Published in final edited form as:

Neuropharmacology. 2008 October ; 55(5): 844–850. doi:10.1016/j.neuropharm.2008.06.072.

Regulation of inhibitory synaptic transmission by a conserved atypical AP2 interaction within GABA_A receptor β and γ subunits

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Abstract

The number of surface and synaptic GABA_A receptors is an important determinant of inhibitory synapse strength. Surface receptor number is in part controlled by removal of receptors from the membrane by interaction with the clathrin adaptor AP2. Here we demonstrate that there are two binding sites for AP2 in the $\gamma 2$ -subunit: in addition to a $\gamma 2$ -subunit specific Yxx ϕ type motif located within a site for tyrosine phosphorylation there is also a basic patch AP2 binding motif, conserved with a similar motif found in GABA_A receptor β -subunits. Blocking GABA_A receptor-AP2 interactions using a peptide that inhibits AP2 binding to GABA_A receptors via the conserved atypical motif increases synaptic responses within minutes, whereas simultaneously blocking both binding mechanisms has an additive effect. These data suggest that a hierarchy of AP2 internalization signals control the levels of surface and synaptic GABA_A receptor levels to regulate synaptic inhibition.

1. Introduction

The GABA_A receptor, a member of the ligand-gated ion channel superfamily, mediates the majority of fast inhibitory synaptic transmission in the central nervous system of mammals. Understanding the molecular mechanisms that regulate GABA_A receptor function is important for our understanding of how neuronal excitability and synaptic inhibition are controlled. GABA_A receptors are hetero-oligomeric pentamers assembled from seven subunit classes ($\alpha 1$ -6, $\beta 1$ -3, $\gamma 1$ -3, δ , ϵ , π and θ) with the majority of GABA_A receptors in the brain assembled from at least 2 α , 2 β and 1 $\gamma 2$ subunit {Kittler, 2002 #14}. The GABA_A receptor $\gamma 2$ subunit regulates receptor pharmacology and membrane trafficking and confers on receptors benzodiazepine sensitivity and selective targeting to inhibitory synapses {Essrich, 1998 #7; Crestani, 1999 #6}. The intracellular domains (ICDs) of GABA_A receptor subunits are a locus for protein interactions and post-translational modifications (including phosphorylation and palmitoylation), which are important for regulating receptor function, intracellular transport and localization {Moss, 1992 #21; Moss, 1995 #22; Wang, 1999 #24; Wang, 2003 #25; Kittler, 2000 #11; Keller, 2004 #8; Rathenberg, 2004 #16}. It has recently been demonstrated that

altered membrane trafficking and endocytosis of GABA_A receptors may contribute to or underlie certain neuropathologies such as increased excitotoxicity in ischemia and the generation of pharmacoresistance and self-sustaining seizures in status epilepticus {Mielke, 2005 #29; Naylor, 2005 #27; Kang, 2006 #28; McNamara, 2006 #50}. Currently the molecular mechanisms and protein interactions that underlie GABA_A receptor internalization under normal or pathological conditions are not fully understood.

The number of surface and synaptic GABA_A receptors is an important determinant of inhibitory synapse strength. Surface receptor number can in part be regulated by endocytosis of receptors and interaction with the AP2 clathrin adaptor protein complex {Kittler, 2000 #11; Kittler, 2005 #18} which is composed of α , β 2, μ 2, and σ 2 adaptin subunits. The μ 2-AP2 subunit binds to defined endocytic motifs in a number of ionotropic neurotransmitter receptors, which include classical Yxx ϕ motifs and atypical basic patch domains, {Owen, 2004 #4; Bonifacino, 2003 #5; Haucke, 2000 #32}. We have shown previously that GABA_A receptor intracellular domains (ICDs) interact with AP2 (Kittler et al., 2000) by directly interacting with the μ 2 subunit of AP2 (μ 2-AP2) {Kittler, 2005 #18; Kittler, 2000 #11}. A detailed understanding of the molecular mechanisms of AP2 binding to GABA_A receptors is important both for understanding normal brain function and for understanding how altered GABA_A receptor membrane trafficking may contribute to altered inhibition in pathology. In the case of GABA_A receptor β -subunits the interaction with μ 2-AP2 occurs via an atypical basic residue interaction motif (basic patch motif {Kittler, 2005 #18}) that has also been identified in some other neuronal membrane proteins including synaptotagmin 1 (syt-1; {Haucke, 2000 #32}) and AMPA type glutamate receptors {Kastning, 2007 #30}{Lee, 2002 #52}. In contrast, in the γ 2 subunit a tyrosine type YXX ϕ motif is a determinant of GABA_AR γ -subunit binding to AP2 {Kittler, 2008 #57}.

In this study we use biochemical and electrophysiological approaches to further characterize the molecular determinants responsible for binding of the AP2 complex to the GABA_A receptor γ 2 subunit. We demonstrate that the γ 2 subunit ICD (residues 302-404 located between TMs III and IV; numbering as for the γ 2 short splice isoform) can interact directly with μ 2-AP2 via two binding motifs. In addition to the recently described Y³⁶⁵GY³⁶⁷ECL³⁷⁰ (Yxx ϕ type) AP2 binding motif that mediates high affinity phospho-dependent binding to μ 2-AP2 {Kittler, 2008 #57} we identify here an additional atypical basic patch AP2 binding motif present in the γ 2 subunit and that is similar to that identified in GABA_A receptor β -subunits. Targeting the basic patch AP2 interaction sites of the GABA_A receptor using interfering peptides increases the synaptic response. Simultaneously targeting both the basic patch and YXX ϕ binding mechanisms, results in an additive effect suggesting that these two AP2 interaction mechanisms can act either separately or in concert to regulate synaptic receptor number.

2. Methods

2.1 Antibodies and cDNA constructs

Mouse anti μ 2-AP2 was from BD Biosciences and mouse anti β -AP2 was from Sigma and were used at 1:250 to 1:500 as described previously {Kittler, 2005 #18; Kittler, 2000 #11}. Plasmids to the GABA_A receptor subunit intracellular domains fused to GST and the subunits of AP2 have been described previously {Haucke, 2000 #32; Kittler, 2005 #18}.

2.2 GST affinity purification assays and pull down experiments from brain extracts

GST affinity purification assays from rat brain lysate, ³⁵S labeled or purified proteins were performed essentially as described in a number of previous studies {Kittler, 2004 #17}{Kittler, 2005 #18; Kittler, 2001 #12; Haucke, 1999 #31; Haucke, 2000 #32}. To produce ³⁵S labeled μ 2 proteins, μ 2 constructs were labeled by quick coupled transcription / translation as described

previously {Haucke, 2000 #32; Kittler, 2005 #18}. For GST pull downs from rat brain, one adult rat brain was extracted with 20ml buffer (50mM HEPES, pH 7.6, 1% Triton, 150mM NaCl, 5mM EDTA, 5mM EGTA, 1mM Na orthovanadate, protease inhibitors). 50 μ g of GST fusion protein was rotated with 2.5mg of brain at 4°C, washed with extraction buffer and eluted in SDS sample buffer. For peptide competition binding assays of purified His₆-tagged μ 300 μ M of the syt-1 pep (CKRLKKKKTTIKK) was pre-incubated with 5 μ g purified His₆- μ 2 (157-435) in 250 μ l binding buffer for 60 min at 4 °C on a rotating wheel. Scrambled sc peptide (RKTKKIKLKTKK) was used as control.. The mixture was then incubated with 20 μ g of GST- β 3 or GST- γ 2AECL immobilized on glutathione agarose for 90 min at 4 °C on a rotating wheel.

2.3 Whole-Cell Recordings

Whole-cell recordings of mIPSCs from striatal neurons in acute slices used standard voltage clamp techniques {Kittler, 2005 #18; Kittler, 2004 #17; Chen, 2006 #19}. Electrodes were filled with the following internal solution: 100 mM CsCl, 30 mM NMG, 10 mM HEPES, 1 mM MgCl₂, 1 mM EGTA, 5 mM QX314, 12 mM phosphocreatine, 5 mM MgATP, 1.5 mM Na₂GTP, 0.1 mM leupeptin, pH = 7.2-7.3, 265- 270 mOsm/liter. The neurons were submerged in continuously flowing oxygenated artificial cerebrospinal fluid containing Tetrodotoxin (0.5 μ M). The cell membrane potential was held at -70mV. Data analyses were performed with Axograph (Axon Instruments, Union City, CA), Kaleidagraph (Albeck Software, Reading, PA) and Mini Analysis program (Synaptospft, Leonia, NJ).

3. Results

3.1 The GABA_A receptor γ -subunits interact directly with μ 2-AP2

We initially investigated if all three GABA_A receptor γ -subunits interact with AP2 using glutathione-S-transferase (GST) fusion protein affinity purification experiments (GST pull downs) from brain lysate. The intracellular domains of γ 1-, γ 2S and γ 3- subunits were fused to GST (to give GST- γ 1, GST- γ 2S and GST- γ 3) and expressed as GST fusion proteins in bacteria, purified and immobilized on glutathione agarose beads. GST- γ 1, GST- γ 2S and GST- γ 3 were exposed to brain lysates and bound complexes were resolved by SDS-PAGE. Bound AP2 was detected by immunoblotting with a monoclonal antibody to μ 2-AP2 (Fig. 1B), which revealed that AP2 from brain associates with the ICDs of γ 1 - γ 3 subunits demonstrating that AP2 binding is conserved across the γ -subunit subfamily.

The AP2 complex is comprised of four subunits: α , β 2, μ 2, and σ 2 adaptins ({Bonifacino, 2003 #5}; Fig. 1A). We performed additional affinity purification experiments to establish whether the different subunits of AP2 can interact directly with the γ -subunit ICDs. GST- γ 1, GST- γ 2S, GST- γ 2L (identical to γ 2S but in addition containing a 8 residue LLRMFSFK splice insert within the ICD) and GST- γ 3 were exposed to individual ³⁵S-methionine-labeled AP2 subunits produced by *in vitro* translation and bound protein detected by SDS-PAGE followed by phosphorimaging. Using this approach, we found that the γ -subunit ICDs bound specifically to the μ 2 subunit of AP2 (Fig. 1C). No significant interaction was observed for any of the fusion proteins of GABA_A receptor γ -subunit ICDs with the α , β 2 or σ 2 subunits of the AP2 complex. These results are in agreement with recent work demonstrating that a peptide containing a tyrosine motif within the GABA_A receptor γ 2 subunit ICD (residues 361-370) can directly interact with μ 2-AP2 (Kittler et al., 2008).

3.2 Identification of two AP2 binding sites within the γ 2-subunit

Tyrosine (Yxx Φ) motifs target a variety of cargo proteins including ion channels for clathrin-mediated endocytosis via direct binding to a pocket within subdomain A of μ 2-AP2 {Roche, 2001 #44; Owen, 2004 #4; Bonifacino, 2003 #5; Lavezzari, 2004 #45; Roche, 2001 #44}. A

conserved putative classical Yxx ϕ motif (YECL; residues numbered 367-370 in $\gamma 2S$; see Fig. 2A, B) absent in GABA_A receptor β -subunits, mediates high affinity phospho-dependent binding to $\mu 2$ -AP2 {Kittler, 2008 #57}. To test whether the Yxx ϕ motif (Y³⁶⁷ECL³⁷⁰) was the sole determinant of AP2 binding to the GABA_A receptor $\gamma 2$ -subunit we mutagenised the Y³⁶⁷ residue to alanine in GST- $\gamma 2$ (to give GST- $\gamma 2^{AECL}$; Y to A mutations disrupt AP2 binding to the $\gamma 2$ subunit YECL tyrosine motif; {Kittler, 2008 #57}). GST- $\gamma 2$ wild type or GST- $\gamma 2^{AECL}$ were exposed to ³⁵S labeled $\mu 2$ -AP2 produced by *in vitro* translation and bound protein detected by SDS-PAGE followed by phosphorimaging. Mutation of the $\gamma 2$ subunit residue Y³⁶⁷ to alanine resulted in a substantial decrease in AP2 binding to the GST- $\gamma 2^{AECL}$ (Fig. 2C). Surprisingly however, some AP2 binding remained suggesting that in addition to the YECL motif, at least one additional AP2 binding site must exist in the $\gamma 2$ subunit ICD. To identify additional AP2 binding signals we first opted to truncate the $\gamma 2$ subunit ICD into two halves, GST- $\gamma 2N$ (containing amino acids in the $\gamma 2$ ICD upstream of the YECL motif) and GST- $\gamma 2C$ (which contains the YECL motif) and analyzed their ability to bind AP2 from brain (see Fig. 2B for truncation sites). Both GST- $\gamma 2N$ and GST- $\gamma 2C$ could interact with AP2 from brain lysate (Fig. 2D) further supporting the existence of two AP2 binding sites in the $\gamma 2$ subunit. Since the classical tyrosine motif is present in the C-terminal half of the $\gamma 2$ ICD, a second AP2 binding site must exist in the N-terminal half of the $\gamma 2$ ICD to account for AP2 interaction to this region. To determine the identity of the second $\mu 2$ -AP2 binding site contained within the N-terminal half of $\gamma 2$, we tested binding to ³⁵S labeled μ -AP2 of a series of deletion constructs (GST T1 - T3 $\gamma 2^{AECL}$; see Fig. 2B) where amino acids from the N-terminal region of the $\gamma 2$ ICD were successively deleted (these experiments were carried out using GST- $\gamma 2$ deletion constructs containing Y³⁶⁷ mutated to alanine A to minimize the contribution of the YECL motif to the AP2 binding signal). GST- $\gamma 2^{AECL}$ deletions were exposed to ³⁵S labeled $\mu 2$ -AP2 and bound complexes resolved by SDS-PAGE and detected by phosphorimaging. The truncation approach revealed that an approximately 20 amino acid stretch (residues 318-337) in the $\gamma 2$ -subunit ICD, proximal to TM3, also contained an AP2 binding site since deletion of this domain abrogated remaining $\mu 2$ -AP2 binding to these GST- $\gamma 2^{AECL}$ T1-T3 deletion constructs (Fig. 2E).

3.3 An atypical AP2 binding mechanism conserved across GABA_A receptor subunits

We have previously demonstrated that GABA_AR β -subunits interact with subdomain B of $\mu 2$ -AP2 (see Fig. 3A) via an atypical $\mu 2$ -AP2 interaction motif, rich in basic amino acids (residues K⁴⁰¹THLRRRSQLK⁴¹² in the $\beta 3$ -subunit; {Kittler, 2005 #18}) homologous to a similar basic patch $\mu 2$ -AP2 interaction motif identified in synaptotagmin 1 (Syt1; {Haucke, 2000 #32} Fig. 3C) and AMPA receptors. Analysis of the 20 amino acid sequence in $\gamma 2$ ICD containing the $\mu 2$ -AP2 binding site proximal to TM3 (ie binding in the absence of the YECL motif) revealed that this region contained a basic patch of lysine and arginine residues with substantial homology to that identified in GABA_AR β -subunits (see Fig. 3C). Our results therefore support the existence of two separate AP2 binding sites within the $\gamma 2$ subunit ICD: a basic patch motif conserved with a similar motif identified in receptor β -subunits and a Yxx ϕ motif (Y³⁶⁷ECL³⁷⁰) specific to γ -subunits. To further confirm that the mechanism of basic patch $\mu 2$ -AP2 binding in the $\gamma 2$ subunit was conserved with that identified in receptor β -subunits {Kittler, 2005 #18}, we also identified the region in $\mu 2$ -AP2 important for binding to the $\gamma 2$ subunit basic patch. Basic patch motifs interact with subdomain B on $\mu 2$ -AP2 whereas Yxx ϕ motifs do not (see Fig. 3A). In agreement with this, GST fusion protein pull downs, with either the GST- $\gamma 2^{AECL}$ mutant or GST- $\gamma 2N$ (both of which only contain the basic patch binding site), with ³⁵S labeled truncations of $\mu 2$ -AP2 revealed that the $\gamma 2$ -subunit basic patch binds to the same region of $\mu 2$ -AP2 (subdomain B, residues 283-394; Fig. 3B) previously identified for basic patch binding of GABA_AR β -subunits {Kittler, 2000 #11}. In contrast amino acids in the C-terminal region in $\mu 2$ -AP2 that are critical for YECL motif binding (Subdomain A: residues 407 – 435; Fig. 3A) were not needed for the basic patch interaction (Fig. 3C).

The similarity between the basic patch AP2 binding motifs found in syt1, GABA_A β -subunits and within γ 2 as shown here (Fig. 3C) and the fact that these proteins bind to the same subdomain B of μ 2-AP2 suggests that all of these interactions occur via the same mechanism (involving μ 2-AP2 subdomain B residues 283-394; Fig. 3A,B). To further confirm this we took advantage of the observation that a peptide from syt1 (syt1-pep) binds with high affinity to the basic patch motif binding site in μ 2-AP2 {Grass, 2004 #33} and would therefore be predicted to significantly compete basic patch dependent μ 2-AP2 binding to GABA_A receptor β - and γ 2- subunits. In agreement with this we found that the interaction of GST- β 3 or GST- γ 2^{AECL} (we used GST- γ 2^{AECL} for these experiments to remove the tyrosine motif binding component of the μ 2-AP2 interaction) with His₆-tagged P2 (residues 157-435; hereafter His- μ 2) was significantly inhibited in the presence of syt1-pep, but not in the presence of a scrambled (random sequence) control peptide (Fig. 3D). These results further support that a conserved basic patch μ 2-AP2 binding mechanism exists in GABA_A receptor β - and γ -subunits.

3.4 Functional consequences of targeting μ 2-AP2 binding to GABA_A receptor subunits via their basic patch binding mechanism

Our results from the above *in vitro* binding studies predicted that the syt1-pep could effectively compete binding of AP2 to $\alpha\beta\gamma$ containing GABA_A receptors by simultaneously targeting basic patch dependent μ 2-AP2 binding to both β and γ subunits. We used this peptide as a tool for investigating the functional consequences of acutely disrupting basic patch motif dependent AP2 recruitment to $\alpha\beta\gamma$ GABA_A receptors. We carried out whole-cell patch clamp electrophysiological experiments to monitor the effects on inhibitory synaptic transmission of dialyzing syt1-pep into neurons via the patch pipette. This approach has the important advantage that the syt-1 peptide can disrupt AP2 binding to the GABA_A receptor ICDs without altering recruitment of any other GABA_A receptor associated proteins which may bind to the receptor via overlapping domains. Since dialysis of the peptide occurs from the patch pipette into the soma of an individual postsynaptic neuron, it is not expected that this peptide will affect the syt1-AP2 interaction because the locus of this protein complex is exclusively at pre-synaptic terminals (and hence not in the recorded peptide dialyzed neuron). We and more recently others have reported that blocking dynamin and AP2-dependent endocytosis of GABA_A receptors with a peptide that targets the function of the GTPase dynamin (P4 peptide) results in an increase in the amplitude of mIPSCs in cultured cortical neurons, GABA_A receptor current in dissociated prefrontal cortical neurons and inhibitory postsynaptic currents (IPSCs) in brain slices {Kittler, 2005 #18; Kittler, 2000 #11; Blanchet, 2003 #46}. We predicted that syt1-pep, which readily competes basic patch motif dependent AP2 binding to the GABA_A receptor, would similarly reduce GABA_A receptor internalization and cause an increase in mIPSC amplitude. As shown in Fig. 4A, C and D neurons dialysed with syt1-pep via the patch pipette caused a sustained increase in mIPSC amplitude over a 60 min time course (pep-Syt1: $16.2 \pm 2.6\%$, $n=5$) compared to a control scrambled peptide (which does not block interaction with AP2) over the same 60 min time course (control: $-1.7 \pm 1.2\%$, $n=5$; Fig. 4B,C and D). Dialysis of the syt1-pep also caused a significant enhancement in mIPSC frequency within 60 min whereas the control peptide was ineffective (syt1-pep: $17.5 \pm 2.4\%$, $n=5$; control: $-3.6 \pm 1.4\%$, $n=5$). Presumably the enhancement of frequency is due to the recruitment of mIPSCs previously below the threshold of detection, owing to an increased number of surface active GABA_A receptors as has been described previously {Kittler, 2000 #11; Kittler, 2005 #18}. Representative cells showing the effect of syt1-pep and control peptide on mIPSC are illustrated in Fig. 4C. We therefore conclude that the identified basic patch μ 2-AP2 binding mechanism, conserved within the ICDs of GABA_A β - and γ -subunits, plays a critical role in regulating the number of surface and synaptic GABA_A receptors.

3.5 Simultaneous targeting of GABA_A receptor basic patch and γ 2 subunit specific YECL μ 2 interactions causes an additive enhancement of mIPSCs

Our results demonstrate that acutely targeting the GABA_A receptor interaction with μ 2-AP2, via either the conserved basic patch mechanism using syt1 pep (Fig 4A-D) or via the γ 2 subunit specific YECL motif (Kittler et al., 2008) results in a rapid increase in mIPSC amplitude suggesting that both these μ 2-AP2 interaction mechanisms are important regulators of the number of surface and synaptic $\alpha\beta\gamma$ containing GABA_A receptors. We also investigated the consequences of targeting both μ 2-AP2 interaction mechanisms on inhibitory synaptic responses by co-dialysing syt1-pep and an inhibitory tyrosine motif peptide (YECL-pep; Kittler et al., 2008), on the size of mIPSCs. As shown in Fig. 5A-D, co-dialysis of syt1-pep and YECL-pep over the same 60 min time course produced a significant additive effect on mIPSC amplitude compared to interfering with GABA_AR-AP2 interactions individually using syt-1 or YECL peptides alone (syt1-pep and YECL-pep: $31.7 \pm 3.2\%$, $n=7$; syt-pep: 16.2 ± 2.6 , $n=5$; YECL-pep: $20.9 \pm 2.4\%$, $n=7$). Representative cells showing the time dependent effect of syt1-pep + YECL-pep and on mIPSC are illustrated in Fig. 5A, B. Together, our results suggest that there are two mechanisms for AP2 binding to GABA_A receptors and that simultaneously inhibiting both mechanisms, results in an additive effect suggesting that these two AP2 interaction mechanisms can act either separately or in concert to regulate synaptic receptor number.

Discussion

Here, we have further investigated the molecular determinants that regulate the binding of the GABA_A receptor γ 2 subunit with the AP2 complex, a critical regulator of GABA_A receptor endocytosis. These studies have revealed two binding mechanisms for AP2 within GABA_A receptor γ 2 subunits: a basic patch AP2 binding motif (in the μ 2S subunit residues 317–333) identical to that previously identified in GABA_A receptor β -subunits and a γ 2 subunit specific Y³⁶⁵GY³⁶⁷ECL motif within the γ 2 subunit consensus site for tyrosine phosphorylation. Electrophysiological and biochemical results in the present study provide supporting evidence that the basic patch interaction mechanism identified in the γ 2 subunit is conserved with that in receptor β -subunits. Both the atypical basic patch mechanism and the classical YECL type γ 2 subunit specific mechanism of AP2 interaction are important determinants of synaptic GABA_A receptor number, which can act either separately or in concert to regulate the inhibitory synaptic response.

An interesting finding of the current study is that there are two binding sites for AP2 within γ 2 subunits. Using GST fusion protein pull downs we demonstrate that in addition to a YECL type γ 2 motif (Y³⁶⁵GY³⁶⁷ECL) a second AP2 binding mechanism must exist since deletion of this motif does not completely abrogate interaction with AP2. In agreement with this a truncation mapping approach reveals a second AP2 binding site within the γ 2 subunit ICD, proximal to TM3. This motif has substantial sequence similarity with an atypical basic patch AP2 interaction motif identified in receptor β -subunits.

We provide several lines of additional evidence to support a conserved basic patch mechanism of AP2 binding across GABA_AR β - and γ -subunits. The basic patch motifs in both subunit families exhibit significant sequence similarity and both exhibit binding to μ 2-AP2 subdomain B (residues 283-394). Binding via this motif in either β - or γ 2- subunits can be effectively competed by the μ 2-AP2 subdomain B interacting Syt-1 peptide {Haucke, 2000 #32; Grass, 2004 #33}. Competing AP2 binding to native GABA_A receptors via the conserved basic patch mechanism using syt-1 peptide increased the amplitude of mIPSCs, an effect that is similar to that previously observed on mIPSCs upon blocking GABA_A receptor internalization by using a peptide that targets the function of the GTPase dynamin or a peptide of the basic patch AP2 binding motif in GABA_A receptor β -subunits (Kittler et al., 2000; 2005). Our current results

therefore, further confirm functionally the role of GABA_A receptor basic patch AP2 binding mechanism in regulating the strength of inhibitory synaptic transmission. Since the syt1-pep competitively inhibits basic patch binding of GABA_A receptors to AP2 by specifically targeting the μ 2-AP2 subdomain B binding pocket, but is not derived from the GABA_A receptor subunit sequence it would not be expected to inhibit binding of GABA_A receptors to any other GABA_A receptor associated proteins. Therefore, the observation that syt1-pep alters inhibitory synaptic transmission further validates our previous interpretation of the critical role of basic patch dependent AP2 binding mechanisms for regulating synaptic GABA_A receptor number {Kittler, 2005 #18}.

We have recently demonstrated that a Yxx ϕ type motif binding mediates AP2 binding in GABA_A receptor γ 2-subunits and is important for the accumulation of synaptic GABA_A receptors. In contrast to the basic patch motifs, the YECL motif interacts with the μ 2-AP2 Yxx ϕ motif binding pocket which is critically dependent on the C-terminal 28 residues in μ 2-AP2 but independent of the basic patch interaction site in subdomain B. Importantly, we demonstrate that the basic patch and YECL motif AP2 binding mechanisms, in addition to acting separately, can also act in concert to modulate the number of synaptic GABA_A receptors. Co-Dialysis of syt1 and YECL peptides into neurons had a significant additive effect on increasing the amplitude of mIPSCs. Thus the number of surface and synaptic GABA_A receptors can be controlled by at least two mechanisms for AP2 recruitment to GABA_A receptors which may allow neurons to regulate the rates of endocytosis of cell surface GABA_A receptor populations by different signaling pathways.

The number and activity of postsynaptic GABA_A receptors is a critical determinant of inhibitory synaptic plasticity during development and in the adult: for example during optimization of somatic inhibition at critical period onset in the mouse visual cortex {Katagiri, 2007 #35}, or for the E-S coupling component of long term potentiation {Lu, 2000 #36}. Dynamic changes in the activity and membrane trafficking of GABA_ARs has also been proposed to underlie pathological alterations in inhibition and neuronal excitability in conditions such as epilepsy and stroke. The characterisation of the critical AP2 interaction mechanisms within GABA_AR β - and γ -subunits will facilitate better understanding of the role of GABA_A receptor internalization in alterations in inhibitory synapse strength during plasticity and in disease .

Acknowledgements

This work was supported by UK Medical Research Council funding to J.T.K. and S.J.M.. V.H gratefully acknowledges support by the German funding agency DFG (SFB 449, TP A11 and Z3).

Figure 1. The γ -subunit intracellular domains interact with the $\mu 2$ subunit of AP2
 (A) Diagram of AP2 tetrameric structure and direct interaction of $\mu 2$ -AP2 with cargo. (B) Interaction of $\mu 2$ -AP2 from brain lysate with GST $\gamma 1$ - $\gamma 3$ subunits but not GST alone. (C) Interaction of ^{35}S labeled $\mu 2$ -AP2 but not ^{35}S labeled $\alpha 2$ -AP2, $\beta 2$ -AP2, $\sigma 2$ -AP2 with GST $\gamma 1$ - $\gamma 3$ subunits. GST alone does not interact with ^{35}S labeled $\mu 2$ -AP2.

Figure 2. Two AP2 binding sites are present in the $\gamma 2$ subunit

(A) Sequence alignment of $\gamma 1$ - $\gamma 3$ subunit intracellular domains showing a conserved basic patch domain (basic residues in blue) proximal to TM3, Yxx ϕ motif (green) and Y³⁶⁷ in red. The 8 amino acid splice insert of $\gamma 2L$ is also shown (grey). T1-T3 denote truncation sites of GST- $\gamma 2$ AECL fusion constructs. (B-E) Mapping AP2 binding sites in the $\gamma 2S$ intracellular domain (ICD). (B) Schematic diagram of GST- $\gamma 2S$ fusion proteins used for mapping experiments (Y³⁶⁷A mutation shown in red). (C) Reduced interaction of GST- $\gamma 2AECL$ mutation. (D) Both halves of the $\gamma 2S$ ICD independently interact with AP2 from brain. (E) Truncation mapping in the absence of the YECL motif (Y³⁶⁷ mutated to A) reveals a second AP2 interaction motif between residues 318-327.

Figure 3. An atypical AP2 binding mechanism conserved across GABA_A receptor subunits
 (A-C) The basic patch in γ2S binds to subdomain B of μ2-AP2 (A shows adaptin constructs used and the location of basic patch and Yxxφ motif interaction domains as determined for other receptors). (B) The Yxxφ motif binding site is unnecessary for GST-γ2N and GST-γ2AECL which can both interact with μ2-AP2 subdomain B. (C) Alignment of basic patch domains in GABA_A receptor β- and γ2- subunits and in synaptotagmin 1 (syt1). (D) Coomassie stained gels showing the effects of inclusion of syt1 peptide or a scrambled control (sc) peptide on binding of His-μ2 to GST-β3 or GST-γ2AECL. Arrows denote GST-fusion protein and bound μ2-AP2.

Figure 4. Consequences of blocking basic patch binding on inhibitory synaptic responses
(A-D) Consequences of dialysis of syt1 pep or control scrambled peptide on inhibitory synaptic responses. (A,B) Cumulative plots of mIPSC amplitude from syt-1 or control sc peptide injected neurons. (C) Representative traces from syt-1 and control scrambled peptide-injected cells at the 3rd and 60th min. (D) bar plot summary showing the differential effects of syt-1 and control scrambled peptide on mIPSC amplitude and frequency

Figure 5. Functional effects of simultaneously targeting basic patch and YECL motif interactions with μ 2-AP2

(A-D) Effects of co-injecting syt-1 and YECL pep on inhibitory synaptic responses. (A,B) Representative cumulative plots (A) and traces (B) from the 3rd and 60th minute in cells co-dialysed with YECL pep and syt-1 pep compared to control. (C) Plot of normalized mIPSC amplitude as a function of time in cells dialyzed with YECL peptide, syt-1 peptide, YECL + syt-1 peptides or scrambled control. Co-dialysis of syt-1 and YECL peptides causes a marked increase in mIPSC amplitude over dialysis of either peptide alone. (D) Bar plot summary showing the differential effects on mIPSC amplitude of YECL, syt-1 peptides alone or co-dialysed together.