The role of GABA<sub>A</sub>R phosphorylation in the construction of inhibitory synapses and the efficacy of neuronal inhibition

Mansi Vithlani and Stephen J. Moss

Department of Neuroscience, Tufts University, 136 Harrison Ave, Boston, MA 02111, U.S.A., and Department of Neuroscience, Physiology and Pharmacology, University College London, Gower Street, London WC1E 6BT, U.K.

Abstract

GABA<sub>A</sub>Rs [GABA (γ-aminobutyric acid) type-A receptors] are heteropentameric chloride-selective ligand-gated ion channels that mediate fast inhibition in the brain and are key therapeutic targets for benzodiazepines, barbiturates, neurosteroids and general anaesthetics. In the brain, most of the benzodiazepine-sensitive synaptic receptor subtypes are assembled from α<sub>1−3</sub>, β<sub>1−3</sub> and γ<sub>2</sub> subunits. Although it is evident that the pharmacological manipulation of GABA<sub>A</sub>R function can have profound effects on behaviour, the endogenous mechanisms that neurons use to promote sustained changes in the efficacy of neuronal inhibition remain to be documented. It is increasingly clear that GABA<sub>A</sub>Rs undergo significant rates of constitutive endocytosis and regulate recycling processes that can determine the efficacy of synaptic inhibition. Their endocytosis is regulated via the direct binding of specific endocytosis motifs within the intracellular domains of receptor β<sub>1−3</sub> and γ<sub>2</sub> subunits to the clathrin adaptor protein AP2 (adapter protein 2). These binding motifs contain major sites of both serine and tyrosine phosphorylation within GABA<sub>A</sub>Rs. Their phosphorylation can have dramatic effects on binding to AP2. In the present review, we evaluate the role that these phospho-dependent interactions play in regulating the construction of inhibitory synapses, efficacy of neuronal inhibition and neuronal structure.

Introduction

Fast synaptic inhibition in the brain is mediated largely via GABA<sub>A</sub>Rs [GABA (γ-aminobutyric acid) type-A receptors], which are chloride-selective ligand-gated ion channels. These proteins are also clinically relevant drug targets for anxiolytic, sedative, anticonvulsant and hypnotic agents, including benzodiazepines, barbiturates and some general anaesthetics. Deficits in the functional expression of GABA<sub>A</sub>Rs are relevant in such neuropsychiatric disorders as autism, anxiety disorders, cognitive deficits, depression, epilepsy, schizophrenia and substance abuse. Given the roles that GABA<sub>A</sub>Rs play in neuronal inhibition, as drug targets and in pathology, there is significant interest in how neurons regulate both the number of these receptors that are expressed on the neuronal cell surface and their activity.

Molecular analysis has revealed that GABA<sub>A</sub>Rs belong to the superfamily of cysteine-loop ligand-gated ion channels that comprises nACh (nicotinic acetylcholine) receptors, strychnine-sensitive glycine receptors and 5-HT<sub>1A</sub> (5-hydroxytryptamine type-3) receptors [1]. Members of this receptor family are heteropentameric glycoproteins composed of homologous subunits that specifically recognize one another and assemble around an intrinsic ion channel. Each subunit has a common structure consisting of a large extracellular ligand-binding N-terminal region and a short, barely extruding C-terminus separated by four highly conserved hydrophobic TM (transmembrane)1–4 domains. In addition, a major cytoplasmic loop lies between TM3 and TM4 [1].

To date, 19 GABA<sub>A</sub> receptor subunits have been identified in the mammalian brain. These can be divided into eight classes, namely α<sub>1−6</sub>, β<sub>1−3</sub>, γ<sub>1−3</sub>, δ<sub>1</sub>, ε<sub>1</sub>, π<sub>1</sub>, θ<sub>1</sub> and ρ<sub>1−3</sub>, and provide the basis for extensive heterogeneity of GABA<sub>A</sub>R structure [2]. However, consensus opinion suggests that most of the synaptic GABA<sub>A</sub>R subtypes are composed of α<sub>1−3</sub>, β<sub>1−3</sub> and γ<sub>2</sub> subunits [3], whereas receptors containing α<sub>4−6</sub>, β<sub>1−3</sub> and δ subunits form specialized populations of extrasynaptic receptors that mediate tonic inhibition [4,5]. Benzodiazepine-sensitive α<sub>1−5</sub>, β<sub>1−3</sub> and γ<sub>2</sub> subunit-containing receptors also mediate tonic inhibition [3].

GABA<sub>A</sub>Rs are dynamic entities on the plasma membrane that undergo constitutive endocytosis and recycling

After assembly within the endoplasmic reticulum, transport-competent GABA<sub>A</sub>Rs traffic through the secretory pathway and are then inserted into the plasma membrane primarily at extrasynaptic sites [6,7]. Extrasynaptic GABA<sub>A</sub>Rs exhibit high rates of lateral mobility, providing a mechanism for
Phospho-dependent binding of the clathrin adaptor AP2 to GABA<sub>R</sub> β subunit isoforms

A critical determinant of membrane protein endocytosis is recruitment into clathrin-coated pits prior to the formation of endocytotic vesicles. This process is facilitated by the clathrin adaptor protein AP2, which forms a link between cargo and clathrin. AP2 is a heterotetrameric complex composed of two large (∼100 kDa) α and β<sub>2</sub> subunits, a medium (50 kDa) μ<sub>2</sub> subunit, and a small (19 kDa) σ<sub>2</sub> subunit. These subunits are, in this context, commonly referred to as adaptins. The α-adaptn is responsible for targeting the protein to the plasma membrane, where the β<sub>2</sub>-adaptn interacts with clathrin to trigger clathrin assembly, forming coated pits. This in turn leads to the activation of μ<sub>2</sub>-adaptn phosphorylation, inducing a conformational change in the subunit that allows the complex to directly bind to endocytotic motifs in cell-surface receptors, clustering the protein cargo into the assembling coated pit [16,17].

Consistent with their high rates of clathrin-dependent endocytosis, GABA<sub>A</sub>Rs are found in clathrin-coated pits and are also intimately associated with AP2 as measured via co-immunoprecipitation [13,18–20]. In vitro binding assays were used to further assess which components of AP2 bind to GABA<sub>A</sub>R subunits. These assays revealed that the μ<sub>2</sub>–AP2, but not the α, β<sub>2</sub> or σ<sub>2</sub> subunit, is capable of binding to the intracellular domains of the GABA<sub>A</sub>R β<sub>1–3</sub> and γ<sub>1–3</sub> subunits, but not to the corresponding regions of the α<sub>1</sub>, α<sub>3</sub> or α<sub>5</sub> subunits [13,21].

Molecular analysis was utilized to delineate the amino acids responsible for μ<sub>2</sub>–AP2 binding in the receptor β subunit isoforms. This revealed a conserved amino acid motif between residues 400 and 412 of these subunits that is sufficient to mediate μ<sub>2</sub>–AP2 binding, namely KTHLRRSSQLK in the case of β<sub>3</sub> [21]. Similar atypical ‘basic patch’ binding motifs for μ<sub>2</sub>–AP2 have been identified in AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors and the vesicle-associated protein Stg1 [22,23]. Intriguingly, this motif also contains the principal phosphorylation sites for both cAMP-dependent PKA (protein kinase A) and PKC (protein kinase C) within the β<sub>3</sub> subunit Ser<sup>408</sup>/Ser<sup>409</sup> [24,25]. Thus phosphorylation of the β<sub>3</sub> subunit on Ser<sup>408</sup>/Ser<sup>409</sup> may be of significance in regulating GABA<sub>A</sub>R binding to μ<sub>2</sub>–AP2 and thus their endocytosis. To study this, the effects of phosphorylating Ser<sup>408</sup>/Ser<sup>409</sup> on the binding of μ<sub>2</sub>–AP2 to the β<sub>3</sub> subunit were analysed. As measured by in vitro binding, phosphorylation of Ser<sup>408</sup>/Ser<sup>409</sup> by either PKA or PKC drastically reduced the binding of μ<sub>2</sub>–AP2 to the β<sub>3</sub> subunit. Likewise, phosphorylation of the β<sub>3</sub> subunit on Ser<sup>409</sup> abolished μ<sub>2</sub>–AP2 binding to the intracellular domain of this receptor subunit. Using surface plasmon resonance, high-affinity binding of μ<sub>2</sub>–AP2 to a peptide corresponding to residues 400–412 of the β<sub>3</sub> subunit was evident (K<sub>D</sub> = 300 nM), which was reduced to 1900 nM for peptides chemically phosphorylated on Ser<sup>408</sup>/Ser<sup>409</sup> [21].

To explore the significance of this phospho-dependent interaction for synaptic inhibition, the effects of a peptide corresponding to residues 400–412 of the β<sub>3</sub> subunit on the properties of mIPSCs (miniature inhibitory postsynaptic currents) have been measured. This peptide produced a time-dependent increase in the amplitudes of mIPSCs in cultured neurons in a manner that was occluded by inhibitors of dynamin without modifying the properties of mIPSCs [21]. In contrast, a peptide corresponding to residues 400–412 chemically phosphorylated on Ser<sup>408</sup>/Ser<sup>409</sup> did not modify the properties of mIPSCs [21].

Consistent with these electrophysiological studies, enhancing the phosphorylation of Ser<sup>408</sup>/Ser<sup>409</sup> by the pharmacological activation of PKC increased the cell-surface expression levels of GABA<sub>A</sub>Rs containing β<sub>3</sub> subunits. In parallel with this enhanced cell-surface expression and phosphorylation, reduced binding of the β<sub>3</sub> subunit to the AP2 adaptin was evident as measured by immunoprecipitation [25a].

It is important to note that phosphorylation of Ser<sup>408</sup>/Ser<sup>409</sup> in the β<sub>3</sub> subunit was subject to dynamic modulation by both neurotransmitter and growth factor receptors that activate both PKA and PKC signalling cascades. These modulatory receptors included D<sub>1</sub> (dopamine type-1) and D<sub>2</sub> and TrkB (tropomyosin receptor kinase B) receptors [24,26,27]. This
functional cross-talk may provide input-specific control of GABA$_\gamma$R phosphorylation and thus affect the efficacy of synaptic inhibition by modulating receptor endocytosis and hence accumulation on the plasma membrane.

**Phospho-dependent binding of the clathrin adaptor AP2 to GABA$_\gamma$R $\gamma$ subunit isoforms**

Approaches similar to those outlined above were used to determine the amino acid residues within the $\gamma_2$ subunit that mediate binding to $\mu_2$–AP2. This resulted in the identification of a classical tyrosine-based binding motif (YXX$\phi$, where $\phi$ is a hydrophobic amino acid) centred on Tyr$^{367}$ in the (YGY$^{367}$ECL) in the $\gamma_2$ subunit [17,28]. Significantly, both Tyr$^{367}$ and the adjacent tyrosine residue Tyr$^{365}$ are the principal sites of phosphorylation for Src family members in GABA$_\gamma$R$_\gamma$ [29,30]. Using surface plasmon resonance and in GABA$_\gamma$R [29,30]. Using surface plasmon resonance coupled with crystallography, it was evident that this motif bound $\mu_2$–AP2 with an affinity of 40 nM, an interaction critically dependent on Tyr$^{367}$ [28]. Phosphorylation of either Tyr$^{365}$ or Tyr$^{367}$ also ablated $\mu_2$–AP2 to the $\gamma_2$ subunit. Introduction of a peptide containing Tyr$^{365}$/Tyr$^{367}$ into neurons produced a large increase in mIPSC amplitude that was accompanied by an increase in the number of receptors on the cell surface [28], an effect not replicated by a peptide in which Tyr$^{365}$/Tyr$^{367}$ had been mutated to alanine residues.

In addition to this tyrosine-based motif, an additional $\mu_2$–AP2 motif was identified between residues 324 and 335 of the $\gamma_2$ subunit, namely RKPSKDSDKKKK. This motif is enriched in basic amino acids, similar to that identified in receptor $\beta$ subunit isoforms [31]. It is notable that this sequence contained Ser$^{237}$, a site of phosphorylation for both PKC and CaMKII (Ca$^{2+}$/calmodulin-dependent protein kinase II) with GABA$_\gamma$Rs [32,33]. It will be interesting to examine the role that phosphorylation of Ser$^{237}$ plays in regulating the binding of GABA$_\gamma$Rs to $\mu_2$–AP2.

**GABA$_\gamma$R endocytosis regulates the number and size of inhibitory synapses and the maturity of dendritic spines**

To examine the significance of GABA$_\gamma$R endocytosis in the construction of inhibitory synapses, imaging studies were performed in neurons expressing fluorescent $\beta_3$ subunits in which Ser$^{297}$/Ser$^{299}$ had been mutated to alanine residues ($\beta_3$ S$^{297}$/S$^{299}$). This mutation mimics the effects of phosphorylation by significantly reducing the binding of the $\beta_3$ subunit to $\mu_2$–AP2 [25a]. As measured by live imaging, GABA$_\gamma$A Rs containing $\beta_3$ S$^{297}$/S$^{299}$ exhibited enhanced levels of cell-surface expression compared with those containing wild-type $\beta_3$ subunits, a phenomenon that was mediated by reduced endocytosis [25a]. In keeping with this, the size and number of inhibitory synapses was enhanced in neurons expressing $\beta_3$ S$^{297}$/S$^{299}$, which was paralleled by a significant increase in both the amplitude and frequency of mIPSCs. Neurons expressing $\beta_3$ S$^{297}$/S$^{299}$ exhibited marked deficits in the number of mature spines together with a reduction in the expression levels of PSD-95 (postsynaptic density 95) at excitatory synapses [34,35]. This deficit in maturity was clearly due to enhanced GABAergic inhibition, as it was reversed by pharmacological blockade of GABA$_\gamma$A Rs. Given the critical role that spines play in excitatory transmission and in information storage, this observation suggests a critical role for GABA$_\gamma$R membrane trafficking in regulating spinogenesis and has profound implications for cognition.

**Conclusions**

Fast synaptic inhibition mediated by GABA$_\gamma$A Rs plays a critical role in neuronal function. Deficits in this process are central to neuropsychiatric disorders ranging from autism to epilepsy. It is increasingly clear that GABA$_\gamma$A Rs undergo significant rates of phospho-dependent endocytosis, a process that can shape the size and number of inhibitory synapses and neuronal excitation. Thus long-term changes in the strength of inhibitory connections by the phospho-dependent modulation of GABA$_\gamma$R endocytosis may contribute to synaptic plasticity and ultimately to behaviour.

**Acknowledgement**

S.J.M. serves as a consultant for Wyeth Pharmaceuticals, a relationship that is regulated by Tufts University and does not have any impact on this work.

**Funding**

S.J.M. is supported by the National Institute of Neurological Disorders and Stroke [grant numbers NS047478, NS048045, NS051195, NS056359 and NS054900].

**References**


