Activity-dependent phosphorylation of GABA$_A$ receptors regulates receptor insertion and tonic current

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The expression of GABA$_A$ receptors and the efficacy of GABAergic neurotransmission are subject to adaptive compensatory regulation as a result of changes in neuronal activity. Here, we show that activation of L-type voltage-gated Ca^{2+} channels (VGCCs) leads to Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylation of S383 within the β3 subunit of the GABA$_A$ receptor. Consequently, this results in rapid insertion of GABA$_A$ receptors at the cell surface and enhanced tonic current. Furthermore, we demonstrate that acute changes in neuronal activity leads to the rapid modulation of cell surface numbers of GABA$_A$ receptors and tonic current, which are critically dependent on Ca^{2+} influx through L-type VGCCs and CaMKII phosphorylation of β3S383. These data provide a mechanistic link between activity-dependent changes in Ca^{2+} influx through L-type channels and the rapid modulation of GABA$_A$ receptor cell surface numbers and tonic current, suggesting a homeostatic pathway involved in regulating neuronal intrinsic excitability in response to changes in activity.

Introduction

GABA$_A$ receptors are responsible for fast inhibitory neurotransmission in the brain, which consists of two components: phasic and tonic inhibition (Farrant and Nusser, 2005). These receptors are the major sites of action for both benzodiazepines and barbiturates, and are heteropentameric Cl$^-$ permeable, ligand-gated ion channels that are assembled from a range of homologous subunits that share a common structure (Rudolph and Mohler, 2004; Olsen and Sieghart, 2009). Insertion and internalization of GABA$_A$ receptors occur outside the synapse (Bogdanov *et al*,

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2006) and receptors are exchanged between synaptic an extrasynaptic regions by lateral diffusion (Jacob *et al*, 2005; Thomas *et al*, 2005; Bogdanov *et al*, 2006), representing a mechanism whereby the efficacy of synaptic inhibition can be modulated (Thomas *et al*, 2005).

Phosphorylation of the intracellular domains between transmembrane domain (TM) 3–4 of the GABA$_A$ receptor β and γ subunits by serine/threonine and tyrosine kinases has been shown to alter receptor function either by a direct effect on receptor properties, such as the probability of channel opening or desensitization, or by regulating trafficking of the receptor to and from the cell surface (Moss and Smart, 2001; Jacob *et al*, 2008). CaMKII phosphorylation sites within GABA$_A$ receptor subunits were initially identified using glutathionie-S-transferase fusion proteins of the large intracellular loops of these receptor subunits (McDonald and Moss, 1994, 1997). In a recombinant system, expression of α1β3 GABA$_A$ receptor subunits in a neuronal cell line led to the identification of β3S383 as the major site for CaMKII phosphorylation and modulation of whole-cell GABA currents (Houston *et al*, 2007). In the hippocampus modulation of GABA$_A$ receptor function by CaMKII has been reported, whereby CaMKII activation potentiates inhibitory post-synaptic currents (IPSCs) and inhibitory post-synaptic potentials (IPSPs) in CA1 pyramidal cells (Wang *et al*, 1995; Wei *et al*, 2004). In other neuronal preparations, CaMKII activation increased the amplitude of whole-cell currents and IPSCs recorded from neurons of the spinal cord dorsal horn (Wang *et al*, 1995), cortex (Aguayo *et al*, 1998) and cerebellum (Houston and Smart, 2006; Houston *et al*, 2008).

GABA$_A$ receptor activation regulates the excitability of neural circuits, and a number of studies in cultures of dissociated neurons have provided evidence that chronic changes (i.e., days) in neuronal activity can lead to homeostatic scaling of GABAergic synaptic strength (Kilman *et al*, 2002; Hartman *et al*, 2006; Swanwick *et al*, 2006; Saliba *et al*, 2007). Additionally, the intrinsic excitability of a neuron (i.e., voltage-dependent conductances) is also subject to homeostatic regulation (Nelson and Turrigiano, 2008). There is evidence now suggesting that the persistent GABA-mediated tonic current plays a role in regulating intrinsic neuronal excitability (Mody, 2005).

Here, we have begun to investigate the effects of L-type VGCC activation on the forward trafficking of GABA$_A$ receptors and tonic current. We find that Ca^{2+} influx through L-type VGCCs induces an increase in β3S383 phosphorylation by CaMKII, which promotes the rapid accumulation of α5/β3 containing GABA$_A$ receptors at the neuronal cell surface and enhances tonic current. Furthermore, CaMKII phosphorylation of β3S383 promotes the insertion of GABA$_A$ receptors into the neuronal membrane without influencing the endocytosis of these receptors. Lastly, our findings show that acute changes in neuronal activity lead to the modulation of GABA$_A$ receptor accumulation at the cell surface and tonic current

and these effects are critically dependent on L-type VGCC activation and phosphorylation of β3S383 by CaMKII.

Results

Ca²⁺ influx through L-type VGCCs increases the cell surface expression of GABA_A receptors and tonic current

We have previously shown that chronic blockade of L-type VGCCs in cultured hippocampal neurons reduces the expression of GABA_A receptors and the efficacy of GABAergic neurotransmission (Saliba *et al*, 2009). As our studies centre on GABA_A receptors in the hippocampus, our experiments will largely focus on the β3 subunit as this is highly abundant in this brain region (Sperk *et al*, 1997). To determine if acute activation of L-type VGCCs has an effect on the cell surface numbers of GABA_A receptors,

we treated mature hippocampal neurons in culture, 16–21 days *in vitro* (div), with the dihydropyridine Bay K 8644, which is a voltage-dependent agonist that stabilizes the open state of L-type VGCCs (Bechem and Hoffmann, 1993). Neurons were incubated with Bay K 8644 (5 μM) for times ranging from 0 to 10 min and cell surface GABA_A receptors were isolated by using a biotinylation assay (Figure 1A). Within 2 min of L-type channel activation, cell surface numbers of GABA_A receptors containing β3 subunits increased by $25.6 \pm 2.2\%$ (Figure 1A). L-type channel activation for 5 and 10 min resulted in a further increase in cell surface GABA_A receptor expression of $44.6 \pm 4.9\%$ and $61.0 \pm 6.0\%$, respectively (Figure 1A). We observed no change in the total expression level of GABA_A receptors at these time points (Figure 1A).

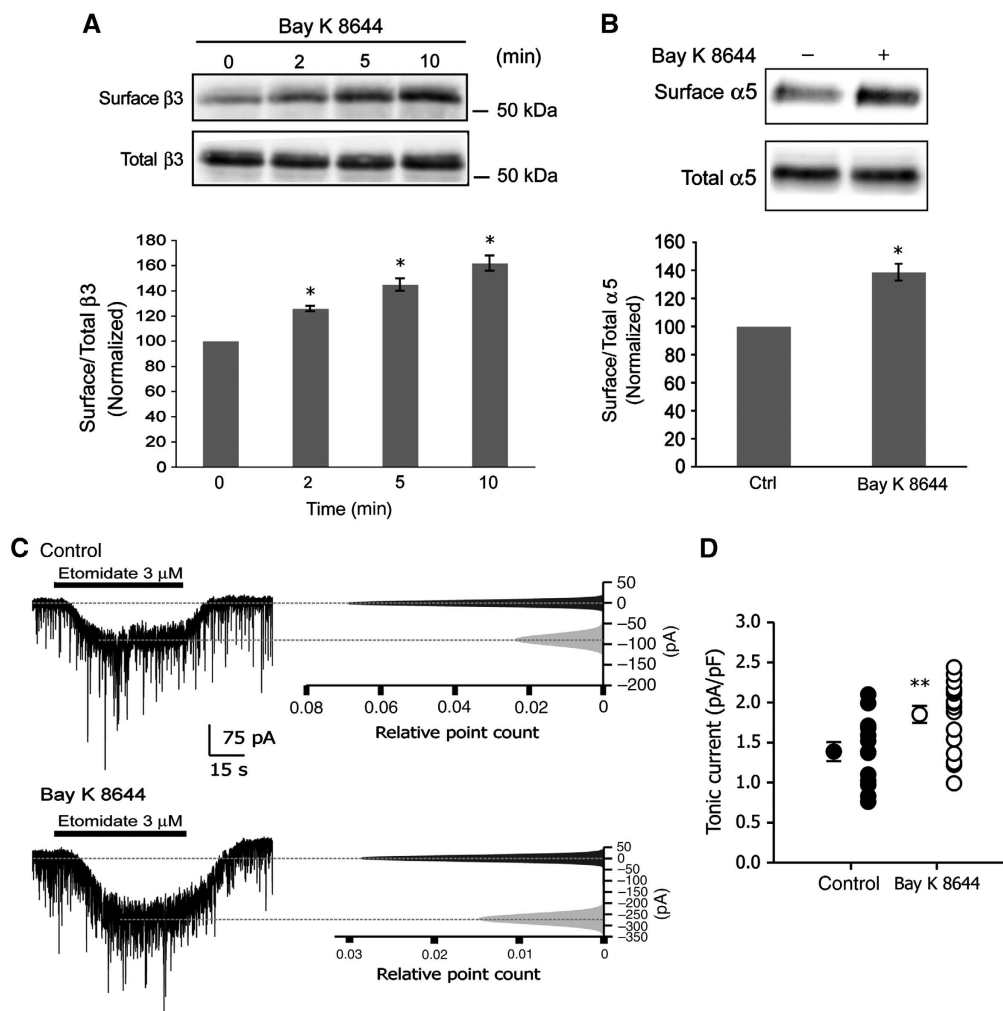GABA_A receptor α5 subunits are abundantly expressed in the CA1 and CA3 region of the hippocampus (Fritschy and

Figure 1 Ca²⁺ influx through L-type VGCCs increases cell surface numbers of GABA_A receptors and the efficacy of tonic current. (**A**) Hippocampal neurons were incubated with Bay K 8644 for 0–10 min. Immunoblots show cell surface (biotinylated) and total levels of GABA_A receptor β3 subunits as indicated. Graph shows the quantification of surface β3/total β3 band intensity ratio normalized to values at time 0 (data are plotted as mean ± s.e. *P<0.05, n=3, t-test). (**B**) Hippocampal neurons were incubated with Bay K 8644 for 10 min and cell surface proteins were isolated and immunoblotted with anti-α₅ IgGs. Graph shows the quantification of cell surface α₅/total α₅ band intensity ratio normalized to control (ctrl) values (*P<0.05, n=3, t-test). (**C**) Left panels show whole-cell voltage-clamp recordings before (top) and after (bottom) application of Bay K 8644. Right panels show corresponding all point histograms of 10-s traces before (■) and after (▨) etomidate application. Histograms were fitted with single Gaussian functions, and means were determined. After subtracting baseline from current evoked by etomidate, tonic current was normalized to cell size. (**D**) Data plotted are values from individual neurons and their mean values ± s.e. (control: n=13 and Bay K 8644: n=14; 3 different cultures; **P<0.01; t-test).

Mohler, 1995; Sperk et al, 1997; Sur et al, 1998) are predominantly extrasynaptic (Fritschy et al, 1998) and are responsible for tonic current in pyramidal cells (Caraiscos et al, 2004). As the GABA_A receptor α5 subunit mainly assembles with the β3 subunit in hippocampal neurons (Sur et al, 1998), we speculated that α5 surface expression may also increase following Ca^{2+} influx through L-type channels. To test this prediction, we incubated hippocampal neurons with Bay K 8644 (5 μM) for 5 min and isolated surface receptors using a biotinylation assay, observing an increase in surface α5 subunits of $38.6 \pm 5.9\%$ (Figure 1B).

We considered the possible consequences of Ca^{2+}-dependent upregulation of cell surface GABA_A receptor α5/β3 subunit expression on the efficacy of tonic current and took recordings from cultured hippocampal neurons (16–21 div) following a 10-min application of 5 μM Bay K 8644 (Figure 1C). We applied etomidate to hippocampal neurons in culture to study the effects of Bay K 8644 on tonic conductance. Etomidate is a positive allosteric modulator selective for GABA_A receptors containing β2 or β3 subunits and preferentially enhances tonic current generated by GABA_A receptors containing the α5 subunit in hippocampal pyramidal neurons (Caraiscos et al, 2004; Cheng et al, 2006). We observed an increase in tonic current, measured as the whole-cell current evoked by bath application of 3 μM etomidate (Figure 1C). Tonic current evoked by etomidate increased by 33.1% from 1.39 ± 0.12 pA/pF in control neurons to 1.85 ± 0.11 pA/pF following a 10-min application of Bay K 8644 (Figure 1D). Taken together, these findings demonstrate that Ca^{2+} influx through L-type VGCCs leads to the rapid accumulation of GABA_A receptors assembled from α5/β3 subunits at the cell surface and enhanced tonic current.

Ca^{2+} influx through L-type VGCCs increases phosphorylation of β3S383 by CaMKII

Previous studies have shown that CaMKII phosphorylates a GST fusion protein of the GABA_A receptor β3 subunit at S383 (McDonald and Moss, 1997) and phosphorylates the same residue in recombinant GABA_A receptor β3 subunits (Houston et al, 2007). To assess changes in phosphorylation following Ca^{2+} influx through L-type channels, we developed a rabbit phosphorylation site-specific antibody to phosphorylated β3S383 using the phospho-peptide CQYRKQSpMPKEG corresponding to the sequence surrounding β3S383. We discovered, using immunoblotting with anti-p-β3S383 IgGs, that under basal conditions myc-tagged β3WT is phosphorylated in a recombinant system using the neuronal cell line SH-SY5Y (Figure 2A) and also in cultures of dissociated hippocampal neurons, were a band of ∼55 kDa was detected (Figure 2B). Expression of myc-tagged phospho-null βS383A in SH-SY5Ys abolished the p-S383 immunosignal (Figure 2A). We increased Ca^{2+} influx into hippocampal neurons using Bay K 8644 (5 μM for 5 min) and observed and increase inthe p-S383 signal (Figure 2B and C). Also pretreatment of immunoblots with λ-phosphatase to remove phosphate groups, abolished the signal generated with anti-p-S383 IgGs (Figure 2B). In addition, preincubation of anti-p-S383 IgGs with a 500 molar excess of immunizing antigen prior to immunoblotting abolished the p-S383 immunosignal (Figure 2B). Collectively, these data show that anti-p-S383 IgGs are specific for phosphorylated β3S383.

To determine whether the Ca^{2+}-dependent increase in cell surface levels of GABA_A receptors is associated with an increase in phosphorylation of β3S383, we activated L-type channels with Bay K 8644 (5 μM). Within 2 min following activation of L-type channels an increase in β3S383 phosphorylation of $69.3 \pm 7.2\%$ was observed which remained stable at 5 min (Figure 3A). To demonstrate the role of CaMKII activity, neurons were incubated with the CaMKII inhibitor KN-93 (4 μM) for 1 min prior to addition of Bay K 8644 (5 μM) for 5 min (Figure 3B). Inhibiting CaMKII activity blocked the Ca^{2+}-dependent rise in phosphorylation of β3S383 (Bay K 8644: $67.0 \pm 8.6\%$ increase relative to control; Bay K 8644 plus KN-93: $4.0 \pm 12.4\%$ decrease relative to control; Figure 3B) and the rise in cell surface numbers of GABA_A receptors (Bay K 8644: $45.3 \pm 5.3\%$ increase relative to control; Bay K 8644 plus KN-93: $8.0 \pm 4.16\%$ decrease relative to control; Figure 3B). In contrast, activation of extrasynaptic and synaptic NMDA receptors by brief application of NMDA to cultures of mature hippocampal neurons results in significant dephosphorylation of β3S383 by calcineurin (Supplementary Figure S1).

To assess whether β3S383 phosphorylation is critical for the observed Ca^{2+}-dependent increase in cell surface numbers of GABA_A receptors, hippocampal neurons were nucleofected with β3WT and the phospho-null mutant β3S383A. Both β3WT and β3S383A are tagged at their N-termini with pHluorin (pH) and the α-bungarotoxin binding site—BBS (Bogdanov et al, 2006; Saliba et al, 2007; Jacob et al, 2009). Nucleofected neurons (14 div) were incubated with Bay K 8644 (5 μM) for 10 min. To assess changes in the cell surface expression of ^pHBBSβ3WT and ^pHBBSβ3S383A, fixed, non-permeabilized neurons were immunolabelled with anti-GFP
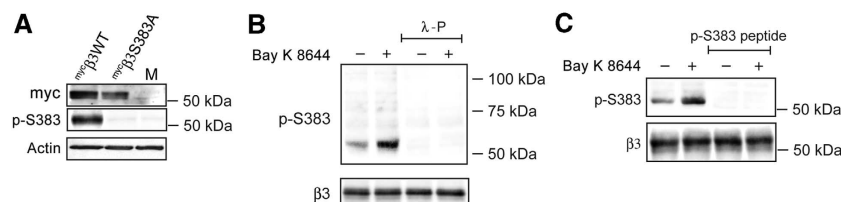

Figure 2 Specificity of anti-phosphorylated β3S383 IgGs. (**A**) SH-SY5Y neuroblastoma cells were nucleofected with myc-tagged β3WT, myc-tagged β3S383A or mock nucleofected (M). Equal amounts of total protein were immunoblotted with rabbit anti-phosphorylated β3S383 (p-S383) IgGs, anti-myc IgGs and anti-actin IgGs, as indicated. (**B**) Hippocampal neurons were incubated with or without Bay K 8644 as indicated and immunoblots show p-S383 and total β3. Replicate blots were treated with λ-phosphatase (λ-P), prior to immunoblotting with anti-p-S383 IgGs as shown. (**C**) Hippocampal neurons were incubated with or without Bay K 8644. Immunoblots show p-S383 and total β3. Replicate bots were also immunoblotted with anti-p-S383 IgGs, which had been preabsorbed with an excess of p-S383 immunizing peptide, as indicated.
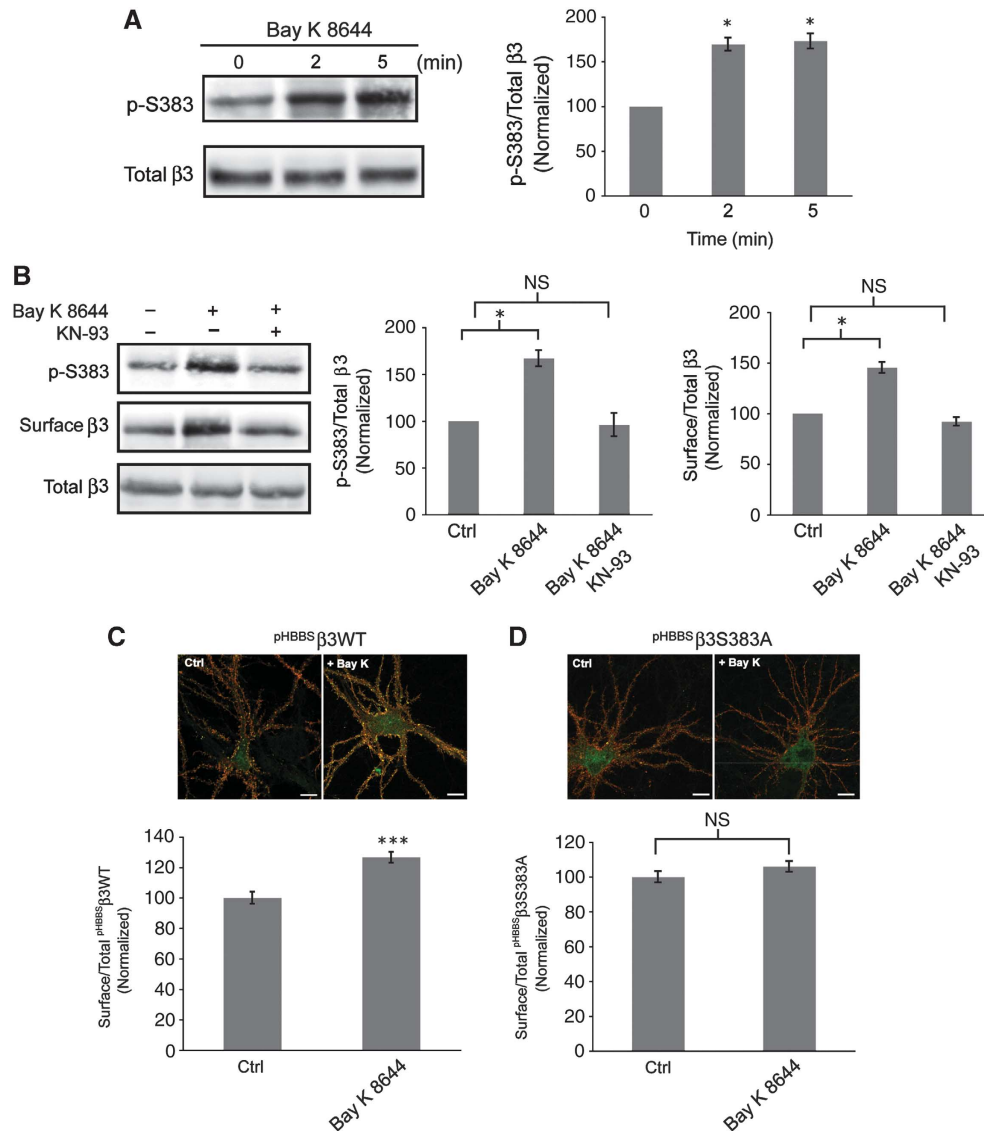
Figure 3 CaMKII phosphorylation of β3S383 is essential for the Ca^{2+}-dependent increase in GABA$_A$ receptor surface expression. (**A**) Hippocampal neurons were incubated with Bay K 8644 and immunoblots show p-S383, surface and total β3. Graph represents the ratio of p-S383 to total β3 band intensities, normalized to values at time 0 (data are plotted as mean ± s.e. *$P<0.05$, $n=3$, *t*-test). (**B**) Hippocampal neurons were incubated with or without Bay K 8644 plus KN-93 as indicated. Immunoblots show p-S383, surface and total β3. First graph represents the ratio of p-S383 to total β3 band intensities, normalized to control (Ctrl) values and the second graph represents cell surface β3 to total β3 band intensity ratio, normalized to control (Ctrl) values (*$P<0.05$, $n=3$, NS: not significantly different, *t*-test. (**C, D**) Nucleofected neurons expressing ^{pHBBS}β3WT (**C**) and ^{pHBBS}β3S383A (**D**) were incubated with Bay K 8644. Cell surface pHluorin-tagged β3 subunits are labelled red and the pHluorin signal (green) represents total ^{pHBBS}β3WT and ^{pHBBS}β3S383A expression (scale bar 10 μm). Graphs represent cell surface/total ratios of ^{pHBBS}β3WT and ^{pHBBS}β3S383A fluorescence intensity normalized to control (Ctrl) values (***$P<0.001$; NS: not significantly different; $n>20$ neurons, three independent cultures; *t*-test).

IgGs (Figure 3C and D). Confocal images were taken of immunolabelled pyramidal cells and cell surface immuno-fluorescence intensity along 2–3 proximal dendrites was measured using the program Metamorph (Saliba *et al*, 2007). Activation of L-type channels increased ^{pHBBS}β3WT surface expression by 25.0 ± 3.5% (Figure 3C) but had no significant effect on ^{pHBBS}β3S383A surface expression (6.0 ± 3.1% increase relative to control; Figure 3D). Taken together, these data reveal that phosphorylation of β3S383 by CaMKII is critical for the observed Ca^{2+}-dependent rise in cell surface GABA$_A$ receptor numbers.

As an alternative to pharmacological blockade of CaMKII activity in assessing the role of phosphorylation of β3S383 in

GABA$_A$ receptor accumulation at the cell surface, we used the CaMKII inactive mutant; CaMKIIK42R in our next set of experiments. Hippocampal neurons (15 div) were transfected with ^{pHBBS}β3WT and CaMKIIWT and inactive CaMKIIK42R using Lipofectamine 2000. Neurons were transfected using double the amount of CaMKIIWT and CaMKIIK42R plasmid DNA compared with ^{pHBBS}β3WT plasmid DNA and surface expression was assayed 24 h later (Figure 4A). Using immunofluorescence labelling of non-permeabilized neurons with anti-GFP IgGs, we observed that co-expression of inactive CaMKIIK42R with ^{pHBBS}β3WT resulted in a reduction in cell surface numbers of GABA$_A$ receptors by 44.0 ± 3.0% (Figure 4B) compared with controls (^{pHBBS}β3WT and
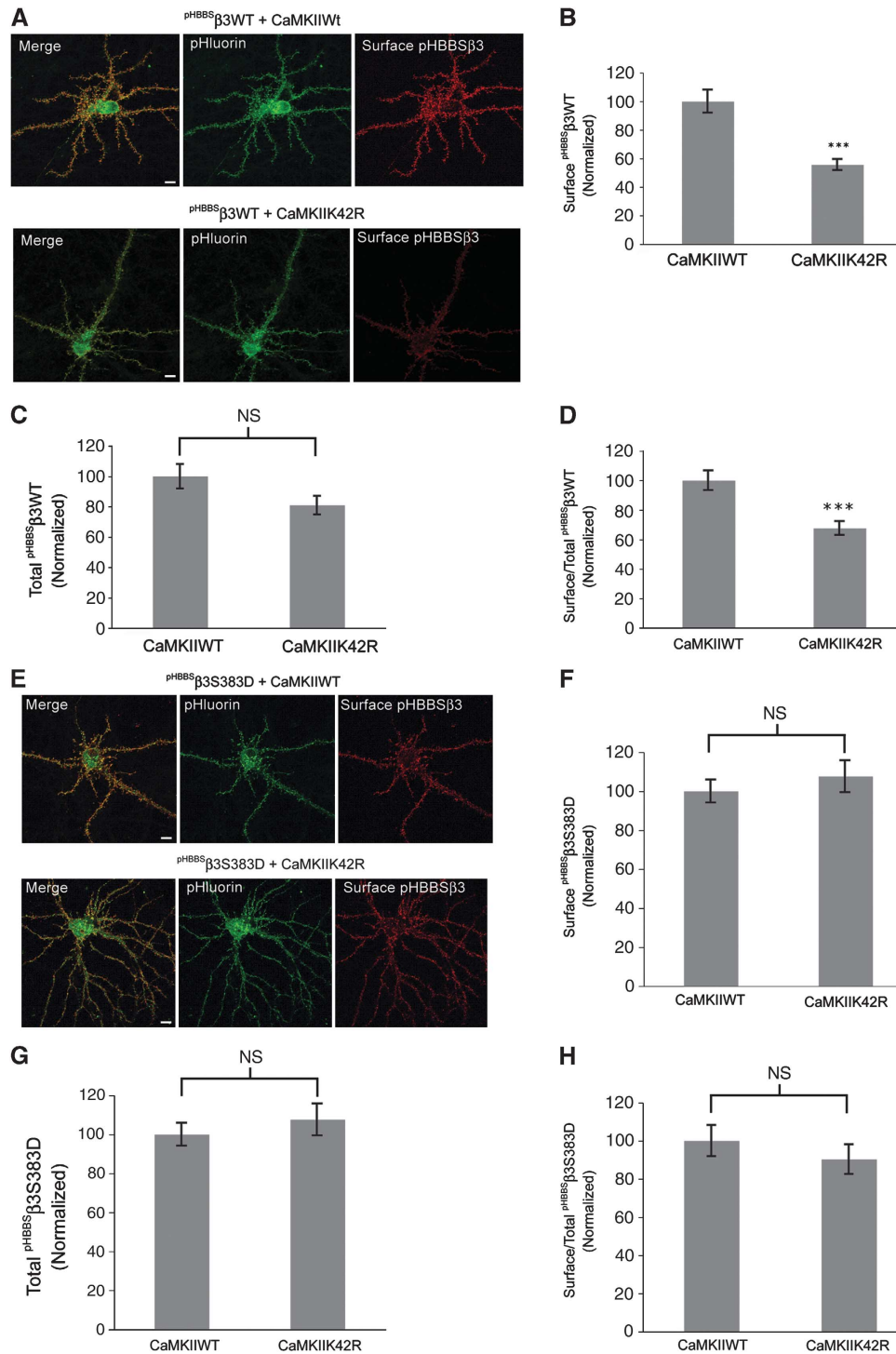
Figure 4 Expression of inactive CaMKIIK42R reduces GABA$_A$ receptor cell surface numbers. (**A**) Hippocampal neurons (15 div) were transfected with ^{pHBBS}β3WT and CaMKIIWT and inactive CaMKIIK42R as indicated. Cell surface population of ^{pHBBS}β3WT subunits is labelled red. The pHluorin (green) signal represents total ^{pHBBS}β3WT expression (scale bar 10 μm). (**B**) Graph represents cell surface immunofluorescence intensity of ^{pHBBS}β3WT + CaMKIIK42R normalized to control (^{pHBBS}β3WT + CaMKIIWT) values (data are plotted as mean ± s.e., ***$P<0.001$; $n>20$ neurons; three independent cultures, t-test). (**C**) Graph represents total pHluorin fluorescence intensity of ^{pHBBS}β3WT + CaMKIIK42R normalized to control (^{pHBBS}β3WT + CaMKIIWT) values (NS: not significantly different, $n>20$ neurons; three independent cultures, t-test). (**D**) Graph represents the cell surface/total fluorescence intensity ratio of ^{pHBBS}β3WT + CaMKIIK42R normalized to control (^{pHBBS}β3WT + CaMKIIWT) values (***$P<0.001$; $n>20$ neurons; three independent cultures; t-test). (**E**) Hippocampal neurons (15 div) were transfected with ^{pHBBS}β3S383D and CaMKIIWT and inactive CaMKIIK42R as indicated. Cell surface population of ^{pHBBS}WTβ3S383D subunits is labelled red. The pHluorin (green) signal represents total ^{pHBBS}β3S383D expression (scale bar 10 μm). (**F**) Graph represents cell surface immunofluorescence intensity of ^{pHBBS}β3S383D + CaMKIIK42R normalized to control (^{pHBBS}β3S383D + CaMKIIWT) values (NS: not significantly different; $n>20$ neurons; three independent cultures; t-test). (**G**) Graph represents total pHluorin fluorescence intensity of ^{pHBBS}β3S383D + CaMKIIK42R normalized to control (^{pHBBS}β3S383D + CaMKIIWT) values (NS: not significantly different; $n>20$ neurons; three independent cultures; t-test). (**H**) Graph represents the cell surface/total fluorescence intensity ratio of ^{pHBBS}β3S383D + CaMKIIK42R normalized to control (^{pHBBS}β3S383D + CaMKIIWT) values (NS: not significantly different, $n>20$ neurons; three independent cultures; t-test.

CaMKII WT) and a small non-significant reduction in total ^{pHBBS}β3WT of 19.0 ± 6.0% (Figure 4C). Additionally, we also observed a reduction in the ratio of cell surface to total ^{pHBBS}β3WT of 32.3 ± 4.6% in neurons expressing inactive CaMKIIK42R (Figure 4D).

To determine if the effect of inactive CaMKIIK42R on GABA$_A$ receptor cell surface expression is specific to β3S383, we co-transfected hippocampal neurons (15 div) with the phospho-mimic ^{pHBBS}β3S383D subunit and CaMKIIWT and inactive CaMKIIK42R (Figure 4E). We observed no significant difference in the surface or total expression levels of ^{pHBBS}β3S383D following co-expression with CaMKIIK42R compared with expression with control CaMKIIWT (surface β3S383D + CaMKIIK42R: 7.6 ± 8.1% increase relative to CaMKIIWT; total β3S383D + CaMKIIK42R: 11.5 ± 9.9% increase relative to CaMKIIWT; surface/total β3S383D ratio + CaMKIIK42R: 9.7 ± 7.7% decrease relative to CaMKIIWT; Figure 4F–H). These experiments indicate that CaMKII phosphorylation of β3S383 plays a pivotal role in the regulation of GABA$_A$ receptor accumulation at the neuronal cell surface and further show that the effect of inactive CaMKII on GABA$_A$ receptor surface expression is specific to β3S383.

Ca^{2+} influx through L-type VGCCs increases the exocytosis of GABA$_A$ receptors

The Ca^{2+}-dependent increase in GABA$_A$ receptor numbers at the cell surface could possibly result from a change in the insertion rate of receptors or result from an altered rate of endocytosis (Kittler et al, 2000, 2004; Jacob et al, 2009). To examine the contribution of insertion to the Ca^{2+}-dependent rise in GABA$_A$ receptor cell surface numbers, we used nucleofected neurons expressing ^{pHBBS}β3WT and assessed insertion of receptors using Alexa-594 labelled α-bungarotoxin (Bgt), as previously described (Saliba et al, 2007, 2009). Briefly, hippocampal neurons (15 div) were incubated with unlabelled α-Bgt at 15°C to block existing cell surface ^{pHBBS}β3WT subunits. Neurons were then incubated at 37°C with Alexa-594-Bgt for a range of time points between 0 and 40 min (Figure 5A). Cell surface levels of ^{pHBBS}β3WT containing GABA$_A$ receptors increased gradually over time and the rate of receptor insertion was best described by a single exponential process with a time constant of 13.1 ± 1.8 min (Figure 5A). To determine if Ca^{2+} influx through L-type channels increases the number of GABA$_A$ receptors being inserted, we incubated hippocampal neurons expressing ^{pHBBS}β3WT with or without Bay K 8644 (5 μM) for 10 min in the presence of Alexa-594-Bgt. We assessed the level of insertion at 10 min by determining the fluorescence intensity of newly inserted ^{pHBBS}β3WT (alexa-594-Bgt). The data were expressed as the ratio of newly inserted to total ^{pHBBS}β3WT pHluorin fluorescence in control and Bay K 8644-treated neurons (Figure 5B). Compared with control levels, we observed a 26.4 ± 5.3% increase in insertion of ^{pHBBS}β3WT containing GABA$_A$ receptors following L-type channel activation for 10 min (Figure 5B).

GABA$_A$ receptors are subject to constitutive endocytosis, which is mediated by the direct binding of clathrin adaptor protein AP2 to specific endocytosis motifs within the intracellular domains of GABA$_A$ receptor β1–β3 and γ2 subunits (Kittler et al, 2000, 2005; Jacob et al, 2009; Vithlani and Moss, 2009). Phosphorylation of S408 and S409 within

the GABA$_A$ receptor β3 subunit (PKC and PKA phosphorylation sites) can modulate the binding of adaptins to GABA$_A$ receptors and thus modulate their rate of endocytosis (Kittler et al, 2005; Jacob et al, 2009; Vithlani and Moss, 2009). To determine if phosphorylation of β3S408/9 changes following activation of L-type channels we incubated hippocampal neurons (16–21 div) with Bay K 8644 (5 μM) for 5 min and assessed phosphorylation of β3S408/9 using western blotting with anti-phosphorylated β3S408/9-specific IgGs (Jovanovic et al, 2004). We found no significant change in the phosphorylation of β3S408/9 following L-type channel activation (Bay K 8644: 6.2 ± 4.5% decrease relative to control; Figure 5C) but observed an increase in β3S383 phosphorylation similar to the data in Figure 3A.

To further support our data that demonstrates Ca^{2+} influx through L-type channels regulates exocytosis of GABA$_A$ receptors, we used myristoylated dynamin inhibitory peptide, to block clathrin-dependent endocytosis, as used in previous studies (Kittler et al, 2000; Bogdanov et al, 2006; Jacob et al, 2009). Both control neurons and neurons exposed to Bay K 8644 were incubated with 50 μM myristoylated dynamin inhibitory peptide for 10 min prior to activation of L-type channels with Bay K 8644 (5 μM) for 5 min. Cell surface GABA$_A$ receptors were isolated using a biotinylation assay and we observed that blocking endocytosis did not prevent the Ca^{2+}-dependent rise in surface GABA$_A$ receptor expression (Bay K 8644: 37.0 ± 7.2% increase relative to control; Figure 5D). Together, these observations suggest that Ca^{2+} influx through L-type channels promotes the insertion of GABA$_A$ receptors without influencing receptor endocytosis.

The effects of phospho-null β3S383A and phospho-mimic β3S383D on GABA$_A$ receptor expression and tonic current

We used the phospho-null (^{pHBBS}β3S383A) and a phospho-mimic (^{pHBBS}β3S383D) mutant to further assess the role of phosphorylation of β3S383 in cell surface expression of GABA$_A$ receptors. In these experiments, we nucleofected hippocampal neurons with ^{pHBBS}β3WT, ^{pHBBS}β3S383A and ^{pHBBS}β3S383D and determined cell surface and total expression levels at 15 div (Figure 6A and B). We observed a decrease in expression of surface (45.5 ± 11.6%) and total (41.3 ± 5.6%) ^{pHBBS}β3S383A compared with ^{pHBBS}β3WT expression levels (Figure 6A). In contrast, the surface and total expression levels of ^{pHBBS}β3S383D were markedly above those of ^{pHBBS}β3WT levels by 43.8 ± 15.0% and 53.0 ± 16.3%, respectively (Figure 6B).

We further assessed changes in surface expression of β3S383 phospho-mutants by measuring tonic current, using whole-cell patch clamp recordings. Bath application of bicuculline (25 μM) dramatically decreased whole-cell holding current in hippocampal neurons expressing ^{pHBBS}β3WT and ^{pHBBS}β3S383D (Figure 6C and E), but only had a minor effect in ^{pHBBS}β3S383A-expressing neurons (Figure 6D). Whole-cell current change, measured as a difference between baseline before and after bicuculline application was 1.36 ± 0.27 pA/pF for ^{pHBBS}β3WT, 2.11 ± 0.75 pA/pF for ^{pHBBS}β3S383D and 0.20 ± 0.06 pA/pF for ^{pHBBS}β3S383A, respectively (Figure 6F). In agreement with higher surface expression of ^{pHBBS}β3S383D containing GABA$_A$ receptors, the benzodiazepine flurazepam (3 μM) greatly potentiated the holding current
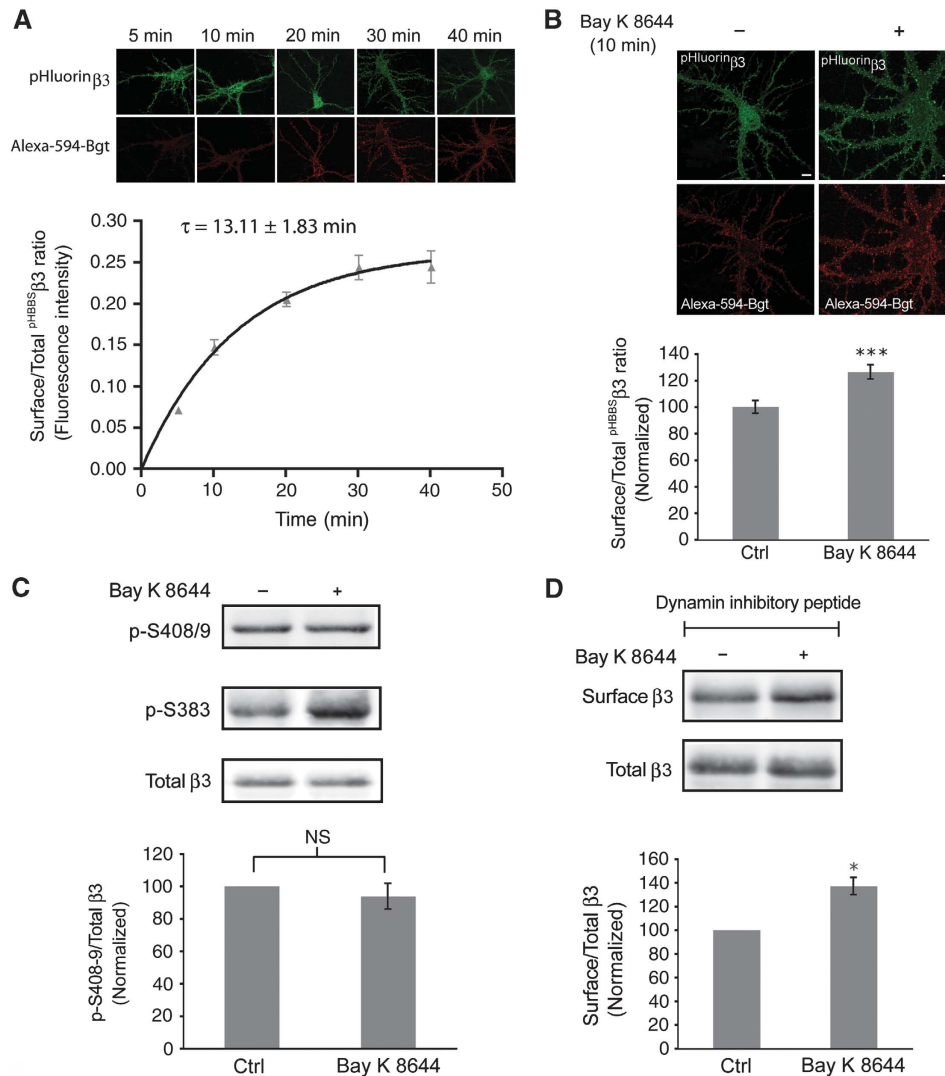
Figure 5 Activation of L-type VGCCs increases insertion of GABA$_A$ receptors. (**A**) Insertion of ^{pHBBS}β3WT containing GABA$_A$ receptors over time. Newly inserted ^{pHBBS}β3WT is labelled red (Aexa-594-Bgt). Graph represents the ratio of newly inserted ^{pHBBS}β3 to total pHluorin fluorescence intensity over time. (**B**) Nucleofected hippocampal neurons expressing ^{pHBBS}β3WT were incubated with Alexa-594-Bgt with or without Bay K 8644 (scale bar 10 μm). Graph shows quantification of newly inserted ^{pHBBS}β3WT with Bay K 8644 expressed as the surface/total ^{pHBBS}β3WT fluorescence intensity ratio normalized to control (Ctrl) values (data are plotted as mean ± s.e. ***$P < 0.001$; $n > 20$; three independent cultures; t-test). (**C**) Hippocampal neurons were treated with Bay K 8644. Lysates were immunoblotted with anti-phosphorylated β3S408/9 IgGs (p-S408/9) and anti-p-S383 IgGs. Graph represents quantification of the ratio of band intensities of P-S408/9 to total β3 normalized to control (Ctrl) values (NS: not significantly different; $n = 4$; t-test). (**D**) Hippocampal neurons were treated with myristoylated dynamin inhibitory peptide prior to incubation with or without Bay K 8644. Immunoblots show total and cell surface levels of β3 subunits as indicated. Graph represents quantification of the ratio of band intensities of cell surface and total β3 subunits, normalized to control (Ctrl) values (*$P < 0.05$, $n = 3$, t-test).

in neurons expressing β3S383D when compared with neurons expressing the ^{pHBBS}β3WT subunit (-0.57 ± 0.09 pA/pF for ^{pHBBS}β3WT; -1.10 ± 0.31 pA/pF, for ^{pHBBS}β3S383D; Figure 6G, I and J). In neurons expressing the phospho-null ^{pHBBS}β3S383A subunit, flurazepam showed a lower potentiation of holding current compared with neurons expressing ^{pHBBS}β3WT (-0.57 ± 0.09 pA/pF, for ^{pHBBS}β3WT; -0.22 ± 0.06 pA/pF, for ^{pHBBS}β3S383A; Figure 6G, H and J). These data indicate that ^{pHBBS}β3 subunits assemble into functional GABA$_A$ receptors containing γ subunits. Furthermore, these results also show that expression of β3S383A and β3S383D have opposite effects on GABA$_A$ receptor expression and tonic current.

Stimulating neuronal activity increases phosphorylation of β3S383, cell surface expression of GABA$_A$ receptors and tonic current

A prolonged increase (24 h) in neuronal activity in cultured neurons has been previously shown to upregulate the expression of GABA$_A$ receptors (Saliba *et al*, 2007). Thus, the question arises whether an acute increase in neuronal activity lasting minutes, as opposed to several hours, has an influence on the expression of cell surface GABA$_A$ receptors. To explore this possibility, we incubated hippocampal neurons (16–21 div) with the weak K$^+$ channel blocker 4-aminopyridine (4-AP, 50 μM) for time points ranging from 0 to 30 min (Figure 7A). Cell surface GABA$_A$ receptors were isolated
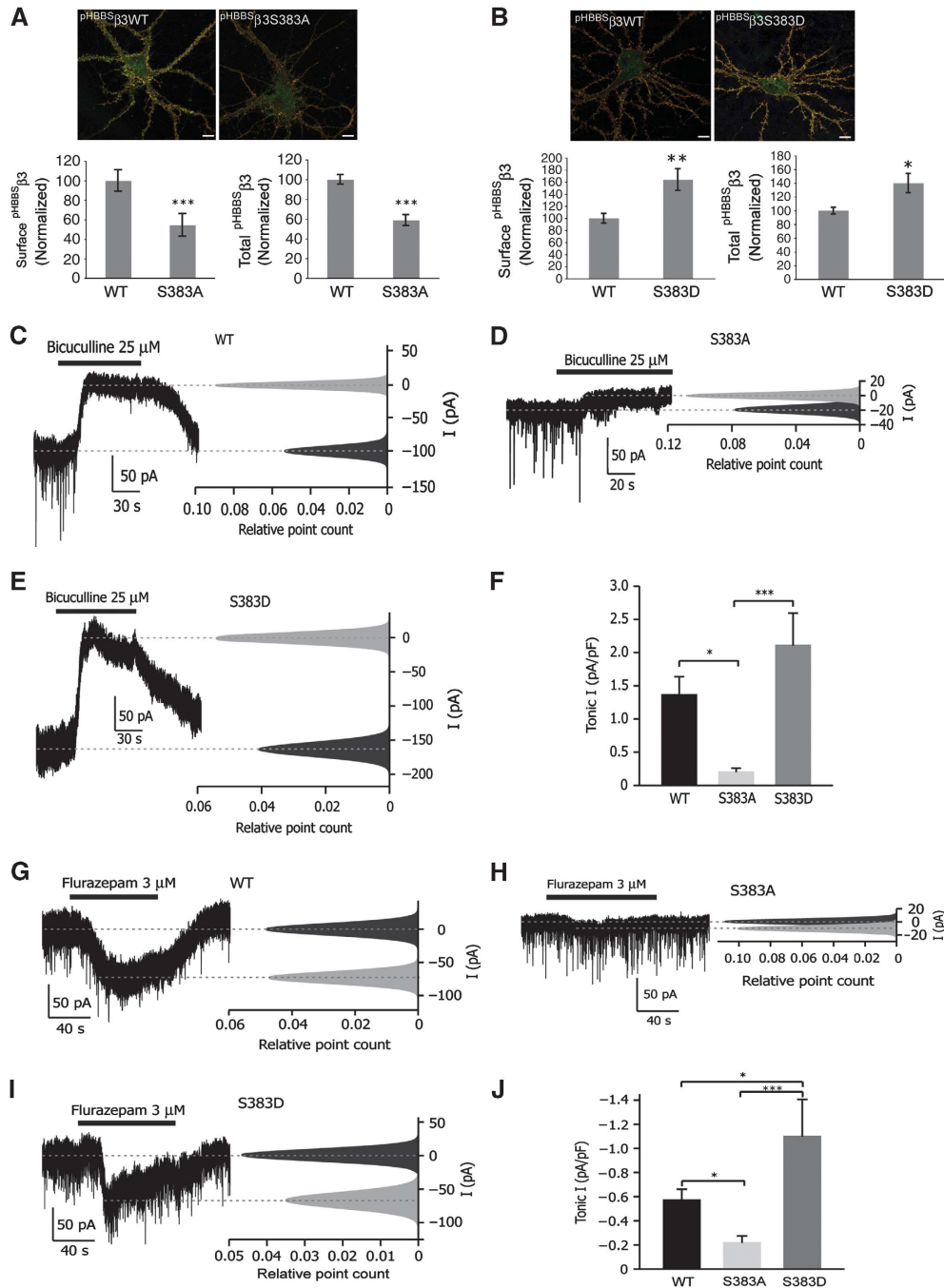
Figure 6 The effects of phospho-null β3S383A and phospho-mimic β3S383D on GABA$_A$ receptor expression and tonic current. (**A**) Cell surface $^{\text{pHBBS}}$β3WT and $^{\text{pHBBS}}$β3S383A subunits were labelled red and the pHluorin signal (green) represents total $^{\text{pHBBS}}$β3 expression (scale bar 10 μm). Graphs represent cell surface and total fluorescence intensity normalized to control ($^{\text{pHBBS}}$β3WT) values as indicated (***$P<0.001$; $n>20$ neurons; three independent cultures; *t*-test). (**B**) Comparison of $^{\text{pHBBS}}$β3WT and $^{\text{pHBBS}}$β3S383D surface and total expression. Cell surface receptors were labelled as in (**A**) (scale bar 10 μm). Graphs represent cell surface and total fluorescence intensity normalized to control ($^{\text{pHBBS}}$β3WT) values as indicated (*$P<0.05$; **$P<0.01$; $n>20$ neurons; three independent cultures; *t*-test). (**C–E**) Representative traces with corresponding all-point histograms from hippocampal neurons (16–21 div) nucleofected with $^{\text{pHBBS}}$β3WT (**C**), $^{\text{pHBBS}}$β3S383A (**D**) and $^{\text{pHBBS}}$β3S383D (**E**) constructs. Tonic current was expressed as the difference in baseline current before and after application of bicuculline (25 μM). The 10-s fragments of recordings before and during bicuculline application were taken and all-point histograms plotted. Histograms were fitted with single Gaussian functions, and means were determined. (**F**) Summary of recordings obtained from 8 ($^{\text{pHBBS}}$β3WT), 8 ($^{\text{pHBBS}}$β3S383A) and 5 ($^{\text{pHBBS}}$β3S383D) hippocampal neurons from three independent cultures. Tonic current was normalized to the cell size. (*$P<0.05$ and ***$P<0.001$; one-way ANOVA with Tukey's *post-hoc* test.) (**G–I**) Representative traces with corresponding all-point histograms from hippocampal neurons (16–21 div) nucleofected with $^{\text{pHBBS}}$β3WT (**G**), $^{\text{pHBBS}}$β3S383A (**H**) and $^{\text{pHBBS}}$β3S383D (**I**) constructs. Tonic current was expressed as the difference in baseline current before and after application of flurazepam (3 μM). The 10-s fragments of recordings before and during flurazepam application were taken and all-point histograms plotted. Histograms were fitted with single Gaussian functions, and means were determined. (**J**) Summary of recordings obtained from 8 ($^{\text{pHBBS}}$β3WT), 8 ($^{\text{pHBBS}}$β3S383A) and 3 ($^{\text{pHBBS}}$β3S383D) hippocampal neurons from three independent cultures. Tonic current was normalized to the cell size. (*$P<0.05$ and ***$P<0.001$; one-way ANOVA with Newman–Keul *post-hoc* test.)
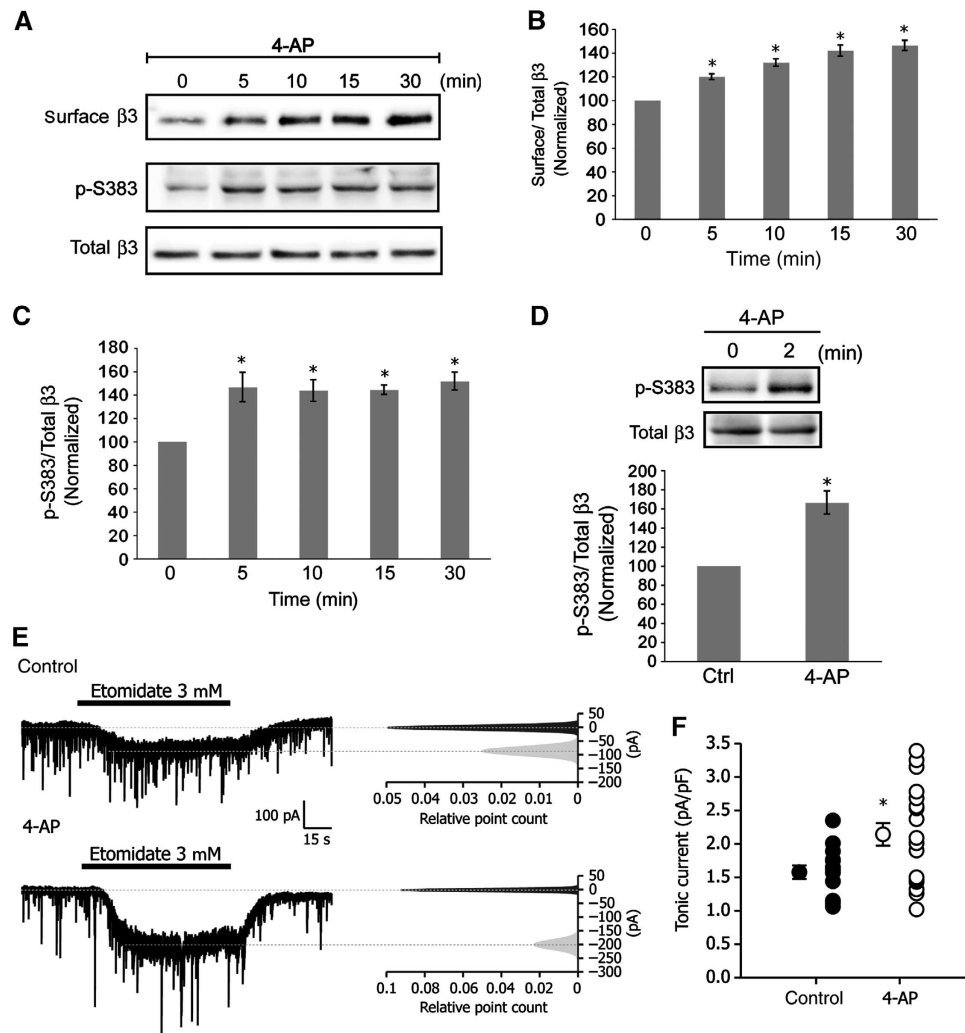
Figure 7 Enhancing neuronal activity increases phosphorylation of β3S383, the number of cell surface GABA_A receptors and tonic current. (**A**) Hippocampal neurons were incubated with 4-AP for times indicated. Immunoblots show p-S383, surface and total β3. (**B**) Graph shows the quantification of cell surface β3/total β3 band intensity ratio normalized to values at time 0 (data are plotted as mean ± s.e. *$P < 0.05$, $n = 3$, *t*-test). (**C**) Graph shows the quantification of the ratio of p-S383 to total β3 band intensities, normalized to values at time 0 (*$P < 0.05$, $n = 3$, *t*-test). (**D**) Acute hippocampal slices incubated with 4-AP. Immunoblots show p-S383 and total β3. Graph shows the quantification of the ratio of p-S383 to total β3 band intensities, normalized to values at time 0 (*$P < 0.05$, $n = 3$, *t*-test). (**E**) Control whole-cell voltage-clamp recordings before (top) and after (bottom) application of 4-AP. Right panels: corresponding all-point histograms of 10-s traces before (■) and after (▨) etomidate application. Histograms were fitted with single Gaussian functions, and means were determined. After subtracting baseline from current evoked by etomidate, tonic current was normalized to cell size. (**F**) Data plotted are values from individual neurons and their mean values ± s.e. (control, $n = 14$, 4-AP, $n = 19$, four independent cultures; *$P < 0.05$; *t*-test).

using a biotinylation assay and surface GABA_A receptors were found to rise gradually over time in the presence of 4-AP with a 20.0 ± 2.5% increase in receptor numbers at 5 min. By 30 min GABA_A receptor cell surface expression levels had increased by 46.3 ± 4.2% compared with levels at time 0 (Figure 7A and B). Given an acute increase in neuronal activity promotes the accumulation of new GABA_A receptors at the cell surface, we speculated that phosphorylation of β3S383 by CaMKII might mediate the effects of neuronal activity on the rise in cell surface receptor numbers. To assess the role of β3S383 phosphorylation in activity-dependent changes in cell surface GABA_A receptor numbers, we incubated hippocampal neurons for 0 to 30 min with 4-AP (50 μM) and determined any changes in phosphorylation (Figure 7A). A rapid increase in phosphorylation was observed (51.0 ± 5.6%) following a 5-min incubation with

4-AP which remained stable throughout the 30-min stimulation (Figure 7A and C). To determine if activity-dependent phosphorylation of β3S383 that we had observed in cultured hippocampal neurons occurs in the intact hippocampus, we incubated acute hippocampal slices with 50 μM 4-AP for 2 min. We assayed the level of β3S383 phosphorylation in lysates and observed a 67 ± 12.5% rise in β3S383 phosphorylation (Figure 7D).

In parallel to an increase in GABA_A receptor surface expression, an acute increase in neuronal activity also augmented tonic current. Following the incubation of cultured hippocampal neurons (16–21 div) with 100 μM 4-AP (30 min), the whole-cell current evoked by bath application of etomidate (3 μM) increased by 37.2% from 1.56 ± 0.10 pA/pF in control neurons to 2.14 ± 0.17 pA/pF following application of 4-AP (Figure 7E and F). Collectively, these experiments show

that acutely stimulating neuronal activity promotes the phosphorylation of β3S383 leading to an increase in GABA$_A$ receptor surface expression and an increase in tonic current.

Ca^{2+} influx through L-type VGCCs mediates activity-dependent CaMKII phosphorylation of β3S383 and increases GABA$_A$ receptor surface expression

Stimulating neuronal activity results in an increase in Ca^{2+} influx via a number of different VGCCs. Given that L-type channel activation induces phosphorylation of β3S383 by CaMKII, we assessed the role of L-type VGCC channel activation in modulating activity-dependent phosphorylation of β3S383 and cell surface numbers of GABA$_A$ receptors. Hippocampal neurons (16–21 div) were incubated with or without 4-AP (50 μM) plus the L-type channel blocker nimodipine (10 μM) for 30 min (Figure 8A). Blocking L-type channels with nimodipine prevented the activity-dependent increase in β3S383 phosphorylation (4-AP: 45.0 ± 8.6% increase relative to control; 4-AP plus nimodipine: 6.4 ± 3.2% decrease relative to control; Figure 8A) and the increase in cell surface levels of GABA$_A$ receptors (4-AP: 40.33 ± 5.9% increase relative to control; 4-AP plus nimodipine: 3.7 ± 8.0% decrease relative to control; Figure 8A). These experiments underscore the critical importance of Ca^{2+} influx through L-type channels in mediating the activity-dependent increase in GABA$_A$ receptor cell surface expression.

Next, we determined whether CaMKII was responsible for the activity-dependent phosphorylation of β3S383 by using the CaMKII inhibitor KN-93. In these experiments, hippocampal neurons (16–21 div) were incubated with or without 4-AP (50 μM) plus KN-93 (4 μM) for 30 min (Figure 8B). KN-93 was added 1 min prior to application of 4-AP. Cell surface GABA$_A$ receptors were isolated and we found that blocking CaMKII activity with KN-93 prevented the activity-dependent increase in β3S383 phosphorylation (4-AP: 51.0 ± 3.7% increase relative to control; 4-AP plus KN-93: 12.7 ± 1.76% decrease relative to control; Figure 8B) and cell surface numbers of GABA$_A$ receptors (4-AP: 43.0 ± 5.4% increase relative to control; 4-AP plus KN-93: 11.0 ± 5.5% decrease relative to control; Figure 8B).

To assess whether β3S383 phosphorylation is critical for the activity-dependent increase in cell surface numbers of GABA$_A$ receptors, hippocampal neurons were nucleofected with [pHBBS]β3WT and [pHBBS]β3S383A (16–21 div) and were incubated with 4-AP (50 μM) for 30 min. Immunofluorescence labelling of the cell surface population of [pHBBS]β3WT and [pHBBS]β3S383A (Figure 8C and D) revealed that stimulating neuronal activity resulted in an increase in [pHBBS]β3WT surface expression of 24.0 ± 2.1% (Figure 8C) with no significant change in [pHBBS]β3S383A surface expression (7.0 ± 3.0% increase relative to control; Figure 8D). Combined, these findings provide direct evidence that the activity-dependent increase in cell surface numbers of GABA$_A$ receptors is dependent on Ca^{2+} influx through L-type channels and phosphorylation of β3S383 by CaMKII.

Blocking neuronal activity reduces phosphorylation of β3S383 and GABA$_A$ receptor cell surface expression

The number of synaptic GABA$_A$ receptors and the strength of GABAergic neurotransmission in dissociated cultured neurons are modulated in an adaptive response to chronic activity blockade, lasting 24 h or more (Rutherford *et al*,

1997; Kilman *et al*, 2002; Swanwick *et al*, 2006; Saliba *et al*, 2007). Since augmenting neuronal activity in cultured neurons enhances phosphorylation of β3S383 we asked whether blockade of neuronal activity has an opposite effect on β3S383 phosphorylation and cell surface numbers of GABA$_A$ receptors. To test this hypothesis, we incubated hippocampal neurons (16–21 div) with 2 μM Tetrodotoxin (TTX) for 30 and 60 min. Immunoblotting lysates with anti-p-S383 IgGs revealed that phosphorylation of β3S383 decreased by 20.5 ± 1.5% at 30 min and by 30.4 ± 2.8% at 60 min (Figure 9A and B) following activity blockade with TTX. Given activity blockade leads to a reduction in β3S383 phosphorylation, we next examined whether an accompanying decrease in the cell surface expression of GABA$_A$ receptors occurs, by incubating hippocampal neurons with TTX (2 μM) for 30 and 60 min. Cell surface levels of GABA$_A$ receptors were determined using a biotinylation assay and we observed a 28 ± 3.2% decrease in cell surface expression of GABA$_A$ receptor β3 subunits following activity blockade for 30 min and a 35.7 ± 2.3% decrease at 60 min (Figure 9A and C).

To determine the significance of β3S383 phosphorylation in regulating cell surface numbers of GABA$_A$ receptors following activity blockade, we incubated hippocampal neurons (16–21 div) expressing [pHBBS]β3WT and [pHBBS]β3S383A with TTX (2 μM) for 60 min. We observed a 29.2 ± 4.1% decrease in cell surface [pHBBS]β3WT expression levels following activity blockade (Figure 9D) but observed no significant change in [pHBBS]β3S383A surface expression (6 ± 4.4% increase relative to control; Figure 9E). Collectively, these results indicate that blocking neuronal activity results in a reduction in CaMKII-dependent phosphorylation of β3S383, which is accompanied by a decrease in the cell surface expression of GABA$_A$ receptors.

Discussion

This body of work has revealed a molecular mechanism regulating GABA$_A$ receptor insertion, which is mediated by Ca^{2+} influx through L-type channels. Activation of L-type VGCCs leads to phosphorylation of β3S383 by CaMKII, which facilitates the accumulation of GABA$_A$ receptors assembled with α5/β3 subunits at the cell surface and enhances tonic current. Furthermore, our studies have revealed important insights into the mechanisms underlying activity-dependent insertion of GABA$_A$ receptors. Our findings suggest that acute changes in neuronal activity lead to the rapid modulation of cell surface GABA$_A$ receptor numbers and tonic current, which is mediated by Ca^{2+} influx through L-type channels and CaMKII phosphorylation of β3S383.

The use of a phospho-specific antibody has allowed us to assess the changes in β3S383 phosphorylation in response to Ca^{2+} influx through L-type channels and has enabled us to demonstrate that β3S383 is phosphorylated under basal conditions in cultured hippocampal neurons and acute hippocampal slices. Furthermore, we were able to show that acute changes in neural activity is accompanied by alterations in the levels of β3S383 phosphorylation by CaMKII which is mediated by Ca^{2+} influx through L-type channels. These findings are in agreement with a previous report showing that depolarization induces phosphorylation of β3S383 by CaMKII using recombinant GABA$_A$ receptors expressed in a
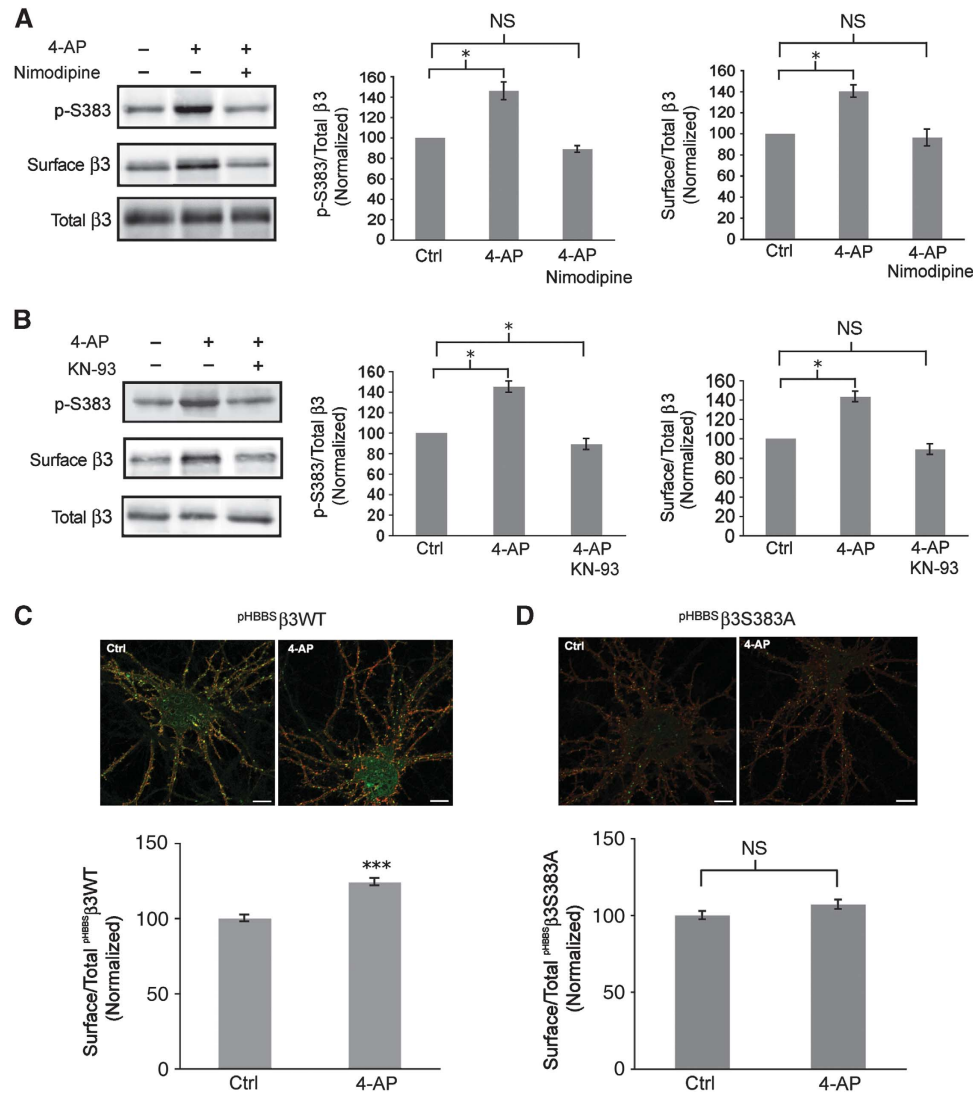
Figure 8 Activation of L-type VGCCs and CaMKII phosphorylation of β3S383 are essential for the activity-dependent increase in surface GABA$_A$ receptor expression. (**A**) Hippocampal neurons (16–21 div) were incubated with or without 4-AP plus nimodipine. Immunoblots show p-S383, surface and total β3. First graph represents p-S383/total β3 band intensity ratio normalized to control (Ctrl) values (data are plotted as mean ± s.e. *$P < 0.05$, NS; not significantly different, $n = 3$, t-test). Second graph represents surface β3/total β3 band intensity ratio normalized to control (Ctrl) values (*$P < 0.05\%$, NS: not significantly different, $n = 3$, t-test). (**B**) Hippocampal neurons (16–21 div) were incubated with or without 4-AP plus KN-93 as indicated. Immunoblots show p-S383, surface and total β3. First graph represents p-S383/total β3 band intensity ratio normalized to control (Ctrl) values (*$P < 0.05$, NS; not significantly different, $n = 3$, t-test). Second graph represents surface β3/total β3 band intensity ratio normalized to control (Ctrl) values (*$P < 0.05$, NS; not significantly different, $n = 3$, t-test). (**C, D**) Nucleofected neurons expressing ^{pHBBS}β3WT (**C**) and ^{pHBBS}β3S383A (**D**) were incubated with or without 4-AP. Cell surface ^{pHBBS}β3WT and ^{pHBBS}β3S383A subunits are labelled red. The pHluorin signal (green) represents total ^{pHBBS}β3WT and ^{pHBBS}β3S383A expression (scale bar 10 μm). Graphs represent cell surface/total ratio of ^{pHBBS}β3WT and ^{pHBBS}β3S383A fluorescence intensity as indicated, normalized to control (Ctrl) values (***$P < 0.001$; NS: not significantly different, $n > 20$ neurons; t-test).

neuronal cell line (Houston and Smart, 2006). In addition, we have shown that phosphorylation of β3S383 is critical for the activity-dependent changes in surface GABA$_A$ expression as revealed by our experiments with β3S383A.

By co-expressing a dominant-negative form of CaMKII (CaMKIIK42R) with the β3 subunit in hippocampal neurons, we have shown that this kinase plays an important role in regulating cell surface expression of GABA$_A$ receptors. Importantly, CaMKIIK42R had no influence on the surface expression of β3S383D, indicating that the effects of dominant-negative CaMKII on GABA$_A$ receptor expression rely solely on β3S383 phosphorylation. These findings are consistent with a report showing that expression of dominant

negative CaMKIIK42R in cerebellar granule cells reduced the amplitude of sIPSCs (Houston *et al*, 2008) further supporting the model that CaMKII regulates GABA$_A$ receptor surface numbers and GABAergic neurotransmission.

Another example of phosphorylation regulating the insertion of GABA$_A$ receptors involves insulin-induced delivery of receptors to the cell surface (Wang *et al*, 2003b). In this study, the authors reported that phosphorylation of S410 within the intracellular loop of the GABA$_A$ receptor β2 subunit by protein kinase B (Akt) is critical for insulin-induced insertion of receptors. In our studies, we have shown using a combination of techniques to demonstrate that that Ca^{2+} influx through L-type channels and CaMKII phosphorylation
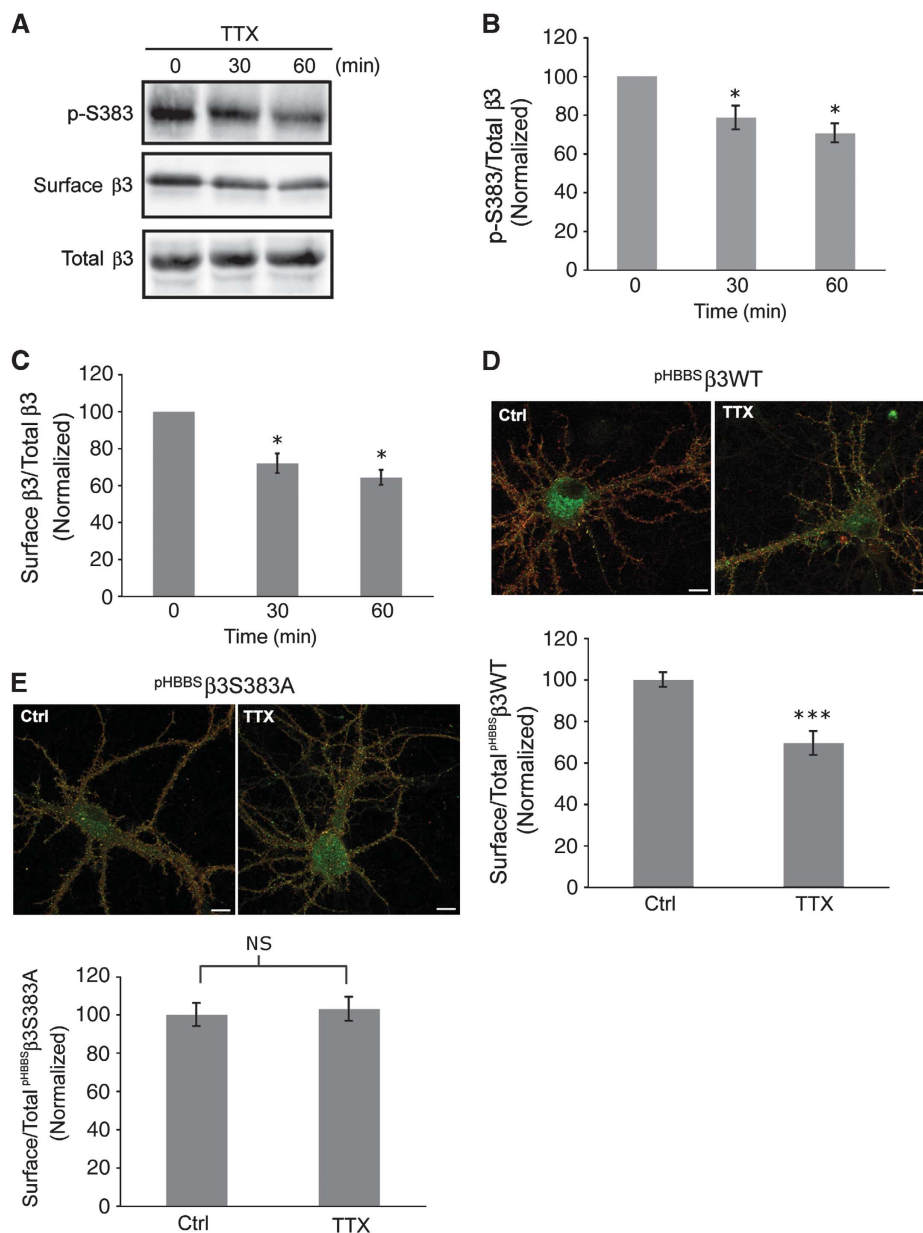
Figure 9 Acute blockade of neuronal activity reduces phosphorylation of β3S383 and cell surface numbers of GABA$_A$ receptors. (**A**) Hippocampal neurons were incubated with TTX for the times indicated. Immunoblots show p-S383, surface and total β3. (**B**) Quantification of p-S383/total β3 band intensity ratio normalized to values at time 0 (data are plotted as mean ± s.e., *$P<0.05$; $n=3$; t-test). (**C**) Quantification of cell surface β3/total β3 band intensity ratio normalized to values at time 0 (*$P<0.05$; $n=3$; t-test). (**D, E**) Hippocampal neurons expressing ^{pHBBS}β3WT (**D**) and ^{pHBBS}β3S383A (**E**) were incubated with TTX for 60 min. Cell surface ^{pHBBS}β3WT and ^{pHBBS}β3S383A subunits are labelled red and the pHluorin signal (green) represents total ^{pHBBS}β3WT and ^{pHBBS}β3S383A expression (scale bar 10 μm). Graphs represent quantification of cell surface/total ^{pHBBS}β3WT and ^{pHBBS}β3S383A fluorescence intensity ratios, as indicated, normalized to control (Ctrl) values (***$P<0.001$; NS; not significantly different, $n>20$; 3 independent cultures; t test).

of β3S383 facilitates GABA$_A$ receptor insertion without influencing receptor endocytosis.

Our findings with the β3S383 mutants that abolish or mimic phosphorylation further support a model whereby phosphorylation of β3S383 is critically involved in regulating the number of GABA$_A$ receptors at the cell surface and tonic current. Furthermore, as the total expression levels of β3S383A and β3S383D are different, this may suggest that phosphorylation of β3S383 may also regulate the endoplasmic reticulum-associated degradation of β3.

The activity-dependent increase in β3S383 phosphorylation and cell surface numbers of GABA$_A$ receptors was completely blocked with nimodipine. These findings strongly suggest that activation of L-type channels is necessary and sufficient to mediate the effects of increased neuronal activity on β3S383 phosphorylation and the numbers of cell surface GABA$_A$ receptors. These observations may also imply that under these experimental conditions, activation of synaptic NMDA receptors appear to have no influence on the forward trafficking of GABA$_A$ receptors.

Recently, it has been reported that an increase in the diffusion coefficient of quantum dot labelled GABA$_A$ receptor molecules occurs within minutes following stimulation of neurons with either 4-AP or NMDA with an accompanying

reduction in the amplitude of miniature IPSCs (Bannai *et al*, 2009). In a recent complimentary study, activation of NMDA receptors causes a rapid increase in surface mobility of individual GABA_A receptors and a rapid dispersal of synaptic receptor clusters (Muir *et al*, 2010). These observations were dependent on dephosphorylation of S327 within the $\gamma 2$ subunit of the GABA_A receptor by calcineurin. In the report by Bannai *et al*, the authors also assessed cell surface numbers of synaptic GABA_A receptors using immunofluorescence labelling, after a 2-h incubation with 4-AP and observed a substantial decrease in synaptic receptor expression. The length of time used in stimulating neural activity is an important factor to consider when assessing changes in cell surface numbers of GABA_A receptors. In our experiments, we used 4-AP for shorter periods (0–30 min) before assaying cell surface expression of GABA_A receptors. However, in agreement with Bannai *et al*, 2009 we also observed a decrease in GABA_A cell surface expression following a 2-h incubation with 4-AP (unpublished observations). In another study activation of NMDA receptors (for 3 min) in a cell culture model of LTD of excitatory synapses (Marsden *et al*, 2007), GABA_A receptor cell surface insertion and expression were reported to increase under these experimental conditions. In this report, however, surface GABA_A receptor numbers were assayed following a 15-min recovery period after NMDA application. It is plausible that during this recovery period, an increase in neuronal activity would be expected, as a result of prior NMDA application leading to activity-dependent activation of L-type VGCCs. In fact, our work shows that a 5-min application of NMDA to neuronal cultures induces a rapid and almost complete dephosphorylation of $\beta 3$S383 by calcineurin. This is in agreement with previous studies showing that NMDA receptor activation promotes dephosphorylation of other GABA_A receptor subunits by calcineurin (Lu *et al*, 2000; Wang *et al*, 2003a; Muir *et al*, 2010). Thus, it appears that activity-dependent regulation of GABA_A receptor insertion is critically dependent on experimental conditions and dependent on the length of time that neuronal activity is stimulated.

Changes in CaMKII-dependent phosphorylation of $\beta 3$S383 may have important implications for the understanding of certain neurological disorders. For example, it has been reported in a number of studies that CaMKII expression or activity is reduced in a series of *in-vivo* and *in-vitro* models of epilepsy (Delorenzo *et al*, 2005). Interestingly, induction of epileptiform activity in cultured hippocampal neurons exposed to low Mg^{2+} results in a marked reduction in $\beta 3$S383 phosphorylation and cell surface expression of GABA_A receptors when assayed 24 h later (unpublished observations). Furthermore, the neurodevelopmental disorder Angelman's syndrome has been linked to compromised CaMKII activity (Weeber *et al*, 2003) in a mouse model of the disorder (Jiang *et al*, 1998) and mice lacking the GABA_A receptor $\beta 3$ subunit express many of the characteristics of Angelman's syndrome and develop epilepsy (DeLorey *et al*, 1998). Thus, it is tempting to speculate that reduced CaMKII phosphorylation of $\beta 3$S383 may be responsible for some of the phenotypic traits observed in Angelman's syndrome.

In addition to synaptic scaling of the strength of excitatory and inhibitory synaptic transmission, the intrinsic excitability of neurons (i.e., voltage-dependent conductances) can also be altered in response to changes in neuronal activity, which represents an additional homeostatic mechanism (Nelson and Turrigiano, 2008). Evidence shows that low concentrations of ambient GABA can continuously activate extrasynaptic GABA_A receptors, to produce an inhibitory tonic conductance (Farrant and Nusser, 2005), therefore regulating the intrinsic excitability of a neuron (Mody, 2005). As our studies have shown that phosphorylation of $\beta 3$S383 can be regulated by acute bi-directional changes in neuronal activity leading to the modulation of GABA_A receptor expression and tonic current, this pathway seems to represent a compensatory homeostatic mechanism maintaining a normal level of intrinsic excitability in response to changes in activity. Finally, the role of extrasynaptic GABA_A receptors in a number of neuropsychiatric diseases is becoming apparent (Brickley and Mody, 2012) and understanding the cellular mechanisms, which modulate tonic current will benefit this expanding field.

Materials and methods

Reagents and antibodies
4-AP, TTX, Bay K 8644, nimodipine and Tubocurarine were purchased from Tocris. Rabbit polyclonal anti-$\beta 3$-specific IgGs and anti-phospho $\beta 3$S408/9 have been described previously (Jovanovic *et al*, 2004). Rabbit polyclonal anti-GFP IgGs were obtained from Synaptic Systems. Secondary peroxidase-conjugated IgGs and secondary Rhodamine Red-X-conjugated IgGs were from Jackson Immuno-Research Laboratories and Alexa-594 α-bungarotoxin was purchased from Invitrogen.

Anti-phosphorylation $\beta 3$S383-specific IgGs and immunoblot analysis
The phospho-peptide CQYRKQSpMPKEG was synthesized and purchased from Alpha Diagnostic International and Cocalico Biologicals raised rabbit anti-sera to CQYRKQSpMPKEG conjugated to keyhole limpet haemocyanin (KLH) from Pierce. Please also see Supplementary data.

Constructs
The GABA_A receptor $\beta 3$ subunit tagged with pHluorin and the BBS has been described previously (Bogdanov *et al*, 2006; Saliba *et al*, 2007). For the generation of ^{pHBBS}S383A and ^{pHBBS}S383D constructs, please see Supplementary data.

Neuronal cell culture, nucleofection and transfection
Hippocampal neurons were obtained as described previously (Saliba *et al*, 2009). Please also see Supplementary data.

Biotinylation
Neurons were biotinylated as described previously (Saliba *et al*, 2007). Please also see Supplementary data.

Immunocytochemistry
Neurons were immunolabelled as described previously (Saliba *et al*, 2009). Please also see Supplementary data.

GABA_A receptor $^{pHBBS}\beta 3$WT insertion Assay
Insertion assays were performed as described previously (Saliba *et al*, 2009). Please also see Supplementary data.

Hippocampal slice preparation
Slices were prepared from C57Bl6 male mice 8–12 weeks old (Terunuma *et al*, 2008). Please also see Supplementary data.

Electrophysiology

Hippocampal neurons were either nucleofected with pHluorin-tagged constructs ([PHBBS]β3WT, [PHBBS]β3S383A and [PHBBS]β3S383D) or not nucleofected and plated on 12-mm glass coverslips (German glass; VWR, Bridgeport, NJ) coated with poly-L-lysine (2 mg/ml; Sigma, St. Louis, MO) and cultured for 2–3 weeks prior to taking recordings. To measure tonic current, coverslips were placed in recording chamber mounted on the stage of inverted microscope and continuously perfused with extracellular solution of the following composition (in mM): NaCl 150, KCl 4.7, CaCl$_2$ 2.5, MgCl$_2$ 1.2, HEPES 10 and glucose 11, adjusted to pH 7.4 by NaOH, osmolality 295–315 mmol/kg. Excitatory and action potentials were blocked by addition of 10 μM DNQX, 25 μM D-AP5 and 0.3 μM TTX (Tocris Bioscience, Ellisville, MO). The bath solution was heated to 32–33°C by in-line heater (Warner Instruments, Hamden, CT). Patch pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) and filled with intracellular solution containing (in mM): CsCl 150, MgCl$_2$ 2, CaCl$_2$ 0.1, HEPES 10, EGTA 1.1, Mg-ATP 2, adjusted to pH 7.2 by CsOH, osmolality 275–290 mmol/kg. For neurons nucleofected with [PHBBS]β3 constructs (Wt, S383A and S383D), the intracellular concentration of chloride ions was lowered to 29.2 mM by replacing 125 mM of CsCl by Cs-methanesulfonate. After establishing whole cell, period of 3 min was left to stabilize recordings prior collecting data. Recordings were made at a holding potential of − 70 mV. The currents were recorded using Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at to 2 kHz, digitized (10 kHz; Digidata 1320A; Molecular Devices) and stored on computer for off-line analysis. Access resistance (<15 MΩ; 65–75% compensation) was monitored throughout the recordings and data from the cell were discarded when change >20% occurred. For tonic current measurements, an all-points histogram was plotted for a 10-s period immediately before and during application of either inhibitors (picrotoxin, bicuculline) or positive allosteric modulators (flurazepam, etomidate) of GABA$_A$ receptors. Tonic currents are represented as the change in baseline amplitude.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Conflict of interest

The authors declare that they have no conflict of interest.

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