α 1 β 3 γ 2, α 1 $\text{pHBBS}\beta$ 3 γ 2 or α 1 $\text{pHBBS}\beta$ 3 γ 2 subunits (Figure 1E and F). Together, these observations suggest that the addition of the BBS and pHluorin reporters to the N-terminus of the β 3 subunit does not compromise receptor functional expression, a finding consistent with prior studies using GABA_A receptors engineered with N-terminal reporters GFP, pHluorin or myc reporters (Connolly *et al*, 1996; Kittler *et al*, 2000b; Taylor *et al*, 2000; Jacob *et al*, 2005).

GABA_A receptors modified with BBS bind Bgt with high affinity

The ability of GABA_A receptors incorporating $\text{pHBBS}\beta$ 3 subunits to bind Bgt was analyzed by exposing live transfected HEK-293 cells to varying concentrations of ^{125}I -Bgt at 37°C for 10 min. After extensive washing, the level of bound Bgt was measured (Figure 2A). This revealed that cells expressing GABA_A receptors incorporating $\text{pHBBS}\beta$ 3 subunits bound Bgt with a K_D of $7.85 \pm 2.41 \times 10^{-9}$ M (mean $\pm$ s.e.m.; $n = 3$), consistent with previous studies on the BBS or AMPA receptors modified with this sequence (Scherf *et al*, 2001; Katchalski-Katzir *et al*, 2003; Sekine-Aizawa and Huganir, 2004). No significant binding was observed in cells expressing GABA_A receptors containing wild-type β 3 subunits (Figure 2A).

To visualize cell-surface GABA_A receptor populations, live expressing HEK-293 cells were stained with rhodamine-conjugated Bgt (Rd-Bgt) and then visualized using confocal microscopy. This imaging revealed robust labelling of the surface of cells exhibiting endogenous green fluorescence, indicating expression of GABA_A receptors incorporating $\text{pHBBS}\beta$ 3 subunits (Figure 2B). No binding was observed in non-transfected cells. To examine the stability of Rd-Bgt binding, cells expressing receptors incorporating $\text{pHBBS}\beta$ 3 subunits were labelled for 10 min with Rd-Bgt, washed and incubated at 15°C to block endo- and exocytosis and subjected to live confocal imaging. The intensity of Bgt staining was then measured at the periphery of expressing cells over time (Figure 2B and C) and compared to the signal seen at zero time, which was given a value of 100%. Rd-Bgt labelling at the surface of HEK-293 cells remained unaltered over a

Figure 2 GABA_A receptors engineered to incorporate pHBBSSβ3 subunits bind Bgt with high affinity. (A) GABA_A receptors containing pHBBSSβ3 subunits bind Bgt with high affinity. HEK-293 cells were transfected with GABA_A receptors α1^{pHBBSS}β3 and γ2 subunit cDNAs. After 48 h, cells were labelled with varying concentrations of ¹²⁵I-Bgtx (1000 Ci/mmol) at 37°C for 10 min before extensive washing. The level of bound Bgt after correction for nonspecific binding was then used to estimate the affinity of Bgt for receptors containing pH^H/BBSβ3 subunits (●) or pHβ3 (□) (calculated using Origin software; data represent mean ± s.e.m.; n = 4). (B–D) Bgt slowly dissociates from GABA_A receptors containing pHBBSSβ3 subunits. Transfected HEK-293 cells were labelled with 5 μg/ml Rd-Bgt for 5 min and washed extensively. Labelled cells were transferred to the stage of a confocal microscope maintained at 15°C and imaged over 180 min. Images of an individual cell at 5 and 180 min are shown in panels B and C, respectively (red = Rd-Bgt, green = pHluorin). (D) The intensity of Rd-Bgt in 2–5 μm² areas of interest was then compared to the initial levels seen at zero time, which was given a value of 100% (each data point represents mean ± s.e.m.; n = 4–6).

200 min time course, suggesting that Rd-Bgt complexes are stable (Figure 2D).

Next we analyzed the ability of Rd-Bgt to detect GABA_A receptors incorporating pHBBSSβ3 subunits when expressed in hippocampal neurons. 8–10-Div neurons were transfected with pHBBSSβ3 and 4–7 days later live neurons were exposed to Rd-Bgt, fixed and subjected to confocal microscopy. Neurons expressing pHBBSSβ3 subunits exhibited endogenous green fluorescence and also significant levels of cell-surface Rd-Bgt binding, whereas the background labelling to non-fluorescent untransfected neurons was negligible (Figure 3A). Rd-Bgt staining in transfected neurons was blocked by excess of unlabelled Bgt (Figure 3B). To further control for possible binding of Bgt to endogenous neuronal AChRs, we examined the effects of curare on Rd-Bgt staining in neurons expressing pHBBSSβ3 subunits. Although curare blocks Bgt binding to neuronal AChRs containing α7 subunits, it does not block binding to the BBS (Schoepfer *et al*, 1988; Sekine-Aizawa and Haganir, 2004). In agreement with this, 100 μM curare did not modify Rd-Bgt binding to neurons expressing pHBBSSβ3 subunits, suggesting that fluorescent Bgt binding sites in our experiments represent GABA_A receptors and not endogenous AChRs (Figure 3E and F). We also examined the stability of Rd-Bgt/GABA_A receptor complexes in hippocampal neurons

Figure 3 Imaging GABA_A receptors incorporating pHBBSSβ3 subunits in hippocampal neurons. (A–D) Visualizing GABA_A receptors incorporating pHBBSSβ3 subunits in neurons. 14-Div hippocampal neurons expressing GABA_A receptor pH^H/BBSβ3 subunits were labelled with 5 μg/ml Rd-Bgt in the absence (A) or presence (B) of 50 μg/ml of unlabelled Bgt. Cells were then fixed and images recorded by confocal microscopy (red = Rd-Bgt, green = pHluorin, yellow arrows = transfected neurons expressing pHBBSSβ3, white = non-transfected neurons; scale bars = 15 μm). Rd-Bgt staining was also performed on neurons expressing pHBBSSβ3 in the absence (C) and presence of 100 μM curare (D). (E–H) Bgt slowly dissociates from neurons expressing pHBBSSβ3 subunits. Transfected neurons were labelled with 5 μg/ml Rd-Bgt for 5 min, washed, transferred to the stage of a confocal microscope maintained at 15°C and imaged for 180 min (red = Rd-Bgt, green = pHluorin). Images of an individual transfected neuron stained with Rd-Bgt at 0 and 180 min are shown in panels E and F. The lower panels represent enlargements of the boxed areas in the upper panels. (G) The intensity of Rd-Bgt staining was measured in 1–2 μm² areas of interest over the entire recording period with levels at zero time being given a value of 100% (data represent mean ± s.e.m.; n = 6). (H) GABA_A receptors incorporating pHBBSSβ3 subunits are targeted to synaptic sites. 14-Div hippocampal neurons expressing pHBBSSβ3 subunits were stained with 5 μg/ml Rd-Bgt for 5 min, washed, fixed, permeabilized, stained with anti-synapsin antibodies and imaged using confocal microscopy (red = Rd-Bgt, green = pHluorin, blue = synapsin). The lower panel represents an enlargement of the boxed area in the upper panel; the white arrowheads indicate synaptic GABA_A receptors, whereas the yellow arrowheads indicate extrasynaptic populations (scale bar = 10 μm).

by incubating Rd-Bgt-stained neurons at 15°C. The level of Rd-Bgt staining in 1–2 µm² areas of interest was measured over a time course of 200 min (Figure 3E and F) with levels evident at zero time being given a value of 100%. No statistically significant loss of Rd-Bgt staining was seen over this time period (Figure 3G), suggesting that Rd-Bgt/GABA_A receptor complexes are stable on the surface of neurons.

The accumulation of GABA_A receptors incorporating pHBBsβ3 at synaptic sites in hippocampal neurons was also addressed. Expressing neurons were labelled with Rd-Bgt and fixed. Presynaptic terminals were then stained with antibodies against synapsin. Consistent with published studies analyzing the distribution of endogenous β3 subunits, Rd-Bgt staining on the surface of hippocampal neurons was evident as distinct clusters in addition to diffuse staining (Danglot *et al*, 2003; Jacob *et al*, 2005). We next calculated the number of these receptor clusters that were apposed to synapsin staining and therefore judged to be synaptic, as it has been documented that clusters containing the β3 subunit are found at both synaptic and extrasynaptic sites (Danglot *et al*, 2003; Jacob *et al*, 2005). A total of $24.6 \pm 6.4\%$ of these clusters were apposed to synapsin staining and were therefore judged to be synaptic (Figure 3H; 250 clusters counted, mean ± s.e.m.; $n = 5$). Similar levels of synaptic targeting for endogenous GABA_A receptors containing the β3 subunit have been previously reported in 12–14-Div hippocampal neurons (Danglot *et al*, 2003; Jacob *et al*, 2005).

Extrasynaptic GABA_A receptors exhibit short residence times on the cell surface

We next compared the relative residence times on the cell surface of synaptic and extrasynaptic GABA_A receptors. Neurons expressing receptor pHBBsβ3 subunits were labelled with Rd-Bgt and incubated at 37°C for up to 90 min in the presence of 50 nM unlabelled Bgt in the external media. At differing time points, neurons were fixed and then incubated with synapsin antibodies to differentiate synaptic and extrasynaptic GABA_A receptors (Kittler *et al*, 2001; Jacob *et al*, 2005). At zero time, abundant diffuse extrasynaptic Rd-Bgt staining was seen in addition to labelled synaptic receptors (Figure 4, panels 1 and 2). However, a consistent finding after 90 min was an extensive loss of extrasynaptic Rd-Bgt labelling (Figure 4, panels 3 and 4).

To quantify this apparent loss of extrasynaptic receptors, we measured Rd-Bgt staining in 1–2 µm² areas of interest representing synaptic receptors and extrasynaptic receptors that were at least 2 µm from the nearest synapses and within the same neuron (Figure 4, panels 3 and 4). The ratio of extrasynaptic-to-synaptic Rd-Bgt staining (Bgt-ES/Bgt-S) was then determined and ratios at zero time were given an arbitrary value of 1.0. This ratio decreased rapidly with time of incubation at 37°C, reaching statistical significance after 10 min and steady state at 60 min (Figure 5A and B). At 90 min, this ratio had significantly decreased to $0.42 \pm 5.2\%$ (Figure 5B; $P < 0.01$; Student's *t*-test; $n = 6$). Given the high stability of Bgt/GABA_A receptors complexes (Figures 2 and 3), our results demonstrate that extrasynaptic GABA_A receptors containing pHBBsβ3 have much shorter residence times on the cell surface as compared to their synaptic counterparts.

Figure 4 Extrasynaptic GABA_A receptors have short residence times at the cell surface. 12–14-Div hippocampal neurons expressing GABA_A receptors incorporating receptor pHBBsβ3 subunits were labelled with 5 µg/ml of Rd-Bgt without (1–4) or with (5–8) 1 h preincubation with myristoylated p4 peptide. Neurons were then incubated at 37°C for between 0 and 90 min. At varying time intervals, samples were removed, permeabilized, stained with antibodies against synapsin and then visualized by confocal microscopy (red = Rd-Bgt, green = pHluorin, blue = synapsin). Images at zero time for an individual neuron are shown in 1, 2, 5 and 6, whereas those in 3, 4, 7 and 8 represent neurons incubated for 90 min at 37°C. The right-hand panels represent enlargements of the boxed areas in the left-hand panels. The yellow and white boxed areas indicate synaptic and extrasynaptic receptor populations respectively (scale bars = 5 µm).

Extrasynaptic GABA_A receptors undergo clathrin-dependent endocytosis

A number of studies have illustrated that GABA_A receptors undergo constitutive clathrin-dependent endocytosis. We therefore sought to determine whether the time-dependent loss of extrasynaptic receptors seen in Figure 4 represents their preferential endocytosis. To do so, hippocampal neurons expressing pHBBsβ3 subunits were preincubated for 1 h with 50 µM myristoylated membrane-permeable p4

peptide (M-p4), a potent inhibitor of GABA_A receptor endocytosis (Kittler *et al*, 2000a, 2005; Chen *et al*, 2005) before labelling with Bgt. Pretreatment of neurons with M-p4 appeared to block the loss of extrasynaptic receptors seen in untreated neurons (Figure 4, panels 5 and 6). After quantifying these results, it was evident that M-p4 blocked the time-dependent decrease in Bgt-ES/Bgt-S ratio seen in controls, an effect that was not replicated with a scrambled myristoylated version of p4 peptide (M-Sc) or non-myristoylated (non-cell permeable) p4 peptide (P4) (Figure 5A and B).

To further analyze the intracellular fate of internalized GABA_A receptors, neurons were labelled for 5 min with Rd-Bgt, washed extensively and then incubated at 37°C for 120 min. After this time period, neurons were finally stained for 5 min with Alx-Bgt before fixation and analysis by confocal microscopy. The majority of the Rd-Bgt label over the incubation period at 37°C accumulated in perinuclear structures with endosomal-like morphology. These intracellular organelles also contained pHluorin fluorescence (Figure 5C–F). In contrast, Alx-Bgt staining was found extensively over the entire plasma membrane as diffuse

staining in addition to clusters (Figure 5C–F). Remaining cell-surface Rd-Bgt staining was also found within these clusters, consistent with its stabilization at synaptic sites, as illustrated in Figure 4. Given the high stability of GABA_A receptor Bgt complexes at 15°C, conditions at which both endo- and exocytosis (Figure 3) are blocked, our results suggest that internalized Bgt-GABA_A receptors are specifically targeted to endosomal structures, where they are capable of rapid recycling back to the plasma membrane or are targeted for lysosomal degradation (Kittler *et al*, 2000a, 2004).

We further analyzed the selective endocytosis of extrasynaptic GABA_A receptors by comparing their distribution with that of the clathrin AP2 adaptin, which directly binds to motifs within the intracellular domains of receptor subunits, facilitating their endocytosis (Kittler *et al*, 2000a, 2005; Chen *et al*, 2005). Neurons expressing ^{pHBBS}β3 subunits were stained with antibodies against AP2 adaptin and presynaptic terminals were labelled with synapsin antibodies and then imaged using confocal microscopy. Punctate staining that incorporated both GABA_A receptor ^{pHBBS}β3 subunits and AP2 immunoreactivity was found on both neuronal cell bodies and processes. Synaptic GABA_A receptors were also evident as measured by the colocalization of ^{pHBBS}β3 fluorescence and synapsin staining (Figure 5C and D). We then calculated the number of synaptic GABA_A receptors that also contained immunoreactivity for the AP2 adaptin. Only $19.2 \pm 1.2\%$ of synaptic GABA_A receptors exhibited detectable levels of AP2 staining (mean $\pm$ s.e.m.; 165 synapses analyzed, $n = 4$). This suggests that GABA_A receptor AP2 complexes are found primarily at extrasynaptic sites, consistent with their preferential endocytosis.

Figure 5 Extrasynaptic GABA_A receptors preferentially undergo clathrin-dependent endocytosis. (A, B) Cell-surface stability of extrasynaptic GABA_A receptors is enhanced by inhibitors of clathrin-dependent endocytosis. The ratio of Rd-ES-Bgt:S-Bgt staining was measured in adjacent synaptic and extrasynaptic domains of RD-Bgt-stained neurons expressing ^{pHBBS}β3 subunits over 90 min as shown in Figure 4 under control conditions (■) or after 1 h pretreatment with M-p4 (■) or M-Sc (■). Data at varying time points were compared to ratios seen at zero time (which was given a value of 1). Each data point represents mean $\pm$ s.e.m. from 44 to 90 synapses ($n = 6-8$). Ratios at 90 min were also compared to the respective levels seen at zero time as shown in panel B (P4 = 1 h pretreatment with membrane-impermeable peptide; *significantly different from control; $P < 0.01$; Student's *t*-test, $n = 6-8$). (C–F) Internalization of Bgt-labelled GABA_A receptors incorporating ^{BBS}β3 subunits. 14-Div neurons expressing ^{BBS}β3 subunits were labelled with 5 μg/ml Rd-Bgt for 5 min at 37°C, washed and incubated at 37°C for 120 min. After incubation, neurons were labelled with 5 μg/ml Alx-Bgt for 5 min at 37°C, washed, fixed and imaged by confocal microscopy; C = Rd-Bgt, D = Alx-Bgt, E = ^{BBS}β3 fluorescence, F = merge. The white arrows represent internalized Rd-Bgt/GABA_A receptors and yellow arrows represent clustered Rd-Bgt staining on the plasma membrane; scale bar = 10 μm. (G, H) Extrasynaptic GABA_A receptors show preferential colocalization with the AP2 adaptin. 14-Div hippocampal neurons expressing ^{pHBBS}β3 were fixed, permeabilized, stained with antibodies against AP2 (red) and synapsin (blue) and visualized by confocal microscopy (green represents endogenous ^{pHBBS}β3 fluorescence; scale bar = 5 μm). The images in panel H are enlargements of the boxed area in panel G. Panel G shows a merged image and the individual channels with green representing pHluorin. The arrows represent extrasynaptic receptors that colocalize with AP2, whereas the arrowheads indicate synaptic GABA_A receptors that colocalize with synapsin.

Imaging newly inserted GABA_A receptors using Bgt staining

To selectively measure GABA_A receptor insertion into the plasma membrane, we first blocked constitutive receptor endocytosis. Previous biochemical experiments have revealed that in excess of 25% of the total cell-surface receptor population are internalized within 15 min at steady state (Kittler *et al*, 2004). To do so, neurons were first treated with M-p4 and incubated with Rd-Bgt at 37°C. At varying time points, neurons were fixed and stained with synapsin antibodies to differentiate synaptic and extrasynaptic GABA_A receptor populations. Using this procedure, Rd-Bgt binding could be detected within 5 min of incubation and appeared to increase dramatically with further incubation at 37°C (Figure 6A–F). The specificity of this new Rd-Bgt staining was measured by coincubating neurons with Rd-Bgt and a large excess of unlabelled toxin. This treatment completely blocked the appearance of new Rd-Bgt staining compared to neurons incubated with Rd-Bgt alone (Figure 6G and H). Newly inserted GABA_A receptors were evident at both synaptic and extrasynaptic sites at 5 min, suggesting that insertion can occur throughout the entire surface (Figure 6B). However, as inhibitory synaptic sites represent only a small fraction of the neuronal surface, the majority of newly inserted receptors will be delivered to extrasynaptic sites.

To provide quantitative information on the delivery of newly inserted receptors to synaptic sites over time, we measured the ratio of newly inserted Rd-Bgt binding sites at synaptic and extrasynaptic sites (S-Bgt:ES-Bgt), with values evident at 5 min (zero time) given a value of 100%. In excess of 85% of all synapses analyzed in our experiments showed increased levels of Rd-Bgt staining, and these were chosen for further analysis, with the ratios seen at zero time being given a value of 1.0. This ratio increased slowly and reached a level of 1.41 ± 0.08 at 30 min (Figure 6J), which was significantly different from zero time ($P < 0.001$; $n = 4$). This suggests that newly inserted receptors accumulate slowly at postsynaptic inhibitory synaptic sites.

Newly inserted extrasynaptic receptors are able to access synaptic sites

The slow accumulation of newly inserted GABA_A receptors at synaptic sites may reflect either slower direct insertion of GABA_A receptors at synaptic sites as compared to extrasynaptic sites or the diffusion of newly inserted extrasynaptic receptors to synaptic sites and their subsequent stabilization. To address these possibilities, we utilized pulse-chase analysis with Bgt conjugated to distinct fluorophores. Neurons were first pretreated with M-p4 peptide to block endocytosis and existing populations of receptors containing ^{pHBB}β3 subunits were blocked with unlabelled toxin. All GABA_A receptors inserted into the plasma membrane over a 5 min time period were then labelled with Rd-Bgt toxin, washed extensively and then incubated at 37°C in toxin-free media. At differing time periods, neurons were fixed and stained with VIAAT antibodies to visualize presynaptic inhibitory terminals and imaged by confocal microscopy. Newly inserted receptors were clearly visible after 5 min incubation with Rd-Bgt (zero time) and also after incubation at 37°C, but there appeared to be increased labelling of synaptic sites at later time points (Figure 7C and D). To quantify these results, we compared the level of Rd-Bgt staining at synaptic and extrasynaptic sites over time as outlined in Figures 4–6, with ratios evident at zero time being given a value of 1.0. This ratio significantly increased

Figure 6 Imaging the insertion of new GABA_A receptors on the surface of hippocampal neurons. 14-Div hippocampal neurons expressing GABA_A receptors incorporating ^{pHBB}β3 subunits were pretreated with M-p4 peptide for 1 h. Neurons were then exposed to 5 μg/ml unlabelled Bgt to label existing cell-surface receptors. After washing, neurons were incubated for 30 min at 37°C in the presence of M-p4. Neurons were then labelled with 10 nM Bgt at various time points, fixed, permeabilized and stained with antibodies against synapsin and imaged using confocal microscopy. (A–D) Neurons incubated for 5 min. (E–H) Neurons incubated for 30 min. (I–L) A neuron incubated with Rd-Bgt, M-p4 and 100 nM unlabelled toxin. In all images, red = Rd-Bgt, blue = synapsin and green = pHluorin. Images C and D, G and H, K and L represent enlargements of the boxed areas in panels A, E and I, respectively. Panels B, F and J show the Rd-Bgt channel only, whereas panels D, H and L show both Rd-Bgt and synapsin staining. The arrowheads indicate synaptic receptor populations, whereas the arrows indicate extrasynaptic receptors. (M) The ratios of newly inserted GABA_A receptors were measured for extrasynaptic and synaptic domains of neurons, as outlined in Figure 5. Each data point represents mean $\pm$ s.e.m. from 60 to 80 synapses; *significantly different from control; $P < 0.01$; Student's *t*-test; $n = 7$ –8.

with time of incubation at 37°C (Figure 7D), suggesting that newly inserted GABA_A receptors at extrasynaptic domains can over time move to synaptic sites.

Discussion

The specific accumulation of GABA_A receptors at postsynaptic sites is a critical determinant of fast synaptic inhibition.

Figure 7 Analyzing the movement of newly inserted extrasynaptic receptors to synaptic sites. 14-Div hippocampal neurons expressing GABA_A receptors were treated with 100 nM unlabelled Bgt for 10 min, washed and then subjected to a second labelling period of 5 min at 37°C with 10 nM Rd-Bgt. They were then incubated at 37°C for varying time periods before fixing followed by immunohistochemistry with anti-VIAAT antibodies. (A) shows a neuron fixed immediately after incubation with Rd-Bgt, whereas (D) shows a neuron incubated for 30 min after labelling with Rd-Bgt. In all images, red = Rd-Bgt, blue = VIAAT and green = pHluorin. Images in (B/C) and (E/F) represent enlargements of the panels in A and D, respectively. Panels C and D show only Rd-Bgt and VIAAT staining. The arrowheads indicate synaptic receptor populations, whereas the arrows indicate extrasynaptic receptors. (G) The ratio of synaptic Bgt to extrasynaptic staining was calculated from neurons expressing $\alpha 1^{\text{pHBBS}}\beta 3$ subunits over time, with the ratio evident at zero time (directly after labelling with Rd-Bgt) being given a value of 1.0. Each data point represents mean $\pm$ s.e.m. from 44 to 90 synapses; *significantly different from control; $P < 0.001$, $n = 4$.

This process is regulated by the relative rates of receptor exocytosis, endocytosis, lateral mobility and receptor stabilization at postsynaptic specializations (Choquet and Triller, 2003; Kittler and Moss, 2003; Luscher and Keller, 2004). Although live imaging studies have provided evidence on the comparative diffusion rates of synaptic and extrasynaptic GABA_A receptors (Jacob *et al*, 2005), measurements on the stability of these distinct receptor populations and their sites of exo- and endocytosis have been hampered by the lack of suitable probes.

To address these issues, we modified GABA_A receptors within their N-terminal cytoplasmic domains with BBS and pHluorin reporters (Scherf *et al*, 2001; Katchalski-Katzir *et al*, 2003). These additions did not compromise the assembly or functional properties of physiologically relevant heteromeric GABA_A receptors composed of $\alpha 1^{\text{pHBBS}}\beta 3$ and $\gamma 2$ subunits; however, these receptors were able to bind Bgt with a K_D of approximately 7 nM and a very slow off-rate, consistent with previous studies on the affinity of the BBS for Bgt and AMPA receptors modified with this 13-amino-acid motif (Sekine-Aizawa and Haganir, 2004). Significantly, no binding was seen in cells expressing GABA_A receptor $\beta 3$ subunits modified with just pHluorin reporters. Recent studies have suggested that recombinant homomeric GABA_A receptors composed of $\beta 3$ subunits can bind Bgt with an approximate affinity of 50 nM when expressed in mammalian cells (McCann *et al*, 2006). However, this finding does not have an impact on our results, as the K_D of Bgt for GABA_A receptors composed of $\alpha 1^{\text{pHBBS}}\beta 3$ and $\gamma 2$ receptors was estimated at 7 nM, and our labelling studies used 10 nM Bgt. Furthermore, this low-affinity-binding Bgt site is abolished on coassembly of $\beta 3$ subunits into heteromeric $\alpha 1^{\text{pHBBS}}\beta 3$ and $\gamma 2$ receptors (McCann *et al*, 2006).

In hippocampal neurons transfected with $\alpha 1^{\text{pHBBS}}\beta 3$, fluorescent Bgt staining was seen only on the cell surface of neurons that exhibited endogenous green fluorescence, demonstrating the expression of GABA_A receptors incorporating $\alpha 1^{\text{pHBBS}}\beta 3$ subunits (Jacob *et al*, 2005). Moreover, Bgt staining in these neurons was insensitive to curare, a potent inhibitor of Bgt binding to AChRs. Together, these results demonstrate that the Bgt sites seen in neurons expressing $\alpha 1^{\text{pHBBS}}\beta 3$ subunits originate from engineered GABA_A receptors and not from endogenous AChRs.

We then utilized staining with Bgt derivatives to compare the relative residence times at the cell surface for extrasynaptic and synaptic GABA_A receptors containing $\alpha 1^{\text{pHBBS}}\beta 3$ subunits. This revealed a rapid loss of extrasynaptic Rd-Bgt staining over a 90 min time course at 37°C. Given the slow off-rate of Bgt for GABA_A receptors incorporating $\alpha 1^{\text{pHBBS}}\beta 3$ subunits, our results directly demonstrate that such extrasynaptic receptors have significantly shorter residence times on the cell surface as compared to synaptic receptors. To assess the mechanism underlying the distinct cell-surface stabilities of synaptic and extrasynaptic GABA_A receptors, we focused on the role of dynamin-dependent endocytosis, a process key in determining receptor number at synaptic sites (Kittler *et al*, 2000a, 2005; Chen *et al*, 2005). Pretreatment of neurons with a membrane-permeable inhibitor of dynamin specifically blocked this loss of extrasynaptic GABA_A receptors. Using immunohistochemistry, it was also evident that only extrasynaptic receptors exhibited high levels of colocalization with the AP2 adaptin, a critical regulator of GABA_A

receptor endocytosis. Our results thus strongly suggest that extrasynaptic GABA_A receptors preferentially undergo clathrin-dependent endocytosis, which has important implications for the construction of inhibitory synapses. Specifically, decreasing the rate of GABA_A receptor endocytosis using inhibitors of dynamin or by blocking the binding of GABA_A receptors to the AP2 adaptin increases receptor number at synaptic sites over a time course of 15–30 min (Kittler *et al*, 2000a, 2005; Chen *et al*, 2005). Given our findings, these effects are likely to reflect the stabilization of extrasynaptic receptors and their subsequent accumulation at synaptic sites.

To further address this issue, we used imaging with Bgt to analyze the sites of GABA_A receptor exocytosis. To do so, we first labelled existing cell-surface populations of receptors incorporating ^{pHBS}β3 subunits with unlabelled Bgt. Newly inserted receptors were then labelled with fluorescent Bgt in the presence of blockers of endocytosis. This revealed that newly inserted receptors were detected within a 5 min labelling period and these receptors were evident over the entire neuronal surface. However, the accumulation of these newly inserted receptors at synaptic sites was slow and did not reach statistical significance until 30 min later. These results may reflect either the movement of extrasynaptic receptors to synaptic sites or their slower direct insertion at synapses. To address these possibilities, we examined the fate of newly inserted receptors over a 5 min labelling period using pulse-chase analysis in the presence of blockers of endocytosis. This revealed that newly inserted receptors at extrasynaptic sites can directly access synaptic sites within a 15 min time period, a process that is facilitated by the high rates of diffusion of extrasynaptic compared to synaptic GABA_A receptors (Choquet and Triller, 2003; Jacob *et al*, 2005).

Together, our results with GABA_A receptors engineered to bind Bgt have provided novel insights into how neurons construct inhibitory synapses. Specifically, they have demonstrated that GABA_A receptor endo- and exocytosis are localized to extrasynaptic membrane domains. Our studies also provide the first direct evidence that synaptic GABA_A receptors are directly recruited from extrasynaptic receptors. Finally, our results reveal a dynamic mechanism for neurons to rapidly modulate both GABA_A receptor number at inhibitory synapses and the efficacy of synaptic inhibition by controlling the availability and stability of extrasynaptic receptors.

Materials and methods

DNA constructs

The ^{pHBS}β3 subunit construct was derived from ^{pH}β3 (Jacob *et al*, 2005). Briefly, ^{pH}β3 was subjected to two rounds of mutagenesis by PCR to introduce the following changes: to introduce the 13-amino-acid BBS tag directly after the pHluorin tag and to add 12 alanines and one proline to separate the BBS tag from the pHluorin tag. The final construct contains the pHluorin tag between amino acids 3 and 4 of the mature β3 subunit. The pHluorin tag is followed by 13-amino-acid alanine/proline spacer.

Cell culture and transfection

Hippocampal neuron cultures were prepared as described previously (Kittler *et al*, 2000a,b). Neurons were transfected with Effectene according to the manufacturer's specifications (Qiagen, Valencia, CA, USA). Briefly, 1 µg of DNA was used for every two 35 mm cell culture dishes containing hippocampal neurons. DNA was added to 150 µl of EC buffer and 8 µl of Enhancer. The solution

was incubated for 5 min at room temperature and 75 µl of Effectene was added. After 10 min incubation at room temperature, the final solution was added to hippocampal neurons. The medium was changed after 6 h. HEK-293 cells were transfected using electroporation, as detailed earlier (Connolly *et al*, 1996, 1999).

Electrophysiology

HEK-293 cells were transfected with equimolar mixes of the relevant expression construct. After 18–48 h, coverslips containing the transfected HEK-293 cells were transferred to a recording chamber mounted on the stage of an inverted microscope and transfected cells identified by their endogenous fluorescence (Kittler *et al*, 2000b; Jacob *et al*, 2005). The external solution contained (in mM) NaCl 140, KCl 4.7, HEPES 10, NaHCO₃ 23, glucose 11, MgCl₂ 1.2 and CaCl₂ 2.5 (adjusted to pH 7.4 with NaOH) continuously oxygenated with a mixture of 95%O₂/5%CO₂. The recording chamber was perfused at a rate of 5–10 ml/min and maintained at 32°C (Kittler *et al*, 2000a, 2005; Chen *et al*, 2005; Jacob *et al*, 2005). The internal solution contained (in mM) KCl 140, CaCl₂ 1.0, HEPES 10, MgCl₂ 2 and ATP (adjusted to pH 7.2 with KOH). Pipettes had a resistance of 2–4.5 MΩ when filled with this internal solution. Patch clamp experiments were performed in the whole cell configuration using an Axopatch 200B amplifier. Series resistance and membrane capacitance were partially compensated (70–80%) and current traces were low-pass filtered at 2 kHz using a four-pole Bessel filter. The holding potential in all experiments was –50 mV. Drugs were rapidly applied to single cells using a modified U-tube, placed 50–100 µm away from the cell of interest (Kittler *et al*, 2000a, 2005; Bedford *et al*, 2001; Chen *et al*, 2005; Jacob *et al*, 2005).

Radiolabelled Bgt binding

Heteromeric GABA_A receptors containing ^{pHBS}β3 subunits were expressed in HEK-293 cells via electroporation (Connolly *et al*, 1996, 1999). After 48 h, cells were labelled with varying concentrations of ¹²⁵I-Bgtx (1000 Ci/mmol) at 37°C for 10 min before extensive washing, as detailed previously (Wells *et al*, 1998). After correcting for nonspecific binding using HEK-293 cells expressing heteromeric receptors containing wild β3 subunits, the level of bound Bgt was measured in a γ counter and then analyzed using the Origin software package to estimate the affinity of Bgt for ^{pHBS}β3 subunits.

Fluorescent Bgt labelling

HEK-293 cells or hippocampal neurons were incubated with Rd-Bgt at a final concentration of 10 nM for 5 min at 37°C. Nonspecific binding was evident in the presence of 100 nM/ml unlabelled Bgt. The cells were then washed extensively and used for immunocytochemistry or live imaging. Blocking of existing cell-surface receptors was performed using 50 µg/ml of unlabelled Bgt for 10 min at 37°C.

GABA_A receptor endocytosis

14-Div hippocampal neurons expressing ^{pHBS}β3 subunits were pretreated with 50 µM myristoylated p4 peptide, scrambled myristoylated p4 peptide, non-myristoylated p4 peptide or vehicle for 1 h. Neurons were then labelled with 10 nM Rd-Bgt for 5 min, washed extensively and placed in the fresh medium containing the peptides supplemented with 50 nM unlabelled Bgt. The neurons were then fixed at varying time points, subjected to immunocytochemistry with anti-synapsin antibodies and examined using confocal microscopy.

Visualizing GABA_A receptor membrane insertion

14-Div hippocampal neurons expressing recombinant GABA_A receptors were pretreated with M-p4 peptide for 1 h. All cell-surface ^{pHBS}β3-containing GABA_A receptors were blocked with unlabelled Bgt for 10 min. The cells were then washed extensively and left to recover in the medium containing M-p4 peptide for 5 min. Newly inserted receptors were labelled with Rd-Bgt and fixed immediately after the recovery period (time point 0) or 15 and 30 min after the recovery period. Fixed cells were stained with antibodies against synapsin.

Immunocytochemistry

HEK-293 cells and transfected hippocampal neurons were processed for immunocytochemistry under either permeabilized or non-permeabilized conditions as described previously (Jacob *et al*,

2005; Kittler *et al*, 2000a, b). The following primary antibodies were used: anti-vesicular polyclonal anti-synapsin 1 at 1:500 (Affinity BioReagents) and anti- β -adaptin (BD Biosciences).

Image acquisition and data analysis

Confocal images of immunostained neurons were taken using a $\times 60$ objective, acquired with Bio-Rad software and analyzed with MetaMorph. A receptor cluster was defined as being approximately 0.5–2 μm in length and approximately two- to three-fold more intense than background diffuse fluorescence. Synaptic clusters were colocalized with or directly apposed to synapsin staining. Clusters further than 1 μm from presynaptic marker staining were considered extrasynaptic (Jacob *et al*, 2005). All channels of an image were first

background subtracted, then thresholded and stacked to determine apposition of postsynaptic receptor subunits with a presynaptic marker (Jacob *et al*, 2005). The threshold value was determined for each culture and used for all images from that culture.

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