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Activity dependent phosphorylation of $GABA_A$ receptors regulates receptor insertion and tonic current

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19 January 2012

Thank you for submitting your manuscript to the EMBO Journal. I have now received the comments from the 3 referees who I had asked to review the paper.

As you can see below, the referees appreciate the insight gained into the regulation of tonic inhibition and the involvement of activity-dependent GABA Receptor phosphorylation in this process. They do raise a number of technical concerns that have to be addressed. Referee #3 also finds that further data in support of the physiological relevance of the described mechanism would be needed for publication here as well as some more causal data to support some of the molecular links. Given the comments provided, I would like to offer to consider a revised manuscript should you be able to address the raised issues in full. I should point out that further physiological relevant data to support the key conclusions of the paper is needed for further consideration here. I should add that it is EMBO Journal policy to allow a single round of revision only and that it is therefore important to address the points raised at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The activity-dependent regulation of GABAA receptor function is a major factor in maintaining the excitatory/inhibitory balance. GABA-A receptors are substrates for CaMKII and the resulting beta3 subunit phosphorylation (S383) is known to be involved in enhancing GABA currents by prolonging IPSCs duration (Houston et al. 2006, 2007, 2009). It was hypothesized that this effect is based on an enhanced GABA-A receptor surface expression.

In a comprehensive study on alpha5 GABAA receptors, the Moss-group now shows that the L-type VGCC-dependent Ca-influx in cultured hippocampal neurons and the CaMKII-dependent increase in beta3S383 phosphorylation leads to an enhanced receptor surface expression (without affecting endocytosis) and thereby enhances tonic GABAergic inhibition.

The experimental evidence demonstrates the selectivity of L-type VGCC activation, the relevance of CaMKII activation (using an inactive CaMKII 42R), the selective targeting of the beta3S383 phosphorylation (using phospho-null mutant beta3S383A and phospho-mimic beta3S383D), the kinetics of receptor insertion, the lack of a contribution from endocytosis, the presence of the gamma2 subunit in the relevant receptors (flurazepam stimulation) and the bidirectional modulation of receptor expression by either enhancing or blocking neural activity. The experiments are very well performed, conclusive and convincing.

The following points need further consideration:

1. The present experimental evidence relates to receptors containing the beta3 subunit as target of CaMKII. The title should therefore be adapted to indicate this fact ("...beta3 GABA-A receptor...").
2. p. 9 last line, Fig. 5: The authors claim that the activity-dependent enhancement of receptor expression includes receptors containing the alpha1, alpha2 or alpha3 subunit. There is no experimental evidence to support this claim in particular as some of these receptors largely contain beta2 subunits and would affect phasic inhibition. However, phasic inhibition was not measured nor was alpha1, alpha2 or alpha3 surface expression determined.
3. Apart from GABA-A receptor containing the alpha5 subunit, receptors containing the delta subunit also contribute to tonic inhibition in hippocampal pyramidal neurons. These receptors are neither mentioned nor considered.
4. In experiments in which CaMKII is stimulated, targets other than beta3S383 are relevant e.g. Ser410 in the beta2 subunit. Indeed, in cerebellar granule cells, a beta2-dependent CaMKII mediated increase in IPSC amplitude was observed (Houston et al. 2008). It would therefore be of interest to determine the potential CaMKII-dependent surface expression of hippocampal beta2 containing receptors as a complement to beta3 receptors.
5. The potentiating effect of CaMKII on GABAA receptors are, in part, mediated by activation of a tyrosine kinase targeting the gamma2 subunit (tyr 365, 367). The potential contribution of this pathway is not addressed.
6. Fig. 4d: Inhibiting endocytosis (in the absence of neural stimulation) would be expected to enhance receptor surface expression per se. The respective data should be given.
7. p. 16 para 2: As the authors showed in an earlier study (Saliba et al. 2009), long term blockade of L-typ VGCC reduces beta3S383 phosphorylation and GABAA receptor surface expression. A deeper discussion of the apparently inverse regulatory mechanisms over time would be welcome.
8. p. 16, line 23: "activation of L-type VGCCs" should presumably read "activity at L-type VGCC".
9. Fig. 1C: Why was etomidate chosen to test tonic inhibition and not a more selective drug e.g. alpha5 partial inverse agonist L-655708.

Referee #2

In this work, the authors examined the role of activity-dependent phosphorylation of γ-aminobutyric

acid (GABA) receptors in tonic inhibition. Using immunoblotting, immunostainings and electrophysiological recordings in cultured rat hippocampal neurons, they showed that the activity-dependent activation of L-type voltage gated Ca2+ channels (VGCCs) results in phosphorylation of serine 383 (S383) of the GABAA receptor β3 subunit by the Ca2+/calmodulin-dependent protein kinase II (CamKII). Phosphorylation of β3S383 leads to rapid insertion of GABAA receptors at the cell surface as demonstrated by biotinylation experiments and immunostainings. As a functional consequence, the amplitude of GABAergic tonic currents increased after the activation of CamKII. While much of the present experimental data support the authors´ interpretation, several major and minor concerns should be addressed.

Major

1. The present study corroborates and extends previous findings, but it should become more clear to the reader (e.g., in the abstract) which essential parts of the proposed mechanism (e.g., identification of β3S383 as the CamKII phosphorylation site) have been described earlier. Some of the findings have been already reviewed by one of the authors and others (Houston et al., 2009, J Physiol 587.10: 2115; Vithlani et al., 2011, Physiol Rev 91: 1009).

2. Fig. 1A shows a strong increase in the intensity of the β3 signal in the biotinylated (surface) fraction. Was a corresponding decrease detectable in the total protein fraction? Can the authors comment on the surface/intracellular protein ratio (with and without Bay K 8644)?

3. It is entirely possible that exposure of the culture to Bay K 8644 induces changes in network activity in addition to the direct effect on L-type VGCCs. That activity-induced changes in the surface expression of the GABAA receptors take place has been convincingly demonstrated in this work. Hence, the experiments in Fig. 1A (and all similar tests) should be carried out in the presence of TTX. On the other hand, nimodipine (and KN-93) may have an effect on network activity in the 4-AP experiments (Fig. 7). Therefore, proper control experiments are needed also in these experiments. Recording and quantifying the activity of the neurons under the various experimental conditions where TTX is not applied (using e.g., Ca2+ imaging) is probably the only way to find out which drug effects are direct and which are based on activity-dependent mechanisms. This confounder is a very serious one because the authors show that even under control conditions, neuronal activity has an influence on GABAAR surface expression (Fig. 8).

4. The authors should discuss the finding that the β3 subunit is primarily expressed at synaptic sites (Herd et al., 2008, J Physiol 586.4: 989).

5. No reference or explanation is provided for the use of etomidate in activating tonic IGABA. What is the evidence that the tonic current does not have an artifactual, drug-induced component (based on changes in the properties of sub-synaptic GABAaRs)?

6. The characterization of β3S383A and β3S383D shown in Fig. 5A and B might become part of Fig. 3. Why was the surface/total ratio shown in the previous figures omitted here?

7. All the data are on α5 and α3 subunits. How do the authors exclude the possibility that trafficking of other subunits is increased as well? Surface expression data for at least the α1 and γ2 subunits is needed to support the idea of a selective effect. Notably, Fig. 6D shows an increase in synaptic GABAAR responses.

8. Was pHluorin always indirectly detected using the anti-GFP antibody or was the pHluorin fluorescence used in some experiments for the normalization of the surface expression to the total protein level? Wouldn´t the authors expect that changes in the surface expression affect the intensity of the pHluorin fluorescence (leading to an underestimation of the effect)?

9. The effect of the dynamin inhibitory peptide (DIP) on surface β3 under control conditions should be shown in Fig. 4 (and not only the effect of Bay K 8644 in the presence of the DIP).

10. What was the liquid junction potential for the two pipette filling solutions? Were corrections done for the holding potential, -70 mV?

11. The development and use of the phosphorylation site-specific anti-P-S383 antibody is crucial for the present manuscript. A proper characterization of the new antibody is essential for the interpretation of the results obtained using this antibody. Therefore, this referee recommends including data shown in supplementary figure 1 in figure 2.

12. It is stated on p. 28 that immunostainings were analyzed blind to experimental conditions. Is this true for the quantification of immunblots, too? Can the authors comment on the difference in the amplitude of the Bay K 8644 effect between biotinylation experiments (Fig. 1) and immunostaings (Fig. 2)?

13. How do the authors explain the decrease in GABAA receptor surface expression observed after a two-hour incubation with 4-AP as stated in the discussion? Did the phosphorylation state of β3S383 change accordingly (i.e., decrease) after prolonged 4-AP incubation?

Minor
- The β3S383A mutant is introduced on p. 6. Therefore, it sounds odd to start on p. 9 with "We generated a phospho-null (β3S383A)...". Please rephrase. It might be also worth citing articles in which the β3S383A has been used before.
- Why was keeping the access resistance within 20% change important? Please provide data on the input R of the neurons and preferably also for the resting Vm before going to voltage clamp.
- The electrophysiology has been done at 32-33 C. What was the temperature in the other types of experiments (p. 28)?
- Why is the specimen recording in Fig. 5D cut much earlier than the other traces?
- Were the cells pre-incubated in the presence of KN-93 or was KN-93 applied together with Bay K 8644 as stated on p. 6?
- Fig. 4C: the P-S383 signal is shown in the figure, but it is not addressed in the figure legend or the text (e.g., with a reference to Fig. 2, where the same effect has been shown).
- p. 16, last paragraph: Much more is known about the functions of tonic IGABA than its role in regulating intrinsic neuronal excitability. Please check more recent papers by Mody and others.
- In most sentences with "tonic inhibition", "tonic current" is the correct expression
- The mode of action of Bay K 8644 should be explained to the reader (gating of L-type VGCCs).
- The specimen recordings in Fig. 1C are not representative of etomidate action, because the effect shown is much larger than the mean change in Fig. 1D. The same applies for Figs. 6D,E.
- That Lipofectamine was obtained from Invitrogen does not belong to the Results section (p. 7).
- The CaMKII KO has probably a wide spectrum of defects, and therefore the 1st paragraph on p. 16 could be made much briefer.
- Abbreviations should be introduced (e.g., CamKII, KLH).
- p. 3: GABAA receptors are not Cl- selective
- p. 4: Figureure
- The readability of the figure legends would improve if the authors would state only once that "data are plotted as mean {plus minus} S.E.".
- antibody vs anti-body
- p-S383 vs P-S383 (Fig. 6)

Referee #3

In this elegant paper, the authors have used a plethora of techniques to dissect the links between activity and phosphorylation and activity of GABAergic receptors and signalling. The topic is obviously important considering the interest in understanding how activity alters the operation of GABA and by this mean also the operation of brain networks in physiological and pathological situations. In spite of these elements,; the study falls short of showing the relevance of these mechanisms in more physiologically relevant situations and whether and how they occur outside the cellular cultures and artificial situations associated with it.

Main issues

i) what happens in vitro in slices and possibly in vivo? Is any of these events possible and can it be observed?

ii) too many speculative correlations, thus the effect of L-type Ca activation of Alpha5 (a5) and beta 3 is just correlation no causal link here

ii) B3 is a component of synaptic GABA receptors. The paper is written in a way that it is easy to think that the effect is specific to tonic currents but this is not shown and ought to be tested
iii) The data in fig 6 show nicely that there is an activity dependent increase in phosphorylation of Beta 3 and increase in tonic currents . Unfortunately the causal link between these two events is not demonstrated. The authors should show this by repeating the experiment in neurons over expressing the s383a mutant.

iv) Both the mutant S383A and S383D have a significant effect on the total protein expression . The significant effect of the mutations could be on posttranslational processing of the protein and thus could affect the interpretation of the demonstration of specific effects on plasma membrane trafficking . This should be discussed in the paper.

In general, the discussion relies on too many assumptions that are not demonstrated causally. More care should be exerted unless the authors find a way to provide direct evidence that synaptic currents and not only exogenous GABA currents are affected preferably with adequate transynaptic stimuli... the shunting actions of GABA are ignored but heavily impact the relevance of the conclusions.

Details and technical
i) As there is a robust time-dependent effect of TTX on Beta 3 surface then special care should be taken to perform the electrophysiological recordings within the same time frame.(e.g. the amplitude of tonic current will depend on the time in blocker mix of excitatory transmission). The time frames use should be given in the methods.
ii) There is a recent paper on role of beta3 on tonic currents by Janssen et al Front. Cell. Neurosci (2011) 5:15.
iii) The effect of S383A and S383Dv mutation on Bay K 8644 a5 surface increase deserves more stringent demonstration
iv) page 9 the s383a and s383d are introduced as new but they were used in previous portion of the paper

1st Revision - authors' response 28 February 2012

Replies to the Referees comments

Referee #1

The activity-dependent regulation of GABAA receptor function is a major factor in maintaining the excitatory/inhibitory balance. GABA-A receptors are substrates for CaMKII and the resulting beta3 subunit phosphorylation (S383) is known to be involved in enhancing GABA currents by prolonging IPSCs duration (Houston et al. 2006, 2007, 2009). It was hypothesized that this effect is based on an enhanced GABA-A receptor surface expression.
In a comprehensive study on alpha5 GABAA receptors, the Moss-group now shows that the Ltype VGCC-dependent Ca-influx in cultured hippocampal neurons and the CaMKII-dependent increase in beta3S383 phosphorylation leads to an enhanced receptor surface expression (without affecting endocytosis) and thereby enhances tonic GABAergic inhibition.
The experimental evidence demonstrates the selectivity of L-type VGCC activation, the relevance of CaMKII activation (using an inactive CaMKII 42R), the selective targeting of the beta3S383 phosphorylation (using phospho-null mutant beta3S383A and phospho-mimic beta3S383D), the kinetics of receptor insertion, the lack of a contribution from endocytosis, the presence of the gamma2 subunit in the relevant receptors (flurazepam stimulation) and the bidirectional modulation of receptor expression by either enhancing or blocking neural activity.
The experiments are very well performed, conclusive and convincing.

The following points need further consideration:

1. The present experimental evidence relates to receptors containing the beta3 subunit as target of CaMKII. The title should therefore be adapted to indicate this fact ("...beta3 GABA-A

receptor...").
This has been amended.

2. p. 9 last line, Fig. 5: The authors claim that the activity-dependent enhancement of receptor expression includes receptors containing the alpha1, alpha2 or alpha3 subunit. There is no experimental evidence to support this claim in particular as some of these receptors largely contain beta2 subunits and would affect phasic inhibition. However, phasic inhibition was not measured nor was alpha1, alpha2 or alpha3 surface expression determined.
This sentence has been changed.

3. Apart from GABA-A receptor containing the alpha5 subunit, receptors containing the delta subunit also contribute to tonic inhibition in hippocampal pyramidal neurons. These receptors are neither mentioned nor considered.
It is possible that the delta subunit surface expression may change in response to changes in activity as this subunit assembles with the β3 subunit. The expression of the delta subunit is restricted to granule cells of the dentate gyrus, were as the α5 subunit is responsible for tonic inhibition in pyramidal cells (Caraiscos et al. PNAS 2004; Bonin et al., J Neurophysiology 2007, Cheng et al., J Neuroscience 2006). In our cultures pyramidal cells predominate, and the delta subunit in our cultures is expressed at low levels making this study technically challenging.

4. In experiments in which CaMKII is stimulated, targets other than beta3S383 are relevant e.g. Ser410 in the beta2 subunit. Indeed, in cerebellar granule cells, a beta2-dependent CaMKII mediated increase in IPSC amplitude was observed (Houston et al. 2008). It would therefore be of interest to determine the potential CaMKII-dependent surface expression of hippocampal beta2 containing receptors as a complement to beta3 receptors.
The focus of this paper is the role that CaMKII downstream of voltage gated Ca+2 channels plays in regulating the activity of GABAAR subtypes containing the β3 subunit. There are numerous other potential CaMKII phosphorylation sites in other GABAAR subunits including S410 in the β2 subunit and within the γ2 subunit. Here we have focused on phosphorylation of the β3 subunit because it is the major β subunit isoform in the hippocampus. Furthermore β2 expression in the hippocampus is comparatively very low (Sperk *et al*., 1997). Clearly and exhaustive analysis of all CaMKII phosphorylation sites within GABAARs is outside the scope of this current manuscript.

5. The potentiating effect of CaMKII on GABAA receptors are, in part, mediated by activation of a tyrosine kinase targeting the gamma2 subunit (tyr 365, 367). The potential contribution of this pathway is not addressed.
This study is mainly focusing on β3S383 phosphorylation and consequential effects on trafficking of GABAA receptors. As mutation of β3S383 completely blocked the effects of Bay K 8644 and 4-AP on the surface levels of GABAA receptors containing the β3 subunit we believe that phosphorylation of Tyr365/367 play minor roles. Carefully analyzing the roles that phosphorylation of γ2-Tyr365/367 play in regulating the membrane trafficking of GABAARs warrants a separate study.

6. Fig. 4d: Inhibiting endocytosis (in the absence of neural stimulation) would be expected to enhance receptor surface expression per se. The respective data should be given.
This has been demonstrated in at least 3 manuscripts form our laboratory (Kittler et al., 2000; Bogdanov et al., 2006; Jacob et al., 2009) and others. Thus due to space restrictions we have simply cited these papers.

7. p. 16 para 2: As the authors showed in an earlier study (Saliba et al. 2009), long term blockade of L-type VGCC reduces beta3S383 phosphorylation and GABAA receptor surface expression. A deeper discussion of the apparently inverse regulatory mechanisms over time would be welcome.
In our earlier study (Saliba et al., 2009) we did not show that phosphorylation of β3-S383 was reduced. We examined the ER associated degradation of β3 subunits in response to chronic Ltype VGCC blockade. Given that there is basal phosphorylation of S383 and Ca2+ influx through L-type channels increases phosphorylation of this residue, acute blockade of L–type channels would most likely lead to a reduction in phosphorylation of β3S383 and reduced cell surface expression of β3 containing GABAA receptors. This has been briefly discussed in our

revised manuscript in the discussion section.

8. p. 16, line 23: "activation of L-type VGCCs" should presumably read "activity at L-type VGCC".
To clarify L-type channels are voltage dependent.

9. Fig. 1C: Why was etomidate chosen to test tonic inhibition and not a more selective drug e.g. alpha5 partial inverse agonist L-655708.
As this study focuses on β3-S383 phosphorylation and trafficking of β3 containing GABAA receptors we used Etomidate as it activates β2/β3 containing GABAA receptors. Furthermore etomidate has been shown to preferentially enhance tonic current mediated by α5 containing GABAA receptors in cultured hippocampal neurons (Cheng et al., *J. Neurosci)*. We have now explained briefly in the results section of the manuscript our reasons for using etomidate.

Referee #2:

In this work, the authors examined the role of activity-dependent phosphorylation of γ-aminobutyric acid (GABA) receptors in tonic inhibition. Using immunoblotting, immunostainings and electrophysiological recordings in cultured rat hippocampal neurons, they showed that the activity-dependent activation of L-type voltage gated Ca2+ channels (VGCCs) results in phosphorylation of serine 383 (S383) of the GABAA receptor β3 subunit by the Ca2+/calmodulin-dependent protein kinase II (CamKII). Phosphorylation of β3 leads to rapid insertion of GABAA receptors at the cell surface as demonstrated by biotinylation experiments and immunostainings. As a functional consequence, the amplitude of GABAergic tonic currents increased after the activation of CamKII.
While much of the present experimental data support the authors´ interpretation, several major and minor concerns should be addressed.

Major

1. The present study corroborates and extends previous findings, but it should become more clear to the reader (e.g., in the abstract) which essential parts of the proposed mechanism (e.g., identification of β2/3S383 as the CamKII phosphorylation site) have been described earlier. Some of the findings have been already reviewed by one of the authors and others (Houston et al., 2009, J Physiol 587.10: 2115; Vithlani et al., 2011, Physiol Rev 91: 1009).
-The introduction and the results section mention the previous findings that the reviewer has pointed out and due to a word limit in the abstract we are prone to think that it would be difficult to refer to these studies here without reducing the relevant information of our study needed in the abstract.

2. Fig. 1A shows a strong increase in the intensity of the β3 signal in the biotinylated (surface) fraction. Was a corresponding decrease detectable in the total protein fraction? Can the authors comment on the surface/intracellular protein ratio (with and without Bay K 8644)?
-Given that the total protein fraction by definition means cell surface and intracellular populations of β3 receptor subunits, we observed no change in total β3 levels at these time points. However, the intracellular pool would be expected to decrease given that there is an approximately 50% increase in cell surface numbers of β3 receptor subunits following activation of L-type channels for 10 minutes. Also we know that following activation of L–type VGCCs intracellular receptors are rapidly inserted into the neuronal membrane (Figure 5B), thus reducing the intracellular pool.

3. It is entirely possible that exposure of the culture to Bay K 8644 induces changes in network activity in addition to the direct effect on L-type VGCCs. That activity-induced changes in the surface expression of the GABAA receptors take place has been convincingly demonstrated in this work. Hence, the experiments in Fig. 1A (and all similar tests) should be carried out in the presence of TTX. On the other hand, nimodipine (and KN-93) may have an effect on network activity in the 4-AP experiments (Fig. 7). Therefore, proper control experiments are needed also in these experiments. Recording and quantifying the activity of the neurons under the various experimental conditions where TTX is not applied (using e.g., Ca2+ imaging) is probably the

only way to find out which drug effects are direct and which are based on activity-dependent mechanisms. This confounder is a very serious one because the authors show that even under control conditions, neuronal activity has an influence on GABAAR surface expression (Fig. 8).
-Given that the action of Bay K 8644 is dependent on a change in membrane voltage, adding TTX to the experiments will block neuronal activity and thus prevent the voltage dependent action of Bay K 8644 on L-type VGCCs. Additionally, increasing network activity of neuronal cultures with NMDA leads to de-phosphorylation of β3S383 by calcineurin (supplementary figure S1), which implies that the effects of Bay K8644 on β3S383 phosphorylation and cell surface numbers of GABAA receptors are specific and not due to indirect effects of an increase in neuronal activity. Also, if the reviewer is implying that nimodipine will decrease network activity there is evidence to the contrary. Addition of nimodipine increases neuronal activity as shown by Thompson et al., 1990: Brain Res. 1990. Therefore if nimodipine increases activity it would not be expected to block the effects of increased neuronal activity on βS383 phosphorylation and receptor cell surface levels as we have shown.

Thompson LT, Deyo RA, Disterhoft JF. (1990). Nimodipine enhances spontaneous activity of hippocampal pyramidal neurons in aging rabbits at a dose that facilitates associative learning *Brain Res*. **3;535(1):**119-30.

4. The authors should discuss the finding that the β3subunit is primarily expressed at synaptic sites (Herd et al., 2008, J Physiol 586.4: 989).
-The study by Herd et al., 2008 focuses on granule cells in the dentate gyrus and the vast majority of neurons in our cultures are pyramidal cells. This report reveals that tonic inhibition is decreased *but not eliminated* in the dentate gyrus of β2 knockout mice. Thus the remainder of the tonic current in the dentate is likely to be mediated by receptors containing β3 subunits. In pyramidal cells β3 is also localized at extra-synaptic sites (Pirker et al., Neurosci: 2000) and several lines of evidence indicate that most α5 GABAA receptors contain the β3 subunit in hippocampal neurons. These facts are mentioned in the results section of the manuscript where experiments assaying cell surface levels of the α5 subunit are introduced.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, and Sperk G (2000). GABAA receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101: 815-850

5. No reference or explanation is provided for the use of etomidate in activating tonic IGABA. What is the evidence that the tonic current does not have an artifactual, drug-induced component (based on changes in the properties of sub-synaptic GABAaRs)?
-We apologize for this oversight. Etomidate has been shown to preferentially activate tonic currents in a number of other studies (Caraiscos et al., *PNAS*: 2004; Cheng et al., *J. Neurosci*: 2006; Belelli, et al., *J. Neurosci*: 2005; Herd et al., *J. Physiol :* 2008, Seymour et al., J Membr Biol: 2009) and we have cited the relevant papers in the manuscript (mainly Caraiscos et al., 2004, and Cheng et al., 2006) and explained our decision to use etomidate.

Belelli D, Peden DR, Rosahl TW, Wafford KA, Lambert JJ (2005). Extrasynaptic GABAA receptors of thalamocortical neurons: a molecular target for hypnotics**.** J Neurosci. 2005 Dec 14;25(50):11513-20.
Herd MB, Haythornthwaite AR, Rosahl TW, Wafford KA, Homanics GE, Lambert JJ, Belelli D (2008). The expression of GABAA beta subunit isoforms in synaptic and extrasynaptic receptor populations of mouse dentate gyrus granule cells. J Physiol. 586(4):989-1004.
Seymour VA, Everitt AB, Tierney ML. 2009Differential drug responses on native GABA(A) receptors revealing heterogeneity in extrasynaptic populations in cultured hippocampal neurons. J Membr Biol. 2009 Feb;227(3):111-22.

6. The characterization of β3A and β3D shown in Fig. 5A and B might become part of Fig. 3. Why was the surface/total ratio shown in the previous figures omitted here?
-We applogize for any confusion. We observed the same changes in the total population of β3S383A and β3S383D (i.e surface + intracellular pools) therefore no significant changes in the surface/total ratio are evident.

7. All the data are on α5 and α3 subunits. How do the authors exclude the possibility that trafficking of other subunits is increased as well? Surface expression data for at least the α1and γ2 subunits is needed to support the idea of a selective effect.
Notably, Fig. 6D shows an increase in synaptic GABAAR responses.
-The focus of this paper is the role that CaMKII downstream of voltage gated Ca+2 channels plays in regulating the insertion of GABAAR subtypes containing the β3 subunit. There are numerous other potential CaMKII phosphorylation sites in other GABAAR subunits including S410 in the β2 subunit and within the γ2 subunit (tyr 365, 367). Here we have focused on phosphorylation of the β3 subunit because it is the major β subunit isoform in the hippocampus. Furthermore β2 expression in the hippocampus is comparatively very low (Sperk *et al*., 1997). Clearly and exhaustive analysis of all CaMKII phosphorylation sites within GABAARs is outside the scope of this current manuscript.
Notably, Fig. 6D shows an increase in synaptic GABAAR responses.
-Previous papers showing sIPSCs are influence by Ca2+ influx and CaMKII activity are cited in the introduction. The focus of this paper is on the role that CaMKII phosphorylation of β3S383 plays in regulating the tonic current, and simply replicating the work of others will not, we believe, enhance the impact of this current study.

8. Was pHluorin always indirectly detected using the anti-GFP antibody or was the pHluorin fluorescence used in some experiments for the normalization of the surface expression to the total protein level?
-Only cell surface pHluorin was detected with anti-GFP IgGs. Cell surface levels (signal from anti-GFP IgGs) were then normalized to the pHluorin signal, which represents the total β3 subunit population (i.e. cell surface + intracellular populations).

Wouldn't the authors expect that changes in the surface expression affect the intensity of the pHluorin fluorescence (leading to an underestimation of the effect)?
To clarify the pHluorin fluorescence signal represents cell surface and intracellular populations of pHBBSβ3 (i.e the total pool of receptor subunits).

9. The effect of the dynamin inhibitory peptide (DIP) on surface β3under control conditions should be shown in Fig. 4 (and not only the effect of Bay K 8644 in the presence of the DIP).
-In this experiment control neurons and neurons treated with Bay K 8644 were both incubated with dynamin inhibitory peptide and have made this clearer in the text. The effects of DIP on cell surface GABAA receptor numbers has been demonstrated in at least 3 manuscripts form our laboratory (Kittler et al., 2000; Bogdanov et al., 2006; Jacob et al., 2009) and others. Thus due to space restrictions we have simply cited these papers.

10. What was the liquid junction potential for the two pipette filling solutions? Were corrections done for the holding potential, -70 mV?
Junction potential for regular solution was ~2 mV, and for low-chloride was ~11.5 mV. In both cases, holding potential was not corrected for that.

11. The development and use of the phosphorylation site-specific anti-P-S383 antibody is crucial for the present manuscript. A proper characterization of the new antibody is essential for the interpretation of the results obtained using this antibody. Therefore, this referee recommends including data shown in supplementary figure 1 in figure 2.
These data have now been made into a main figure and is now Figure 2.

12. It is stated on p. 28 that immunostainings were analyzed blind to experimental conditions. Is this true for the quantification of immunblots, too? Can the authors comment on the difference in the amplitude of the Bay K 8644 effect between biotinylation experiments (Fig. 1) and immunostainings (Fig. 2)?
Quantification of immunoblots was not performed blind. In figure 1 we are assaying cell surface levels of the endogenous β3 subunit. In Figure 3 neurons are expressing the pHluorin tagged β3 construct. The expression of pHBBSβ3 is driven by a strong CMV promoter leading to high levels of expression of pHBBSβ3. As a result there must be a limit to the numbers of new receptors, which realistically can be inserted. Therefore this disparity between endogenous β3 and exogenously expressed pHBBSβ3 levels following activation of L-type channels is a reflection

on the differing expression levels of β3 subunits in the two types of experiments.

13. How do the authors explain the decrease in GABAA receptor surface expression observed after a two-hour incubation with 4-AP as stated in the discussion? Did the phosphorylation state of β3 change accordingly (i.e., decrease) after prolonged 4-AP incubation?
Phosphorylation of β3S383 at 2 hours was not assayed. Here we are concerned with the effects of acute changes in neuronal activity on GABAA receptor cell surface expression that are evident within a few minutes.

Minor

- The β3A mutant is introduced on p. 6. Therefore, it sounds odd to start on p. 9 with "We generated a phospho-null (β3A)...". Please rephrase. It might be also worth citing articles in which the β3A has been used before.
These corrections have been made. However, pHluorin/BBS tagged βS383A has not been used before, only the myc-tagged βS383A construct has been used in a previous study (Houston et al., 2007).

- Why was keeping the access resistance within 20% change important? Please provide data on the input R of the neurons and preferably also for the resting Vm before going to voltage clamp.
Discarding cells after change of access resistance >20% is standard procedure assuring quality of the collected data not particularly targeted for this study. Access resistance was kept bellow 15 MOhm. Data on resting membrane potential were not collected since we used a cesiumbased intracellular solution. Since cesium blocks potassium channels it's difficult to assess the resting membrane potential.

The electrophysiology has been done at 32-33 C. What was the temperature in the other types of experiments (p. 28)?
Biochemical and immunofluorescence experiments using cultured hippocampal neurons were performed at 37ºC; this is now stated in the methods section. Biochemical experiments using hippocampal slices were performed at 32°C as stated in the methods section

- Why is the specimen recording in Fig. 5D cut much earlier than the other traces?
The time scale for this recording is shorter that the other traces and as a result the trace is actually longer.

- Were the cells pre-incubated in the presence of KN-93 or was KN-93 applied together with Bay K 8644 as stated on p. 6?
We apologize for this oversight. KN-93 was added 1 minute prior to application of Bay K 8644 and 4-AP and is now stated in the results section.

- Fig. 4C: the P-S383 signal is shown in the figure, but it is not addressed in the figure legend or the text (e.g., with a reference to Fig. 2, where the same effect has been shown).
We have added a description of pS383 in the results section.

- p. 16, last paragraph: Much more is known about the functions of tonic IGABA than its role in regulating intrinsic neuronal excitability. Please check more recent papers by Mody and others.
A recent review by Mody has been cited.

- In most sentences with "tonic inhibition", "tonic current" is the correct expression
The phrase tonic current has been adopted where appropriate.

- The mode of action of Bay K 8644 should be explained to the reader (gating of L-type VGCCs).
A brief explanation of the action of Bay K 8644 has been given, mentioning the voltage dependence of its action.

- The specimen recordings in Fig. 1C are not representative of etomidate action, because the effect shown is much larger than the mean change in Fig. 1D. The same applies for Figs. 6D,E.
The data in the raw traces cannot be compared to the mean change because the mean is

corrected for cell size and is expressed as pA/pF.

- That Lipofectamine was obtained from Invitrogen does not belong to the Results section (p. 7).
This has been moved to the methods section

The CaMKII KO has probably a wide spectrum of defects, and therefore the 1st paragraph on p. 16 could be made much briefer.
This is true - the CaMKII KO may well have a wide spectrum of defects not just an epileptic phenotype.

- Abbreviations should be introduced (e.g., CamKII, KLH).
We have explained the abbreviations CaMKII and KLH

- p. 3: GABAA receptors are not Cl- selective
The term permeable has been used.

- p. 4: Figureure
We have amended this typo.

- The readability of the figure legends would improve if the authors would state only once that "data are plotted as mean {plus minus} S.E.".
This has been amended as requested. “data are plotted as mean {plus minus} S.E.".
has only been mentioned once in each figure legend.

- antibody vs anti-body
We have amended this typo.

- p-S383 vs P-S383 (Fig. 6)
We have amended this typo.

Referee #3

In this elegant paper, the authors have used a plethora of techniques to dissect the links between activity and phosphorylation and activity of GABAergic receptors and signalling. The topic is obviously important considering the interest in understanding how activity alters the operation of GABA and by this mean also the operation of brain networks in physiological and pathological situations. In spite of these elements,; the study falls short of showing the relevance of these mechanisms in more physiologically relevant situations and whether and how they occur outside the cellular cultures and artificial situations associated with it.

Main issues

i) what happens in vitro in slices and possibly in vivo? Is any of these events possible and can it be observed?
We believe that our paper provides strong evidence that phosphorylation of β3S383 occurs in neurons which regulates insertion of GABAA receptors and is important in determining the efficacy of tonic inhibition. To satisfy the curiosity of the reviewer we have examined the phosphorylation of β3S383 in hippocampal slices. Consistent with our studies in culture we demonstrate that application of 4-AP significantly increases β3S383 phosphorylation with in 2 minutes and this data is now included in Figure 7D.

ii) too many speculative correlations, thus the effect of L-type Ca activation of Alpha5 (a5) and beta 3 is just correlation no causal link here
We respectfully disagree with the referee, we believe there is strong casual evidence in the paper linking the activation of L-type VGCCs to enhanced CaMKII dependent phosphorylation of β3S383, a rise in cell surface expression of GABAA receptors and an increase in tonic current, based on the following observations.
a) The effect of L–type channel activation on GABAA receptor surface expression is completely blocked by expression of the β3-S383A mutant (Figure 3C and 3D).

b) The effect of L–type channel activation on β3S383 phosphorylation and GABAA receptor surface expression is blocked by the CaMKII inhibitor KN-93 (Figure 3B).
c) Expression of inactive CaMKII reduces cell surface expression of GABAA receptors but has no influence on β3S383D expression.
d) Nimodipine blocks the effects of increased neuronal activity (with 4-AP) on β3S383 phosphorylation and GABAA receptor surface expression.
e) The effect of increased neuronal activity (with 4-AP) on GABAA receptor surface expression is completely blocked by expression of the β3-S383A mutant.

ii) B3 is a component of synaptic GABA receptors. The paper is written in a way that it is easy to think that the effect is specific to tonic currents but this is not shown and ought to be tested.
Previous papers showing sIPSCs are influence by Ca2+ influx and CaMKII activity are cited in the introduction. The focus of this paper in on the role CaMKII plays in regulating insertion of GABAA receptors containing β3 and tonic current, and simply replicating the work of others will not, we believe, enhance the impact of this current study.

iii) The data in fig 6 show nicely that there is an activity dependent increase in phosphorylation of Beta 3 and increase in tonic currents. Unfortunately the causal link between these two events is not demonstrated. The authors should show this by repeating the experiment in neurons over expressing the s383a mutant.
Again we apologize for any confusion; We have shown using immunofluorescence labeling that the cell surface expression of β3S383A containing GABAA receptors in hippocampal neurons is unaffected by an increase in neuronal activity (using 4-AP). Please see Figure 8C and 8D.

iv) Both the mutant S383A and S383D have a significant effect on the total protein expression. The significant effect of the mutations could be on posttranslational processing of the protein and thus could affect the interpretation of the demonstration of specific effects on plasma membrane trafficking. This should be discussed in the paper.
These issues have been discussed in our revised manuscript (pg 15 line 6).

In general, the discussion relies on too many assumptions that are not demonstrated causally. More care should be exerted unless the authors find a way to provide direct evidence that synaptic currents and not only exogenous GABA currents are affected preferably with adequate transynaptic stimuli... the shunting actions of GABA are ignored but heavily impact the relevance of the conclusions.

Details and technical

i) As there is a robust time-dependent effect of TTX on Beta 3 surface then special care should be taken to perform the electrophysiological recordings within the same time frame.(e.g. the amplitude of tonic current will depend on the time in blocker mix of excitatory transmission). The time frames use should be given in the methods.
The time frames of TTX action are much longer than the time course of the experiments here. Incubation times of at least 30 mins are required, to see effects on β3 subunit expression while the effects of L type channel activation/CaMKII are seen with in a few minutes. Also, TTX and excitatory transmission blockers were applied in the moment that gigaseal was achieved, therefore, for a very short time and no more than one cell per cover slip was recorded.

ii) There is a recent paper on role of beta3 on tonic currents by Janssen et al Front. Cell. Neurosci (2011) 5:15.
Thank you for bringing this to our attention, but due to the limited number of references we can list we are unable to cite this paper.

iii) The effect of S383A and S383D mutation on Bay K 8644 a5 surface increase deserves more stringent demonstration.
The focus of this paper is on the β3 subunit and we believe that extending this to other GABA(A) receptor subunits warrants an independent study

iv) page 9 the s383a and s383d are introduced as new but they were used in previous portion of the paper

We have revised the respective part of the text.

2nd Editorial Decision 14 March 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been re-reviewed by the three referees and their comments are provided below. As you can see, the referees appreciate the introduced changes and they support publication here. Referee #2 has a few minor issues that I would like to ask you to respond to in a final revision. Once we receive the revision, we will proceed with its acceptance here.

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

I have read the revised manuscript and the accompanying letter. The manuscript now deserves to be published.

Referee #2

This is an improved version of the previous manuscript. The authors have added additional data and most of my concerns have been addressed.
The study now makes an interesting contribution to the field by pointing out the importance of Ca2+ influx through L-type voltage gated Ca2+ channels and the CaMKII-mediated phosphorylation of the GABAAR subunit β3 (at position S383) for the tonic current elicited by GABA.
However, the authors might still find it appropriate to address some points (see below) in the next version of the manuscript.

1. It is stated on p. 28 that immunostainings were analyzed blind to experimental conditions. Is this true for the quantification of immunblots, too? Can the authors comment on the difference in the amplitude of the Bay K 8644 effect between biotinylation experiments (Fig. 1) and immunostainings (Fig. 2)?

Quantification of immunoblots was not performed blind. In figure 1 we are assaying cell surface levels of the endogenous β3 subunit. In Figure 3 neurons are expressing the pHluorin tagged β3 construct. The expression of pHBBS β3 is driven by a strong CMV promoter leading to high levels of expression of pHBBS β3. As a result there must be a limit to the numbers of new receptors, which realistically can be inserted. Therefore this disparity between endogenous β3 and exogenously expressed pHBBS β3 levels following activation of L-type channels is a reflection on the differing expression levels of β3 subunits in the two types of experiments.

I am not as convinced as the authors that the difference "is a reflection on the differing expression levels of β3 subunits in the two types of experiments". According to the data shown in Fig. 6B, expression of the WT receptor subunit under the control of the CMV promoter doesn´t bring the trafficking machinery to the limit. Expression of the S383D mutant under the same promoter results in a much higher surface expression.

2. Why is the specimen recording in Fig. 5D cut much earlier than the other traces?

The time scale for this recording is shorter that the other traces and as a result the trace is actually longer.

After looking at the figure (incl. the time scale) again, this reviewer is still convinced that the trace (now panel 6D) is actually shorter and the question remains open.

3. The specimen recordings in Fig. 1C are not representative of etomidate action, because the effect shown is much larger than the mean change in Fig. 1D. The same applies for Fig. 6 D,E.

The data in the raw traces cannot be compared to the mean change because the mean is corrected for cell size and is expressed as pA7pF.

Does it mean the authors found it impossible to show a representative pair of cells that does not differ in cell size so much?

Referee #3

The authors have responded to my queries.

2nd Revision - authors' response 20 March 2012

This is an improved version of the previous manuscript. The authors have added additional data and most of my concerns have been addressed.
The study now makes an interesting contribution to the field by pointing out the importance of Ca2+ influx through L-type voltage gated Ca2+ channels and the CaMKII-mediated phosphorylation of the GABAAR subunit β3 (at position S383) for the tonic current elicited by GABA.
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Fair point. However, we did not suggest that the trafficking machinery is saturated in cells expressing pHBBSβ3Wt following application of Bay K 8644, only that the numbers of GABA(A) receptors are tightly regulated in response to activity dependent changes in Ca2+ influx, due to their influence on neuronal excitability. The S383D subunit is a phosphorylation mimic, and the phosphorylation dependent trafficking of S383D cannot be regulated by changes in CaMKII activity. As a result we believe that in this case over saturation of the trafficking machinery may occur leading to greater total and cell surface expression of S383D compared to pHBBSβ3Wt. In the experiments with Bay K 8644, CaMKII phosphorylation of β3S383 will also be tightly regulated in response to changes in Ca2+ influx perhaps limiting the numbers of pHBBSβ3Wt subunits that are targeted for phosphorylation by CaMKII.

2. Why is the specimen recording in Fig. 5D cut much earlier than the other traces?

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After looking at the figure (incl. the time scale) again, this reviewer is still convinced that the trace (now panel 6D) is actually shorter and the question remains open.
Recordings from individual cells were terminated at different times. The important point here in these experiments was the effect of the drug reaching a plateau, which the authors believe is the case in Fig. 6D. The difference in the length of the trace does not hold any special or significant meaning.

3. The specimen recordings in Fig. 1C are not representative of etomidate action, because the effect shown is much larger than the mean change in Fig. 1D. The same applies for Fig. 6 D,E.
The data in the raw traces cannot be compared to the mean change because the mean is corrected for cell size and is expressed as pA7pF.
Does it mean the authors found it impossible to show a representative pair of cells that does not differ in cell size so much?
Traces in Fig.1C have been changed following the reviewer's suggestion. Control cell - Cm=70 and current density 1.37 pA/pF (mean=1.38 pA/pF), Bay K 8644 treated cell - Cm = 88 pF, current density 1.99 pA/pF (the same as the mean).
As, for figure 6D and 6E – Since the capacitance of cells varied – the authors found it hard to find cells matching in size as well as current density, representative of mean current densities (as the authors did not have the liberty to choose any cell for recording in these experiments – but only cells that were transfected with the pHluorin tagged β3 constructs, and only transfected cells which could be, with a high degree of certainty, be identified as a pyramidal cell. However, the current density of the selected neuron expressing S383A in figure 6D matches the mean current density.
The traces for S383D and WT cells BOTH had higher current densities than the mean values – but the authors deliberately chose these cells so as to match the degree of difference between S383D and WT.

