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The role of GABA_AR phosphorylation in the construction of inhibitory synapses and the efficacy of neuronal inhibition

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Abstract

γ -aminobutyric acid type-A receptors (GABA_ARs) 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 anesthetics. In the brain the majority of benzodiazepine-sensitive synaptic receptor subtypes are assembled from $\alpha 1$ –3, $\beta 1$ –3 and $\gamma 2$ subunits. Whilst it is evident that the pharmacological manipulation of GABA_AR function can have profound effects on behavior, 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_ARs undergo significant rates of constitutive endocytosis and regulate recycling processes that can determine efficacy of synaptic inhibition. Their endocytosis is regulated via the direct binding of specific endocytosis motifs within the intracellular domains of receptor $\beta 1$ –3 and $\gamma 2$ subunits to the clathrin adaptor protein AP2. These binding motifs contain major sites of both serine and tyrosine phosphorylation within GABA_ARs. Their phosphorylation can have dramatic effects on binding to AP2. In this review we evaluate the role that these phospho-dependent interactions play in regulating the construction of inhibitory synapses, the efficacy of neuronal inhibition and neuronal structure.

Keywords

γ -aminobutyric acid receptor; synapse inhibition

Introduction

Fast synaptic inhibition in the brain is mediated largely via GABA_ARs, 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 anesthetics. Deficits in the functional expression of GABA_ARs are relevant in such neuropsychiatric disorders as autism, anxiety disorders, cognitive deficits, depression, epilepsy, schizophrenia and substance abuse. Given the roles that GABA_ARs 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_ARs belong to the superfamily of cysteine-loop ligand-gated ion channels that is comprised of nicotinic acetylcholine (nACh) receptors, strychnine-sensitive glycine receptors, and 5-hydroxytryptamine type-3 (5-HT₃) 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 transmembrane (TM)1–4 domains. In addition, a major cytoplasmic loop lies between TM3 and TM4 [1].

To date, nineteen GABA_A receptor subunits have been identified in the mammalian brain. These can be divided into eight classes: α (1–6), β (1–3), γ (1–3), δ , ϵ , π , θ , and ρ (1–3), and provide the basis for extensive heterogeneity of GABA_AR structure [2]. However consensus opinion suggests that the majority of synaptic GABA_AR subtypes are composed of α 1–3, β 1–3 and γ 2 subunits [3], whilst receptors containing α 4/6, β 1–3 and δ subunits form specialized populations of extrasynaptic receptors that mediate tonic inhibition [4,5]. Benzodiazepine-sensitive α 1–5, β 1–3 and γ 2 subunit-containing receptors also mediate tonic inhibition [3].

GABA_ARs are dynamic entities on the plasma membrane that undergo constitutive endocytosis and recycling

After assembly within the endoplasmic reticulum transport-competent GABA_ARs traffic through the secretory pathway and are then inserted into the plasma membrane primarily at extrasynaptic sites [6,7]. Extrasynaptic GABA_ARs exhibit high rates of lateral mobility, providing a mechanism for newly-inserted receptors to access synaptic sites where they are stabilized via interaction with the inhibitory post-synaptic scaffold [7] [8]. [9]. Of central importance in stabilizing GABA_ARs at these subcellular specializations is the inhibitory scaffold protein gephyrin. Gephyrin binds to specific amino acid motifs within the intracellular domain of the α 2 subunit, linking GABA_ARs to the actin cytoskeleton and microtubules [10] [11]. Extrasynaptic GABA_ARs are rapidly removed from the plasma membrane via clathrin-dependent endocytosis and, using biotinylation, it has been estimated that in excess of 20% of the total cell surface population of GABA_ARs is internalized within 15 min [7] [12]. Over short time courses endocytosed receptors are rapidly recycled back to the plasma membrane for re-insertion, whilst over longer time periods they can be directed to lysosomes for degradation [12]. This sorting decision is regulated via a direct interaction of GABA_ARs with Huntingtin-associated protein (HAP)-1, which preferentially targets receptors for recycling [12]. Consistent with this mice devoid of HAP1 have reduced numbers of cell surface GABA_ARs and deficits in synaptic inhibition [13].

To examine the consequences of this rapid exchange of GABA_ARs between the cell surface and intracellular compartments the effects of global inhibitors of endocytosis on the efficacy of synaptic inhibition have been examined. Blockade of dynamin activity with P4 peptide, an accepted inhibitor of endocytosis, results in a significant increase in the amplitude of miniature inhibitory synaptic currents (mIPSCs; approximately 100%) over a time course of 30 min in hippocampal neurons [14] [15] [16]. This result suggests that blockade of endocytosis results in an increase in the number of functional GABA_ARs at synaptic sites. Consistent with this imaging studies have revealed that P4 peptide also dramatically increases the residence time of GABA_ARs on the plasma membrane [7].

Phospho-dependent binding of the clathrin adaptor AP2 to GABA_AR 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 2 subunits, a medium (50 kDa) μ 2 subunit, and a

small (19 kDa) $\sigma 2$ subunit. These subunits are, in this context, commonly referred to as adaptins. The α adaptin is responsible for targeting the protein to the plasma membrane, where the $\beta 2$ adaptin interacts with clathrin to trigger clathrin assembly, forming coated pits. This in turn leads to the activation of $\mu 2$ adaptin phosphorylation, inducing a conformational change in the subunit that allows the complex to directly bind to endocytic motifs in cell surface receptors, clustering the protein cargo into the assembling coated pit [17] [18].

Consistent with their high rates of clathrin-dependent endocytosis GABA_ARs are found in clathrin-coated pits and are also intimately associated with AP2 as measured via co-immunoprecipitation [19–21] [14]. *In vitro* binding assays were used to further assess which components of AP2 bind to GABA_AR subunits. These assays revealed that the $\mu 2$ -AP2, but not the α , $\beta 2$ or $\sigma 2$ subunit, is capable of binding to the intracellular domains of the GABA_AR $\beta 1$ –3 and $\gamma 1$ –3 subunits but not to the corresponding regions of the $\alpha 1$, $\alpha 3$ or $\alpha 6$ subunits [14] [22].

Molecular analysis was utilized to delineate the amino acids responsible for $\mu 2$ -AP2 binding in the receptor β subunit isoforms. This revealed a conserved amino acid motif between residues 400–412 of these subunits that is sufficient to mediate $\mu 2$ -AP2 binding - KTHLRRRSSLK in the case of $\beta 3$ [22]. Similar atypical “basic patch” binding motifs for $\mu 2$ -AP2 have been identified in AMPA receptors and the vesicle-associated protein Stgl [23,24]. Intriguingly this motif also contains the principle phosphorylation sites for both cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) within the $\beta 3$ subunit serine residues 408 and 409 (S408/9) [25,26]. Thus phosphorylation of the $\beta 3$ subunit on S408/9 may be of significance in regulating GABA_AR binding to $\mu 2$ -AP2 and thus their endocytosis. To study this, the effects of phosphorylating S408/9 on the binding of $\mu 2$ -AP2 to the $\beta 3$ subunit were analyzed. As measured by *in vitro* binding phosphorylation of S408/9 by either PKA or PKC drastically reduced the binding of $\mu 2$ -AP2 to the $\beta 3$ subunit. Likewise phosphorylation of the $\beta 1$ subunit on S409 abolished $\mu 2$ -AP2 binding to the intracellular domain of this receptor subunit. Using surface plasmon resonance high affinity binding of $\mu 2$ -AP2 to a peptide corresponding to residues 400–412 of the $\beta 3$ subunit was evident ($K_d = 300$ nM), which was reduced to 1900 nM for peptides chemically phosphorylated on S408/9 [22].

To explore the significance of this phospho-dependent interaction for synaptic inhibition the effects of a peptide corresponding to residues 400–412 of the $\beta 3$ subunit on the properties on mIPSCs 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 miniature excitatory post-synaptic currents [22]. In contrast a peptide corresponding to residues 400–412 chemically phosphorylated on S408/9 did not modify mIPSC properties [21].

Consistent with these electrophysiological studies enhancing the phosphorylation of S408/9 by the pharmacological activation of PKC increased the cell surface expression levels of GABA_ARs containing $\beta 3$ subunits. In parallel with this enhanced cell surface expression and phosphorylation reduced binding of the $\beta 3$ subunit to the AP2 adaptin was evident as measured by immunoprecipitation (Jacob et al., 2009).

It is important to note that phosphorylation of S408/9 in the $\beta 3$ subunit was subject to dynamic modulation by both neurotransmitter and growth factor receptors that activate both PKA, and PKC signaling cascades. These modulatory receptors included dopamine type-1 (D_1) and D_3 and TrkB receptors [27] [25] [28]. This functional cross talk may provide input-specific control of GABA_AR 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_AR γ 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 ϕ , where ϕ = a hydrophobic amino acid) centered on Y367 in the (YGY³⁶⁷ECL) in the $\gamma 2$ subunit [18,29]. Significantly both Y367 and the adjacent tyrosine residue Y365 are the principal sites of phosphorylation for Src family members in GABA_ARs [30] [31]. 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 Y367 [29]. Phosphorylation of either Y365 or Y367 also ablated $\mu 2$ -AP2 to the $\gamma 2$ subunit. Introduction of a peptide containing Y365/7 into neurons produced a large increase in mIPSC amplitude that was accompanied by an increase in the number of receptors on the cell surface [29], an effect not replicated by a peptide in which Y365/7 had been mutated to alanine residues.

In addition to this tyrosine-based motif an additional $\mu 2$ -AP2 motif was identified between residues 324–335 of the $\gamma 2$ subunit - RKPSKDKDKKK. This motif is enriched in basic amino acids similar to that identified in receptor β subunit isoforms [32]. It is notable that this sequence contained serine 327, a site of phosphorylation for both PKC and calcium/calmodulin-dependent type II protein kinase (CaMKII) with GABA_ARs [33] [34]. It will be interesting to examine the role that phosphorylation of S327 plays in regulating the binding of GABA_ARs to $\mu 2$ -AP2.

GABA_AR endocytosis regulates the number and size of inhibitory synapses and the maturity of dendritic spines

To examine the significance of GABA_AR endocytosis in the construction of inhibitory synapses imaging studies were performed in neurons expressing fluorescent $\beta 3$ subunits in which S408/9 had been mutated to alanines ($\beta 3^{S408/9A}$). This mutation mimics the effects of phosphorylation by significantly reducing the binding of the $\beta 3$ subunit to $\mu 2$ -AP2. As measured by live imaging GABA_ARs containing $\beta 3^{S408/9A}$ exhibited enhanced levels of cell surface expression compared to those containing wild-type $\beta 3$ subunits, a phenomenon that was mediated by reduced endocytosis (Jacob et al., 2009). In keeping with this, the size and number of inhibitory synapses was enhanced in neurons expressing $\beta 3^{S408/9A}$, which was paralleled by a significant increase in both the amplitude and frequency of mIPSCs. Neurons expressing $\beta 3^{S408/9A}$ exhibited marked deficits in the number of mature spines together with a reduction in the expression levels of post-synaptic density protein-95 at excitatory synapses [35] [36]. This deficit in maturity was clearly due to enhanced GABAergic inhibition as it was reversed by pharmacological blockade of GABA_ARs. Given the critical role that spines play in excitatory transmission and in information storage this observation suggests a critical role for GABA_AR membrane trafficking in regulating spinogenesis and has profound implications for cognition.

Conclusions

Fast synaptic inhibition mediated by GABA_ARs 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_ARs 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_AR endocytosis may contribute to synaptic plasticity and ultimately to behavior.

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Abbreviations

GABA_AR γ-aminobutyric acid receptor

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