Novel Actions of Neurosteroids on $GABA_A$ Receptors

A dissertation

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ABSTRACT

Gamma-aminobutyric acid type A receptors ($GABA_ARs$) are the principal mediators of inhibitory transmission in the mammalian central nervous system. $GABA_ARs$ can be localized at postsynaptic inhibitory specializations or at extrasynaptic sites. While synaptic $GABA_ARs$ are activated transiently following the release of GABA from presynaptic vesicles, extrasynaptic $GABA_ARs$ are activated continuously by resting GABA concentrations and thus mediate tonic inhibition. In the hippocampus and thalamus, extrasynaptic $GABA_ARs$ are predominantly composed of α4 β2/3 and δ subunits. The tonic inhibitory currents mediated by extrasynaptic $GABA_ARs$ control neuronal excitability and the strength of synaptic transmission. However, the mechanisms by which neurons control the functional properties of extrasynaptic $GABA_ARs$ had not yet been explored.

Phosphorylation of residues within synaptic $GABA_AR$ subunits is critical for the assembly, trafficking and cell surface stability of synaptic $GABA_AR$ subtypes. Here we have identified serines 443 in the α4 and serines 408/409 in the β3 subunit as the principal sites for PKC phosphorylation in extrasynaptic $GABA_AR$ subtypes.

Steroid metabolites of progesterone and deoxycortisone (known as neurosteroids) have been shown to be potent positive allosteric

modulators of extrasynaptic $GABA_A$Rs. However, the exact mechanisms by which neurosteroids alter extrasynaptic $GABA_A$Rs function are not well understood. Previous experiments have suggested that Protein Kinase C (PKC) activity is required for neurosteroid-mediated modulation of $GABA_A$Rs. We show that the deoxycortisone metabolite, Tetrahydrodeoxycorticosterone (THDOC) induces the phosphorylation of the α4 $GABA_A$R subunit on serine 443 (S443) dependent upon the ability of this agent to activate PKC. We analyzed the functional significance of THDOC induced phosphorylation of the α4 subunit using patch-clamp recording and TIRF microscopy. Collectively these approaches revealed that THDOC increases the cell surface stability of α4-containing $GABA_A$Rs by promoting their insertion into the plasma membrane, a phenomenon critically dependent on S443 in the α4 subunit and on S408/409 within the β3 subunit when co-expressed. Significantly, the ability of THDOC to increase the expression levels of $GABA_A$Rs was independent of the δ subunit and glutamine 246 (Q246) in the α4 subunit, a site critical for allosteric neurosteroid potentiation.

Collectively we have identified a novel mechanism by which neurosteroids can induce long-term changes in the expression levels of $GABA_A$Rs via PKC dependent phosphorylation of the α4 subunit.

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LIST OF COMMONLY USED ABREVIATIONS

ACSF-Artificial Cerebrospinal Fluid

AP2-clathin adaptor protein 2

BBS- α-bungarotoxin binding site

Bgt-α-bungarotoxin

BIG2-Brefeldin A-inhibited Guaninie Nucleotide-exchange protein

COS-7-CV-1 monkey cell line in origin containing SV40 genetic material

CRH-Corticotropin Releasing Hormone

ER-Endoplasmic Reticulum

GABA- γ-aminobutyric acid

$GABA_ARs$-γ-aminobutyric acid type A receptors

$GABA_BRs$-γ-aminobutyric acid type B receptors

GABARAP-$GABA_AR$ associated protein

GFX-GF 109203X

GODZ-The Golgi-specific DHHC zinc finger protein

HEK293-Human Embryonic Kidney 293 cells

HPA-Hypothalamic-Pituitary-Adrenal Axis

NHS-SS-biotin-succinimidyl 2-(biotinamido)-ethyl-1,3′ dithiopropionate

NSF-*N*-ethylmaleimide-sensitive factor

PDBu-phorbol 12,13-dibutyrate

pHluorin-pH sensitive green fluorescent protein

PKC-Protein Kinase C

RFP-Red Fluorescent Protein

STAR-Steroidogenic Acute Regulatory Protein

THDOC- Allotetrahydrodeoxycorticosterone

THP

TIRF-Total Internal Reflection Fluorescence

TM-Transmembrane domain

3β-HSD-3β Hidroxysteroid Dehydrogenase

3α-HSD-3α-Hidroxysteroid Dehydrogenase

CHAPTER 1

INTRODUCTION

GABA: Main mediator of Neuronal Inhibition in the CNS

The neurotransmitter γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system (CNS). GABA is synthesized in the brain from glutamate through the action of the enzyme L-Glutamic acid Decarboxilase (GAD), which catalyzes the decarboxylation of glutamic acid to form GABA (Erlander et al. 1991). In mammals, GAD exists as two isoforms: GAD65 and GAD67 (with molecular weights of 65 and 67kDa respectively). These two isoforms are encoded by different genes and have a distinct regional distribution throughout the brain (Erlander et al. 1991, Sheik et al.1999). GAD65 is membrane-bound and primarily responsible for vesicular GABA production, whereas GAD67 is located in the cytoplasm and is responsible for cytoplasmic GABA production (Erlander et al. 1991)

After release from pre-synaptic vesicles, GABA is removed from the synaptic cleft by specialized membrane-bound transporters. GABA uptake is a sodium- and chloride-dependant process mediated by a group of genetically related GABA transporters, GAT-1 to GAT-4 (Liu et al. 1993). In the mammalian brain, GABA uptake is primarily mediated by action of GAT-1 (Minelli et al. 1995). GAT-1 is localized in GABAergic axons and nerve terminals and can also be expressed in glial cells (Minelli et al. 1995).

The inhibitory effects of GABA are mediated by two main classes of GABA receptors: Metabotropic G-coupled receptors (GABA type B receptors-$GABA_BR$) and GABA-gated chloride ion channels (GABA type A receptors -$GABA_AR$).

$GABA_BR$ mediate the slow inhibitory actions of GABA

$GABA_BR$ are widely distributed within the CNS as well as in autonomic presynaptic terminals (Castelli et al. 1999, Bettler and Tiao 2006). They are G-protein coupled receptors that inhibit adenylyl cyclase via the $G_{\alpha i/o}$ subunits of activated G-protein (Hill, 1985). $GABA_BR$ belong to the C family of G-protein coupled metabotropic receptors (Reviewed by Bräuner-Osborne et al. 2007). The members of this family have a large extracellular amino-terminus and seven transmembrane domains (Bräuner-Osborne et al. 2007). $GABA_BR$ assemble into heteromers composed of a $GABA_{B1}$ (which can exist as isoforms $GABA_{B1a}$ or $GABA_{B1b}$) and a $GABA_{B2}$ subunit, both of which are required for receptor activity (Couve et al. 1998, Mohler and Fritchy 1999).

$GABA_BRs$ located at presynaptic sites suppress neurotransmitter release by inhibiting voltage sensitive Ca^{2+}-channels (Thomson et al. 1993, Mintz and Bean 1993), whereas $GABA_BRs$ located at postsynaptic sites induce slow postsynaptic currents via the activation of Kir3-type K^+-channels,

resulting in membrane hyperpolarization and shunting of excitatory currents (Lushner et al 1997, Zilibert et al. 1999). In addition, activation of $GABA_B$Rs inhibits the activity of adenylate cyclase to reduce the efficacy of activity cAMP-dependent signaling pathways (see Bettler and Tiao 2006, Chalifoux and Carter 2011).

$GABA_A$R mediate the fast inhibitory actions of GABA

$GABA_A$Rs are the main mediators of fast synaptic inhibition in the CNS. $GABA_A$Rs are chloride permeable channels (Figure 1) that belong to the cys loop ligand gated ion channel super family. Members of this family include nicotinic acetylcholine receptors (nAChRs), glycine receptors, the serotonin (5-hydroxytryptamine) 5-HT3 receptor and the zinc-activated channel (ZAC) (Reviewed by Conolly and Wafford 2004). For this ion channel superfamily, ligand binding is followed by a change in conformation of the channel protein that allows a net inward or outward flow of ions through the membrane-spanning pore of the channel, depending on the electrochemical gradient of the ion. During early developmental stages, $GABA_A$Rs are primarily depolarizing due to the high intracellular chloride concentrations compared to extracellular chloride levels. In the adult brain, the intracellular chloride concentration is lower compared to the extracellular chloride levels. Therefore in mature neurons, $GABA_A$Rs are generally hyperpolarizing (Rivera et al. 2005). This chloride gradient is maintained primarily by the activity of the K+/Cl- co-

transporter 2 (KCC2). In the majority of adult neurons, activation of $GABA_AR$s results in a rapid chloride ion influx that results in the hyperpolarization of the cell membrane and thus a reduction in the probability for an action potential to be generated. Therefore, $GABA_AR$s play a pivotal role in regulating cellular and network excitability in the CNS, which underlies all physiological and behavioral processes.

The members of cys-loop family form heteropentamers assembled from a wide range of heterologous subunits. To date, 19 $GABA_AR$ subunits have been identified. These subunits are divided into eight classes according to sequence homology; α1–6, β1–3, γ1–3, δ, ε, θ, ρ(1-3), and π (Sieghart and Sperk 2002).

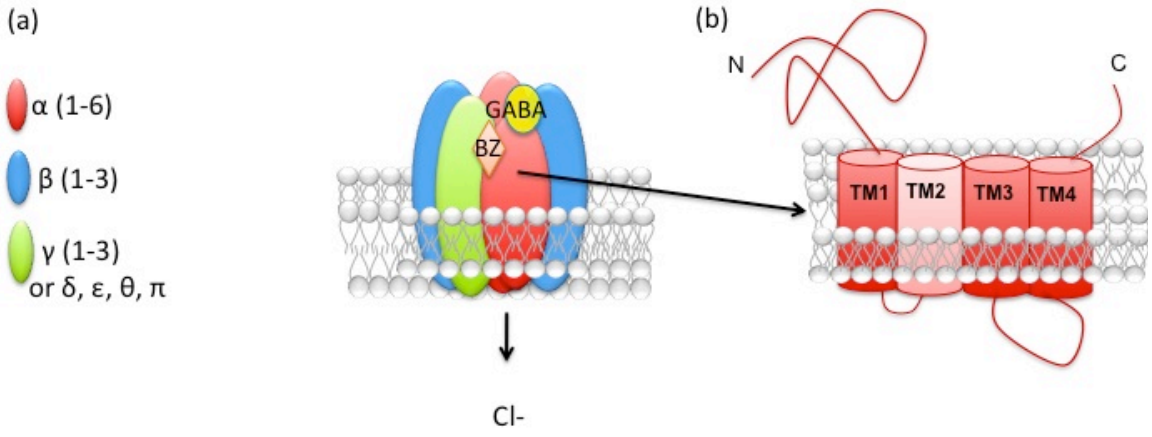

Figure 1: Structure of $GABA_AR$

$GABA_AR$ are composed of five subunits assembled as a heteropentamer. ***(A)*** *The prototypical $GABA_AR$ is composed of two α, two β, and one γ or δ. 19 $GABA_AR$ subunits have been identified to date and they are divided into seven groups on the basis of sequence similarity.* ***(B)*** *The common molecular $GABA_AR$ subunit structure is composed of a large N-terminal extracellular domain, four transmembrane domains, a short C-terminal extracellular domain and a long intracellular loop domain between TM3 and TM4. The intracellular domain contains critical sites for post-translational modifications and protein-protein interactions that modulate receptor activity.*

The genomic location of the 19 genes encoding $GABA_AR$ subunits has been determined, and 14 of them are arranged in $GABA_AR$ gene clusters (Russek 1999). There are gene clusters on chromosomes 4 (α2, α4, β1 and γ1) and 5 (α1, α6, β2 and γ2) as well as on chromosomes 15 (α5, β3 and γ3) and X (α3,θ and ε). The gene for the π subunit is also located on chromosome 5 but at a site distant from the α1,α6 β2 γ2 cluster. Subunits ρ2 and ρ3 are mapped together on chromosome 6, whereas the δ and ρ3 subunits are located on their own on chromosomes 1 and 3 respectively. Each gene cluster contains gene encoding for α,β and γ/ε class (Russek 1999, Darlison et al. 2005). This gene organization has been proposed to be a result of early gene duplication events from a single ancestral αβγ $GABA_AR$ subunit cluster (Russek 1999, Darlison et al. 2005).

Like all other members of the cys-loop family, all $GABA_AR$ subunits share a common structure: they have a large extended amino-terminal extracellular domain containing a cysteine loop signature, four highly conserved transmembrane domains (TMs), and a large intracellular loop domain between TM3 and TM4 (Conolly and Wafford 2004, Arancibia-Cárcamo and Kittler 2009-See figure 1). Using homology modeling based on the structure of the nicotinic acetylcholine receptor (Unwin 1995,O'Mara et al. 2005), it is believed that the lining of the water-filled channel pore is formed by the alignment of TM2 domains when subunits are arranged as a pentameric structure, and they extend through the membrane as alpha-helixes bending outwards (Corringer et al. 2000). TM1, TM3 and TM4 compose the interface with lipids and are thought to isolate TM2 lining from the hydrophobic membrane environment (Connolly and Wafford 2004).

Sites within the extracellular amino-terminal are important for oligomerization of the protein and for subunit-subunit interactions (Jacob et al. 2008, Arancibia-Cárcamo and Kittler 2009). The binding site for GABA is located at the interface between α and β subunits (Connolly and Wafford 2004-See figure 1).

The intracellular domain is the most genetically divergent part of each

individual $GABA_AR$ subunit. This domain is a critical site for cytoplasmic protein interactions with regulatory and signaling molecules including microtubule-binding proteins, cytoskeletal proteins, kinase-anchoring proteins and neurotransmitter transporters (Reviewed by Jacob et al. 2008, Lushner et al. 2011). In addition, the intracellular loop has multiple sites for post-translational modifications such as ubiquitination, palmitoylation and phosphorylation (Reviewed by Kittler and Moss 2003, Jacob et al. 2008). These protein interactions and post-translational modifications modulate receptor activity and trafficking.

Regulation of $GABA_AR$ oligomerization, transport, maturation and trafficking

The heterogeneity of $GABA_AR$ subtypes is restricted primarily by regulation governing the proper assembly of the receptor (Sieghart and Sperk 2002). Although in theory a large number of $GABA_AR$ subtypes could be possible, studies suggest that only a limited number of $GABA_AR$ subunit combinations can oligomerize and reach the neuronal cell membrane (Arancibia-Cárcamo and Kittler 2009).

The oligomerization of $GABA_ARs$ occurs in the Endoplasmic Reticulum (ER). This process is mediated by critical assembly-domains located within the amino-terminals of $GABA_AR$ subunits and occurs within five minutes after translation (Gorrie et al. 1997, Reviewed by Kittler et al.

2002 and Jacob et al. 2008-Figure 2). Receptors must be assembled and reach conformational maturity in the ER before transport to the plasma membrane. Studies in cell lines have shown that most single subunits are not capable of leaving the ER, and are targeted for proteosomal degradation (Reviewed by Moss and Smart 2001, Kittler et al. 2002, Jacob et al. 2008) Although some subunits (β1, β3 and γ2) are able to reach the cell membrane, they are unable to form functional GABA-sensitive channels and are rapidly targeted for degradation (Siegel et al. 1989, Sanna et al. 1995, Krishek et al. 1996, Connolly et al. 1999).

$GABA_A$Rs are typically composed of two α, two β and one γ or δ subunit. However, co-expression of the two α and two β subunits alone can form functional GABA-gated receptors that respond to most $GABA_A$R modulators including bicuculline, picrotoxin and barbiturates (McDonald et al. 1995). $GABA_A$Rs composed of only α and β subunits can form functional receptors in heterologous cell lines.

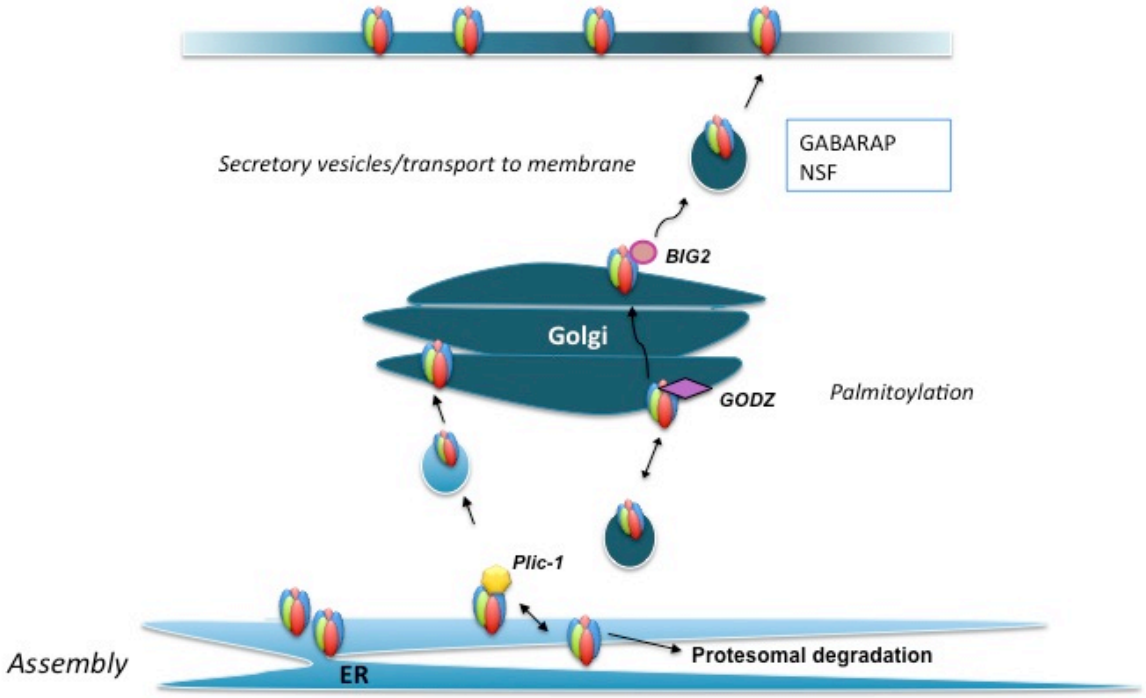

Figure 2: Assembly, maturation and trafficking of $GABA_AR$s

(A) *$GABA_AR$s are assembled as pentamers in the ER. This process is primarily regulated by the interaction of the ER-chaperones BiP and calnexin with residues located in the extracellular domain of $GABA_AR$ subunits. Improperly folded and unassembled $GABA_AR$ subunits are targeted for ubiquitination/proteosomal degradation. The ubiquitin-like protein Plic-1 inhibits degradation via interaction with sites located within the intracellular domain of α and β subunits. Plic-1 promotes $GABA_AR$ transport from the ER to the Golgi apparatus.* ***(B)*** *In the Golgi, the palmitoytransferase GODZ palmitoylates a cysteine residue located within the intracellular loop γ subunit. Palmitoylation promotes $GABA_AR$ transport through the Golgi towards inhibitory synapses in the cell membrane.* ***(C)*** *The translocation of $GABA_AR$ from the Golgi to the cell surface is believed*

to be mediated via BIG2 interaction with the intracellular domain of β subunits as well as other regulatory proteins including GABARAP and NSF.

Several studies have suggested that αβ-$GABA_AR$ exist in small numbers within native environments and they might mediate tonic inhibition in neurons where expressed (Brinkley et al. 1999, Mortensen and Smart 2006). Combinations of αγ and βγ are mostly retained in the ER and when α, β and γ are co-expressed together the formation of αβγ-$GABA_AR$ is strongly favored over receptors composed of only α and β subunits (Angelotti and McDonald 1993).

The assembly and maturation within the ER are regulated by mechanisms involving ER-resident chaperones including Calnexin and Binding inmmunoglobin protein (BiP) (Connolly et al. 1996, Jacob et al. 2008-Figure 2) (Taylor et al. 2000, Klausberg et al. 2001, Ehya et al. 2003, Sarto-Jackson et al. 2006). Calnexin and BiP are involved in regulating quality control of proteins within the secretory pathway (Reviewed by Jacob et al. 2008, Kittler et al. 2009).

After oligomerization in the ER, $GABA_ARs$ are then trafficked to the Golgi apparatus to be sorted into vesicles before insertion in the neuronal plasma membrane. This process is regulated by receptor-associated

proteins that interact with the intracellular loop of $GABA_AR$ subunits (Reviewed by Jacob et al. 2008). Plic-1 (Protein that links integrin-associated protein with the cytoskeleton-1) is involved in $GABA_AR$ stability in the ER and in the transport of the receptor through the secretory pathway. Plic-1 regulates $GABA_AR$ transport and maturation through the secretory pathway via interaction with all isoforms of α and β subunits (Belford et al. 2001). The Plic-1 protein contains ubiquitin-like proteosome binding domains as well as ubiquitin-associated domains and is therefore able to inhibit ubiquitin-mediated proteolysis of $GABA_ARs$ (Walters et al. 2002)

When in the Golgi, γ2-containing $GABA_ARs$ undergo palmitolation via interaction of the Golgi-specific DHHC zinc finger protein (GODZ) with cytoplasmic serine residues within the γ2 subunit. GODZ palmitolation of the γ2 subunit is critical for the subsequent accumulation of synaptic $GABA_ARs$ at inhibitory synapses (Rathenberg et al. 2004)

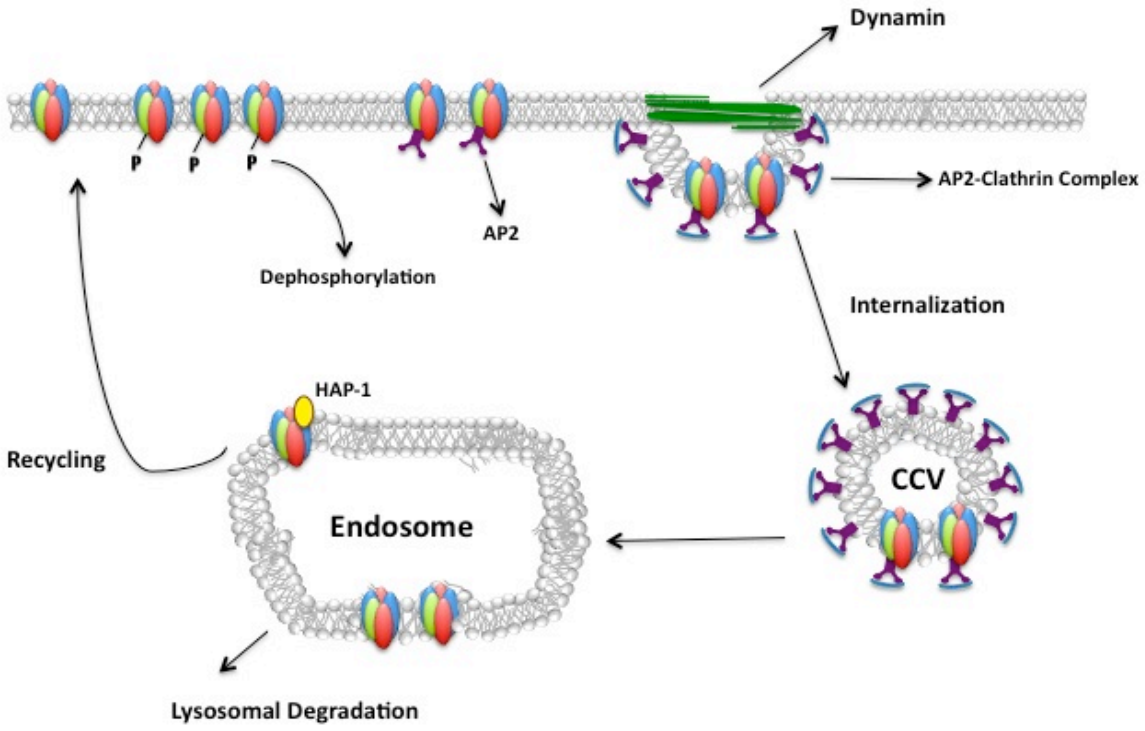

Figure 3: $GABA_AR$ endocytosis and recycling

$GABA_AR$ endocytosis occurs primarily via mechanisms dependant on the formation of clathrin-coated vesicles. Clathrin-mediated endocytosis is regulated in a phospho-dependant manner via the interaction of specific motifs within the intracellular loop of the β and γ subunits with the μ subunit of the AP2 protein. Phosphorylation of residues within the intracellular domain (by kinases PKC/PKA or Fyn/Src) interferes with this interaction and therefore stabilizes $GABA_ARs$ in the cell membrane. When endocytosed, $GABA_ARs$ can be ubiquitinated and degraded via lysosomal degradation. Alternatively, the receptors can interact with regulatory lysosomal proteins such as HAP1, which promotes the transport and recycling of receptors back to the cell surface (Arancibia-Cárcamo et al.,

2009).

$GABA_AR$ endocytosis and recycling

Proper modulation of inhibition through $GABA_ARs$ is dependant on receptor cycling between the cell surface and intracellular compartments (Jacob et al. 2008). To date, a number of regulatory proteins have been implicated in the cycling of $GABA_ARs$ between the cell membrane and intracellular pools. These include BIG2 (Brefeldin-A inhibited GDP/GTP exchange factor 2), GABARAP ($GABA_AR$ associated protein), and components of the endocytotic machinery. BIG2 mediates the $GABA_ARs$ exit from the Golgi towards the cell surface as well as $GABA_AR$ endocytic recycling via interaction with a sequence motif within the intracellular domain of β subunits (Shin et al. 2004, Charych et al. 2004b). GABARAP is an ubiquitin-like protein that interacts with microtubules and with the intracellular loop of all γ subunits (Wang et al. 1999). It is involved in the translocation of $GABA_ARs$ from intracellular compartments to the cell membrane (Leil et al. 2004, Kittler et al. 2004, Terunuma et al. 2004).

Endocytosis of $GABA_ARs$ occurs primarily via mechanisms dependant on dynamin and clathrin (See figure 3). These processes are mediated by the interaction of the clathrin adaptor protein AP2 with residues located within the cytoplasmic intracellular loop of the β and γ subunits (Kittler 2000, 2005, 2008). Work by Kittler et al. 2005, 2008 identified a ten amino acid

motif within the cytoplasmic domain of all β subunits, as critical for mediating the $GABA_AR$ interaction with the μ2 subunit of the AP2 protein. This motif contains a major phosphorylation site (serines 408 and 409) conserved among all β subunits (Kittler et al. 2005,2008).

The interaction of the μ2 subunit with this motif within the β subunit is negatively regulated by phosphorylation at serines 408 and 409. That is, AP2 can bind the $GABA_AR$ and trigger receptor internalization only when this site is dephosphorylated (Kittler et al. 2005). Serines 408 and 409 can be phosphorylated by Protein Kinase A (PKA), Protein Kinase C (PKC), Calcium/Calmodulin-dependant Kinase II (CAMKII) and AKT (McDonald and Moss 1994, McDonald et al. 1998, Brandon et al. 2000, Brandon et al. 2002, Brandon et al. 2006, Xu et al. 2006).

The AP2 μ2 subunit also interacts with two motifs within the intracellular domain in the γ subunit. One consists of a twelve amino acid domain analogous to the AP2 binding site in the β subunit (Smith et al. 2008). The second is a high affinity γ-specific site YGYECL motif that contains a phosphorylation site at tyrosines 365/367 (Smith et al. 2008, Kittler et al. 2008) These residues can be phosphorylated by Fyn kinase and other members of the Src-family of tyrosine kinases (Jurd et al. 2010) and the phosphorylation of these sites interferes with AP2 binding and hence prevents $GABA_AR$ internalization (Boehm et al. 2004, Tretter et al. 2009).

If internalized, $GABA_A$Rs in endosomes can become ubiquitinated and targeted for lysosomal degradation (Arancibia-Cárcamo et al. 2009). Alternatively, regulatory proteins such as the Huntingtin-associated protein 1 (HAP1-Kittler et al. 2004b, Twelvetrees et al. 2010) and the Calcium Modulating Cyclophilin Ligand (CAML- Yuan et al. 2008) can interact with the cytoplasmic domains of the β and γ subunits respectively, and in this way facilitate vesicular transport and recycling back to the cell membrane.

Subcellular localization of $GABA_A$R subtypes

The heterogeneity of $GABA_A$R subunit composition is also carefully regulated by regional and temporal specificity in the patterns of expression in the CNS. This heterogeneity allows for $GABA_A$Rs with different physiological and pharmacological properties as well as differential expression throughout the brain (Rudolph *et al.* 2001, Sieghart and Sperk 2002). $GABA_A$Rs of different subunit composition have different subcellular localization (Connolly *et al.* 1996). $GABA_A$Rs composed of the α1–3, β1–3 and γ2 are predominantly located at synaptic sites (Figure 2-Rudolph and Mohler 2006), whereas $GABA_A$Rs composed of the α4-6, β2/3 and δ subunits are primarily localized at sites distant from synapses or extrasynaptic sites (Figure 4-Farrant and Nusser 2005, Chandra et al. 2006, Zheleznova et al. 2009).

The cell membrane distribution of synaptic and extrasynaptic $GABA_A$Rs is dynamically regulated via the interactions with sub-synaptic scaffold molecules. During synaptic $GABA_A$R membrane targeting, receptors are first introduced to the neuronal membrane at extrasynaptic sites (Bogdanov et al. 2006), followed by subsequent lateral membrane diffusion to synaptic sites (Bogdanov et al.2006).

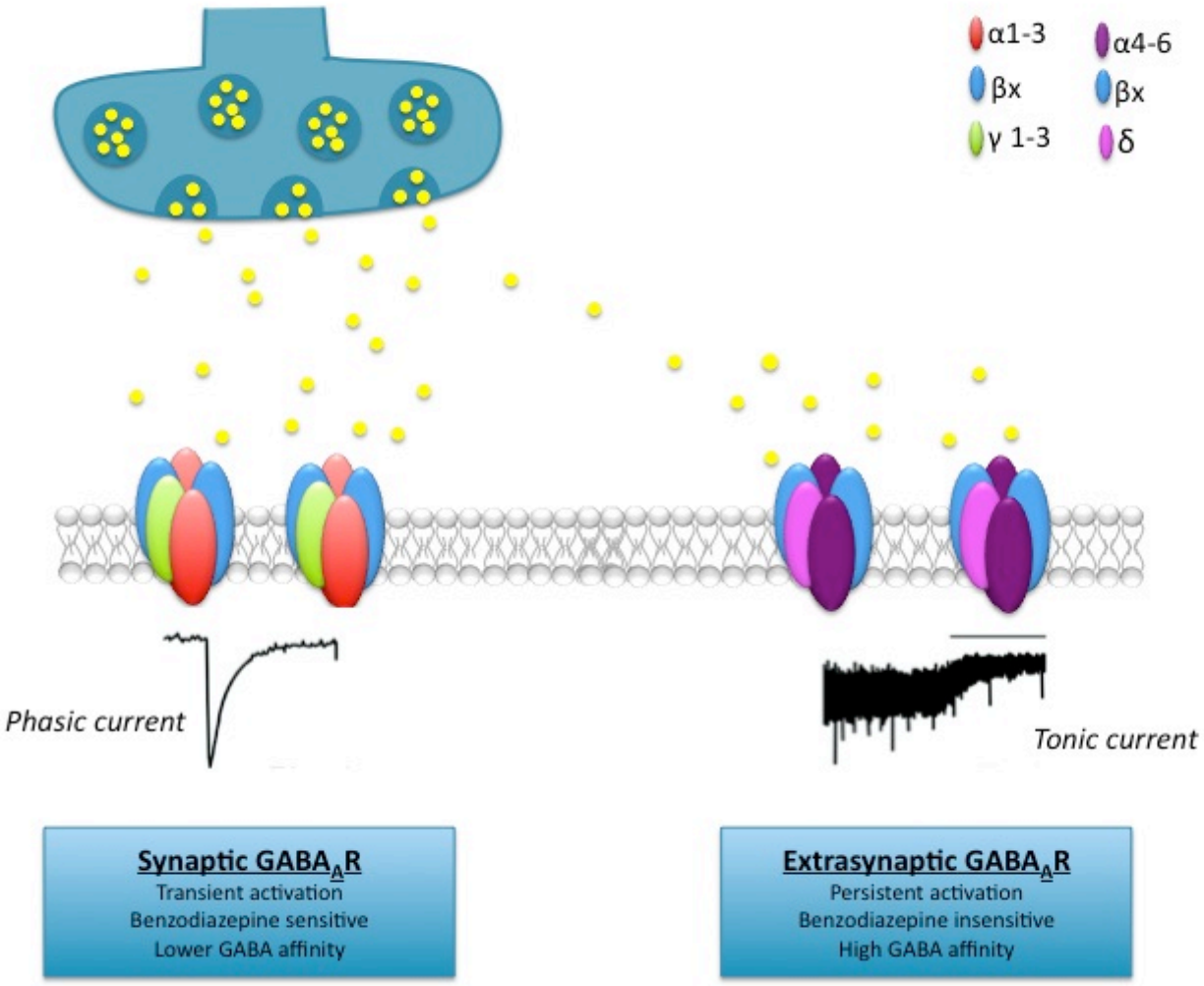

Figure 4: Subcellular localization and function of GABA$_A$R subtypes

Synaptic GABA$_A$Rs are primarily composed of α1–3, β1–3 and γ2 subunits and they are activated in a transient (phasic) manner by high concentrations of GABA released from directly opposed pre-synaptic vesicles. Synaptic GABA$_A$Rs mediate a short-lived hyperpolarization that transiently reduces the probability for an action potential to occur. Extrasynaptic GABA$_A$Rs are primarily composed of α4-6, β2/3 and δ subunits and they are activated in a persistent manner by low

concentrations of GABA that escape from the synapse to the extracellular space. (or released from glia/neurogliaform cells). Extrasynaptic $GABA_ARs$ mediate an uninterrupted (tonic) form of inhibition that persistently reduces the action potential probability.

Scaffold proteins located at post-synaptic localizations bind and cluster surface synaptic $GABA_ARs$ at sites directly opposite GABA releasing axon terminals (Reviewed by Jacob et al. 2008). The sub-membrane scaffold protein gephryrin has a critical role in regulating the clustering of synaptic $GABA_ARs$ at inhibitory synapses (Levi et al. 2004, Mukerjee et al. 2011). Gephryn is the principal scaffolding molecule for both glycinergic and GABAergic synapses (Fritschy et al. 2008, Mukerjee et al. 2011). It interacts with proteins that regulate microfilament dynamics (such as profiling I and II) and with microtubules (Kirsh et al. 1995, Mammoto et al. 1998). The Gephryn-microtubule/microfilament interactions allow for the formation of a hexagonal protein lattice that allows for the organization of synaptic $GABA_ARs$ distribution in the cell membrane (Lushner et al. 2011). Synaptic adhesion molecules play an important role in the maturation and stabilization of inhibitory synapses (Ullrich et al. 1998, Jamain et al. 2008, Cheng et al. 2010). Tran-synaptic complexes of pre-synaptic β-neurexins and post-synaptic Neuroligin 2 (NL2) contribute to the proper alignment of pre- and post- synaptic molecules and structural maturation of inhibitory synapses (Chang et al. 2010).

The cytoskeletal interactions that involve the anchoring at specific membrane localizations for those $GABA_AR$ located at extrasynaptic sites are less understood than for synaptic receptors. The cytoskeletal protein Radixin (a member of the ERM-ezrin, radixin and moesin family) has been implicated in the actin cytoskeleton anchoring of the predominantly extrasynaptic α5 subunit (Loebrich et al. 2006). However, the functional relevance of α5-containing $GABA_AR$ clustering at extrasynaptic sites is unknown. The expression of a dominant-negative radixin construct in neurons abolishes the membrane clustering of α5-containing $GABA_AR$ but has no effect on GABA-mediated currents (Loebrich et al. 2006).

Synaptic $GABA_ARs$ mediate phasic inhibition

$GABA_ARs$ located at synaptic and extrasynaptic sites are activated in a different manner and mediate distinct forms of inhibition. Synaptic $GABA_ARs$ are activated in a transient manner after brief exposure to high concentrations of GABA released from the presynaptic membrane (See Figure 4). This transient activation of synaptic $GABA_ARs$ results in phasic inhibition (Jacob et al. 2008). Activation of synaptic $GABA_ARs$ results in a rapid chloride ion influx that creates a transient or phasic, but significant reduction in the probability for an action potential to be generated due to rapid hyperpolarization of the plasma membrane (Macdonald et al. 1994).

Extrasynaptic $GABA_AR$s mediate tonic inhibition

Extrasynaptic $GABA_AR$s can be activated in a persistent, less temporally restricted manner by low ambient concentrations of GABA (See Figure 4) that either escapes from the synapse into the extracellular space or is released from non-synaptic sites (such as neurogilaform cells and astrocytes-Kozlov et al. 2006,Olah et al 2009). When activated, extrasynaptic $GABA_AR$s generate an uninterrupted form of conductance that is referred to as tonic inhibition. The tonic inhibition mediated by extrasynaptic $GABA_AR$s results in a persistent reduction in the cell's input resistance. This results in a reduction in both the size and duration of excitatory post-synaptic potentials and will in turn reduce the temporal and spatial frame for synaptic integration. Ultimately, tonic inhibition persistently reduces the likelihood for an action potential to occur.

Tonic currents mediated by extrasynaptic $GABA_AR$s were described for the first time in layer III cells of the somatosensory cortex where application of Bicuculline methiodide (a $GABA_AR$ antagonist) resulted in a reduction in the holding current or the current required to hold the cell at a fixed potential (Salin and Prince 1996). Tonic currents have also been described in cerebellar and dentate granule cells (Brickley et al. 1996, Overstreet and Westbrook, Nusser and Mody 2002), hippocampal interneurons and pyramidal cells (Semyanov et al. 2003, Bai el al 2001),

embryonic and developing neurons (Loturco et al. 1995, Ge et al. 2006), neocortical layer 2/3 pyramidal cells (Drasbek and Jensen 2006, Drasbek et al. 2007), the spinal cord (Cronin et al. 2004), and in several sub cortical structures including hypothalamic (Sergeeva et al. 2005, Sarkar et al. 2011), thalamocortical neurons (Cope et al. 2005, Belleli et al. 2005) and medium spiny neurons of the striatum (Ade et al. 2008, Santhakumar et al. 2010).

The fact that $GABA_AR$-mediated tonic inhibition occurs in many brain regions, changes during different developmental stages (Loturco et al. 1995, Ge et al. 2006) and exhibits cell-type specific differences in magnitude suggests critical physiological roles for tonic inhibition. Indeed, tonic inhibition appears to be essential for the control of firing frequency (Rossi et al. 2003), for modulation of the firing threshold (offset), and the gain of transmission (Hamman et al. 2002, Mitchell and Silver 2003, Pavlov et al. 2009), all of which ultimately modulate network excitability (Farrant and Nusser, 2005, Vida et al. 2006). During development, depolarization mediated by tonic currents has a critical role in neuronal migration, dendritic arborization, and the formation of synapses (Represa et al. 2005. Ge et al. 2006).

Extrasynaptic $GABA_AR$ properties

There are several important properties of extrasynaptic $GABA_A$Rs that make them uniquely suited for tonic inhibition. They exhibit a high affinity for GABA and have little or no desensitization (Hass and McDonald 1999, Farrant and Nusser 2005). In addition, extrasynaptic $GABA_A$Rs exhibit different pharmacological properties from their synaptic counterparts; they are largely insensitive to benzodiazepines (Nusser and Mody 2002, Cope et al. 2005) and they are highly sensitive to THIP (4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridin-3-ol/Gaboxadol) a selective $GABA_A$R agonist (Brown et al., 2002; Wohlfarth and Macdonald, 2002). Low concentrations of the $GABA_A$ antagonist Gabazine (SR95531) abolish inhibitory post synaptic currents while having a small or no effect on $GABA_A$R-mediated tonic conductance (Stell and Mody 2002, Semyanov et al. 2003). Furthermore, extrasynaptic $GABA_A$Rs are targets for various endogenous and exogenous molecules, including anticonvulsants, anesthetics and neurosteroids (Belleli et al. 2002, Bianchi and MacDonald 2003, Stell et al .2003).

Extrasynaptic $GABA_A$Rs are uniquely sensitive to neurosteroids

Neuroactive steroids can originate as metabolites of systemically produced progesterone or deoxycorticosterone, but can also be synthesized *de novo* in the brain by neurons and glia (Compagnone and Mellon 2000, Belleli and Herd 2003, Mellon et al. 2004, Maguire and Mody 2005). The enzymes and steroid mitochondrial transporters necessary for

de novo synthesis of pregnane neuorosteroids are present in many CNS regions (Figure 5-Mellon et al. 2001, 2004). The P450cc mitochondrial cholesterol side-chain cleavage enzyme (P450cc) catalyzes the rate limiting step in *de novo* neurosteroid synthesis in which cholesterol is converted to pregnenolone (Figure 5-Mellon et al. 2005).

In addition, the enzymes 5α-reductase and 3α-hydroxysteroid dehydrogenase, which are required for the synthesis of 3α-hydroxy-5α-pregnane-20-one/Allopregnanolone (THPROG- from progesterone) and 3α,5α-3,21-dihydroxypregnan-20-one/Tetrahydrodeoxycorticosterone (THDOC- from deoxycorticosterone), have been shown to be expressed in the brain in a region and cell-type specific manner (Mellon et al. 2004, Agís-Balboa et al. 2006). Unlike classical steroids, which act via their nuclear receptors to regulate gene expression, neurosteroids rapidly alter neuronal excitability via non-genomic mechanisms. Pregnane steroids containing a 3-α hydroxy ring have been shown to be potent steroselective allosteric modulators of $GABA_A$Rs, having anxiolytic, anticonvulsant sedative and anesthetic effects (Paul and Purdi 1992, Majewska 1992, Olsen and Sapp 1995, Gasior et al. 1999, Goodchild et al. 2001)

Extrasynaptic $GABA_A$Rs containing the δ are the most sensitive to neurosteroid modulation (Belleli et al. 2002, Bianchi and MacDonald 2003,

Stell et al .2003). Low physiological concentrations (10-100nM) of 3α,5α-THDOC greatly enhance the tonic conductance mediated by extrasynaptic $GABA_A$Rs with little or no effect on the phasic conductance mediated by synaptic $GABA_A$Rs, in both dentate gyrus and cerebellar granule cells (Stell et al. 2003). Neurosteroids increase the open duration and the frequency of $GABA_A$R channel openings with no effect on the single channel conductance (Callachan et al. 1987, Lambert et al. 1987, Twyman and MacDonald 1992).

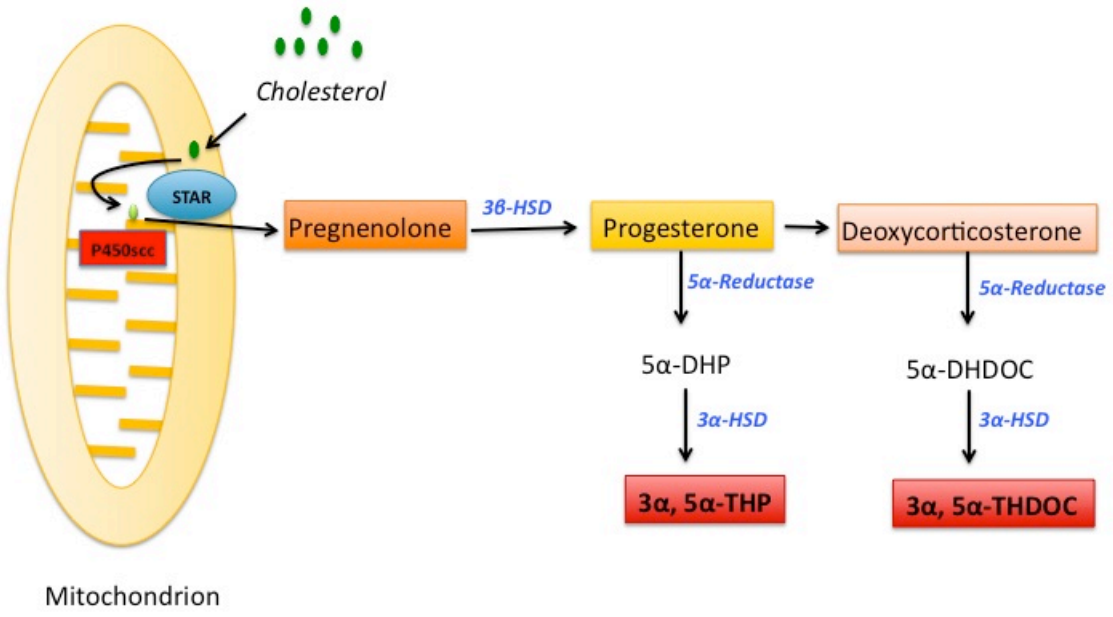

Figure 5: Neurosteroid Synthesis

*Biosynthesis of THDOC and Allopregnanolone from **(A)** cholesterol or **(B)** metabolized from deoxycortisone and progesterone. **(A)** The first limiting step in neurosteroid synthesis is the conversion of cholesterol to pregnenolone by the P450cc Mitochondrial cholesterol side-chain cleavage enzyme (P450cc). P450cc mediates the 20α hydroxylation and the scission of the C20-22 bond. The steroidogenic acute-regulatory protein (STAR) mediates the transport of cholesterol across the mitochondrial membrane. **(B)** THDOC and Allopregnanolone can be synthesized de novo from cholesterol or can be metabolized from systemically produced deoxycorticosterone and progesterone via the actions of the enzymes 5α-reductase and 3α-hydroxysteroid*

dehydrogenase. All these enzymes are expressed in a region specific manner in the adult brain.

Neurosteroids enhance $GABA_AR$s via a distinct binding pocket

The stereoselectivity of the potent interaction between neurosteroids and native $GABA_AR$s strongly suggested early on the possibility of a neurosteroid modulatory site on the receptor protein (Wittmer et al. 1996, Callachan et al. 1997). Electrophysiological and radioligand binding experiments provided evidence that the modulatory site for neurosteroids on $GABA_AR$s was distinct from the binding site for benzodiazepines and other known allosteric recognition modulators (Callachan et al. 1987, Peters et al. 1988, Review Hosie et al. 2007). Subsequent homology modeling studies coupled to the use of $GABA_AR$ chimeras between steroid insensitive *Drosophila*-RDL subunits and α subunits led to the identification of critical residues for neurosteroid modulation. These studies revealed the importance of residues with side chains that could form hydrogen bond interactions with neurosteroid molecules. These residues were found to be located within TM1 and TM4 of the α subunit and are conserved among all α subunit isoforms (Hosie et al. 2006, reviewed in Hosie *et al.* 2007).

For α4β3δ $GABA_AR$, which are predominantly expressed at extrasynaptic sites, Hosie et al. showed that co-expression of the β_3 and δ subunits along with an α_4 where the neurosteroid modulatory site was mutated

($\alpha4^{Q246L}$), significantly decreased or abolished THDOC enhancement of the receptor (Hosie et al. 2009). Furthermore, they provided evidence that the δ subunit does not contribute to the neurosteroid modulation site and is likely to be regulating the efficacy of neurosteroid potentiation after the initial binding to the $GABA_AR$ (Hosie et al. 2009).

Tonic inhibition, Neurosteroids and Disease

Disturbances in the tonic inhibition mediated by extrasynaptic $GABA_ARs$ has been observed in a wide range of psychiatric and neurological conditions including some forms of epilepsy, addiction, cognitive impairments, sleep disorders, anxiety disorders, post-partum depression and stress-related disorders (McDonald et al. 2010, Maguire and Moody 2008, Maldonado-Avilés 2009, Uhlass and Singer 2010, Kato, 2007,Sequeira et al. 2007, Maguire et al. 2005, Nie et al. 2011, Rewal et al. 2009). Mutations within the δ and other extrasynaptic $GABA_AR$ receptor subunits have been implicated in some forms of epilepsy (McDonald et al. 2010), childhood onset of some mood disorders (Feng et al. 2010), Schizophrenia (Maldonado-Avilés et al. 2009) and depression (Kato 2007).

Many of these disorders also involve changes in the levels of neurosteroids, that occur following physiological changes in the levels of ovarian and adrenal cortex hormones. For example, Catamenial epilepsy

(a form of epilepsy in which female patients show a cyclic variation in seizure intensity depending on the menstrual cycle phase) has been linked to changes in tonic inhibition during the ovarian cycle (Maguire et al. 2005). Ganaxolone (an analogue to the neurosteroid allopregnanolone) is currently being used in clinical trials for the treatment of this disorder and has been shown to be an effective anticonvulsant in an animal model (Reddy et al. 2000). Anxiety linked to Premenstrual dysmorphic disorder (PMDD) has been associated with neurosteroid-mediated changes in tonic inhibition in animal models (Smith et al. 1998, Maguire et al. 2005).

Work by Maguire and Moody 2008, linked discrepancies in the number of δ-containing extrasynaptic $GABA_A$Rs and the neurosteroid allopregnanolone (progesterone metabolite) with post-partum depression. Elevated allopregnanolone levels during pregnancy (as a direct result of the 100-fold increase in progesterone during this period) results in a compensatory reduction in the number of δ-containing $GABA_A$Rs. In a mouse model, delay in restoring δ-containing $GABA_A$Rs number to pre-pregnancy levels, resulted in a severe depression-like phenotype (Maguire and Moody 2008).

Of particular relevance for stress-related disorders, a recent study has shown that Corticotropin-releasing hormone (CRH) neurons of the hypothalamus are modulated by the stress hormone metabolite THDOC

through its actions on δ-containing $GABA_A$Rs. CRH neurons are a critical component of the hypothalamic-pituitary-adrenal (HPA) axis, which mediates the physiological response to stress. Under normal physiological conditions, THDOC decreased HPA axis activity by potentiating the inhibitory effects of GABA on CRH neurons. However, during acute stress conditions THDOC activates the HPA axis. This is due to a collapse in the chloride gradient that occurs following the dephosphorylation of KCC2. THDOC actions on δ-containing $GABA_A$Rs seem to constitute a positive feedback mechanism onto CRH neurons, which is required for the proper physiological response to stress (Sarkar et al. 2011).

Neurosteroids regulate changes in $GABA_A$R subunit expression

Neurosteroids dynamically regulate changes in $GABA_A$R subunit cell surface expression. For example, the δ and α4 subunits have been shown to undergo marked changes in expression in response to fluctuating steroid levels (Maguire and Moody 2007 2009,Gulinello et al. 2001, Hsu et al. 2003). The levels of steroid hormones can fluctuate in a wide range of physiological states including stress, puberty, pregnancy and the menstrual cycle. Steroid-induced fluctuations in $GABA_A$R subunit expression result in alterations in neuronal excitability and are implicated in many neurological disorders. However, the molecular mechanisms by which neurosteroids alter $GABA_A$Rs subunit expression and function are not well understood.

For synaptic $GABA_AR$s, the trafficking mechanisms and protein interactions that regulate receptor cell surface localization are essential for the changes in synaptic strength mediated by GABA receptor signaling (Jacob et al. 2008). Endocytosis is a critical process for the regulation of the number of surface synaptic neurotransmitter receptors. Our laboratory has shown that phosphorylation of residues within synaptic $GABA_AR$ subunits regulates the interaction of $GABA_AR$ subunits with protein complexes that mediate endocytosis (See $GABA_AR$ endocytosis section above- Reviewed by Tretter and Moss 2008, Jacob et al. 2008, Lushner et al. 2011). Whether extrasynaptic $GABA_AR$ cell surface expression is modulated in a similar manner had not yet been addressed. The work presented in this thesis aimed to elucidate how the phosphorylation state of extrasynaptic $GABA_AR$ subunits regulates the membrane insertion of these receptors. In addition, we have uncovered a novel phospho-depenent mechanism by which neurosteroids selectively modulate the cell surface expression of extrasynaptic $GABA_AR$s in a phospho-dependant manner.

Chapter 2

PROTEIN KINASE C PHOSPHORYLATION REGULATES MEMBRANE INSERTION OF $GABA_A$ RECEPTOR SUBTYPES THAT MEDIATE TONIC INHIBITION

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CONTRIBUTIONS TO THIS PAPER

For this manuscript, I conducted all of the electrophysiological experiments and contributed to writing and editing.

ABSTRACT

Tonic inhibition in the brain is mediated largely by specialized populations of extrasynaptic receptors, γ-aminobutyric acid receptors ($GABA_AR$s). In the dentate gyrus region of the hippocampus, tonic inhibition is mediated primarily by $GABA_AR$ subtypes assembled from α4β2/3 with or without the δ subunit. Although the gating of these receptors is subject to dynamic modulation by agents such as anesthetics, barbiturates, and neurosteroids, the cellular mechanisms neurons use to regulate their accumulation on the neuronal plasma membrane remain to be determined. Using immunoprecipitation coupled with metabolic labeling, we demonstrate that the α4 subunit is phosphorylated at serine 443 by protein kinase C (PKC) in expression systems and hippocampal slices. In addition, the β3 subunit is phosphorylated on serine residues 408/409 by PKC activity, whereas the δ subunit did not appear to be a PKC substrate. We further demonstrate that the PKC-dependent increase of the cell surface expression of α4 subunit-containing $GABA_AR$s is dependent on serine 443. Mechanistically, phosphorylation of serine 443 acts to increase the stability of the α4 subunit within the endoplasmic reticulum, thereby

increasing the rate of receptor insertion into the plasma membrane. Finally, we show that phosphorylation of serine 443 increases the activity of α4 subunit-containing $GABA_A$Rs by preventing current run-down. These results suggest that PKC-dependent phosphorylation of the α4 subunit plays a significant role in enhancing the cell surface stability and activity of $GABA_A$R subtypes that mediate tonic inhibition.

INTRODUCTION

γ-Aminobutyric acid type A receptors ($GABA_A$Rs) constitute the major inhibitory ligand-gated receptors in the adult central nervous system and are responsible for both phasic and tonic forms of inhibition (Farrant and Nusser 2005). These receptors are pentameric, anion-selective ion channels that can be assembled from eight subunit classes: α(1–6), β(1–3), γ(1–3), δ, ϵ, θ, π, and ρ(1–3)(2–3). This large number of receptor subunits provides the basis for a significant degree of heterogeneity of $GABA_A$R structure and function. However, previous studies suggest that in the brain, the majority of phasic inhibition is dependent upon a few $GABA_A$Rs subunits, namely the α, β, and γ2 subunits located within synaptic sites (Rudolph et al. 2001, Sieghart and Sperk 2002). In the adult brain, these receptors are specific targets for brief exposures to high concentrations of GABA, resulting in short lived, but significant, hyperpolarization. In contrast, tonic inhibition is characterized by a sustained reduction in the cell's input resistance, effectively reducing the probability of action potential generation (Farrant and Nuser 2005, Mody

2005). Tonic inhibition is the result of persistent activation by $GABA_A$Rs consisting primarily of α, β, and δ subunits located within peri- or extrasynaptic sites (Farrant and Nuser 2005). With respect to specific brain regions, extrasynaptic $GABA_A$Rs that mediate tonic inhibition in the thalamus and dentate gyrus of the hippocampus are composed of the α4 and β2/3 subunits with or without the δ subunit (Brunig et al. 2002,Sun et al. 2004,Jia et al. 2005,Bencsits et al. 1999, Mortensen and Smart 2006). Verification of the role that the α4 subunit plays in mediating tonic inhibition comes from α4 subunit knock-out mice, which have substantially lower levels of tonic inhibition in these brain areas (Chandra et al. 2006, Liang et al. 2008).

Changes in tonic inhibition associated with $GABA_A$Rs containing the α4 subunit have been implicated in a number of normal and pathological states in which the thalamus and the hippocampus play a role. It is apparent that tonic inhibition is essential for dynamically regulating the neuronal output, frequency of firing, and gain control of neurotransmission (Hamann et al. 2002, Mitchell and Silver 2003,Semyanov et al. 2003, Chadderton et al. 2004, Cope et al. 2005, Bright et al. 2007, Rothman et al. 2009). In addition, extrasynaptic $GABA_A$Rs have been further shown to be targets for a wide range of endogenous and pharmacological agents, such as neurosteroids, anesthetics, ethanol, and anticonvulsants (Wallner et al. 2003, Cheng et al. 2003, Caraiscos et al. 2004, Maguire and Mody 2008). Finally, modifications in the efficacy of tonic inhibition arise under

pathological conditions including stress, fragile X syndrome, aberrant brain activity associated with menstrual cycle, postpartum depression, schizophrenia, and temporal lobe epilepsies (Maguire and Mody 2008, Maguire and Mody 2007,D'Hulst et al. 2006,Maldonado-Aviles et al. 2009, Feng et al. 2006, Zhang et al. 2007).

Little is known about the endogenous mechanism by which neurons control the functional properties of $GABA_AR$ subtypes that mediate tonic inhibition. It has long been established that a direct relationship exists between the number of synaptic $GABA_ARs$ at the cell surface and the strength of inhibition at the synapse (Nusser et al. 1997, Nusser et al. 1998). Therefore, modulating the insertion and removal rate of $GABA_ARs$ into or from the cell membrane has a marked affect on the amplitude of inhibitory synaptic currents (Kittler et al. 2007). One way in which modulation occurs is via posttranslational modifications of the synaptic $GABA_AR$. Specifically, the phosphorylation of key residues on synaptic $GABA_AR$ subunits regulates the extent to which the $GABA_AR$ will interact with protein complexes responsible for endocytosis from and insertion to the cell membrane; however, the significance of these regulatory processes for subtypes that mediate tonic inhibition remains largely unknown (Tretter and Moss 2008, Tang et al. 2010). Phosphorylation plays a role in regulating the functional expression of $GABA_ARs$ containing α4 subunits. Our results reveal that the α4 subunit is phosphorylated on

serine 443 within the intracellular loop between transmembrane domains 3 and 4 (TM3 and TM4) in a protein kinase C (PKC)-dependent manner. Activating PKC also resulted in higher steady state cell surface accumulation of $GABA_AR$s containing the α4 subunit that was dependent on enhanced insertion into the plasma membrane when expressed in a mammalian cell line. Consistent with this, PKC-dependent phosphorylation of serine 443 produced a robust enhancement in GABA-induced currents in this expression system. Finally, we also observed that PKC activity increased both the phosphorylation and cell surface stability of the α4 subunit in hippocampal slices, a phenomenon that should be correlated with an increase in tonic inhibition. Together, these experiments establish a crucial role for PKC in regulating the functional expression of $GABA_AR$ subtypes that mediate tonic inhibition via direct phosphorylation of the α4 subunit.

EXPERIMENTAL PROCEDURES

Antibodies and Expression Constructs

Polyclonal rabbit anti-α4 and anti-δ antibody was graciously provided to us by Dr. Verena Tretter and Dr. Werner Sieghart from Medical University Vienna. β3 and phospho-β3(phospho-S408A/S409A) antibodies were designed by the Moss laboratory (Jovanovic et al. 2004). Peroxidase-

conjugated IgG secondary antibody was from Jackson ImmunoResearch Laboratories. Fluorescently labeled α-bungarotoxin (α-Bgt) was purchased from Invitrogen. Wild-type and mutant α4 subunit and wild-type β3 cDNAs were cloned into the mammalian cytomegalovirus (CMV) promoter for transgene expression. For fluorescence experiments, α4 subunit protein was conjugated with red fluorescent protein and the Bgt binding site (BBS) (Bogdanov et al. 2006). Briefly, dsRed monomer fluorescent protein (RFP) was introduced after the 4th amino acid of the mature α4 subunit followed by the BBS sequence (WRYYESSLEPYPD). The RFP and the BBS were separated by a 12-alanine/1-proline linker ($^{RFP\text{-}BBS}\alpha4$ and $^{RFP\text{-}BBS}\alpha4^{S443A}$ (mutant described below)). All constructs were generated using standard molecular biology cloning techniques and sequenced fully.

Site-directed Mutagenesis

Mutation of the α4 subunit was carried out using the QuikChange site-directed mutagenesis kit (Stratagene). The mutagenesis primers used to introduce an alanine in place of a serine at site 443 were CCTTTGCGGTCGGCGGCTGCTCGCCCGGCATTT and AAATGCCGGGCGCAGCCGCCGACCGCAAAGG. All mutations were verified by DNA sequencing.

Expression of $GABA_AR$ Subunit Constructs in COS-7 Cells

α4 subunit cDNAs (mutant and tagged versions) and β3 subunit cDNA (where specified) were transfected into COS-7 cells using electroporation with 2 μg of plasmid DNA per construct. COS-7 cells incubated in 60-mm

dishes with 4 ml of Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen) plus 10% fetal bovine serum (FBS) at 37 °C with a 95% O_2, 5% CO_2 gas mixture and were utilized 48 h after transfection (Conolly et al. 1999).

COS-7 and HEK-293 Cell Culture and Transfection

COS-7 and Human embryonic kidney (HEK-293) cells were cultured in medium composed of Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells were electroporated (110 V, Bio-Rad Gene Pulser Xcell) with equal ratios of cDNA encoding for $GABA_A$ receptor subunits along with GFP cDNAs (in pCDM8). Cells were used 24–72 h after transfection. Successful transfection of the cells was determined by fluorescence microscopy to identify GFP-labeled cells.

Hippocampal Slice Preparation

Hippocampal slices (350 μm thick) from 10–11-week-old C57BL/6 mice were prepared with a microslicer (Leica VT1000S) and pooled in ice-cold oxygenated artificial cerebral spinal fluid (ACSF). ACSF solution contents differed depending on the experiment being conducted. For ^{32}P labeling experiments (described below), ACSF contained the following: 125 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 1.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, and 10 mM glucose, pH 7.4. For slice biotinylation experiments (described below),

ACSF contained the following: 124 mM NaCl, 3 mM KCl, 25 mM $NaHCO_3$, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 1.1 mM NaH_2PO_4, and 10 mM glucose, pH 7.4. The slices were transferred individually to a solution containing fresh ACSF, gassed with a mixture of 95% O_2, 5% CO_2, and equilibrated in a 30 °C water bath for 1 h. Afterward, slices were utilized for either ^{32}P labeling or slice biotinylation.

Cell Lysis and Immunoprecipitation

Samples collected from either COS-7 cell cultures or hippocampal slices were lysed in lysis buffer containing the following: 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM sodium pyrophosphate, 1% Triton X-100, and 0.1% SDS. In addition, the following protease inhibitors were added: 250 µg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; 10 µg/ml leupeptin, 1 µg/ml pepstatin, and 10 µg/ml antipain. Samples were then sonicated and spun at 16,000 × g. The supernatant was collected and then subjected to a protein assay using a standard Bradford protocol. 100–200 µg of protein were loaded per immunoprecipitation sample along with 3 µg of indicated antibody and 40 µl of protein A-Sepharose beads (1:1 slurry) (GE Healthcare). Samples were allowed to conjugate for 18–24 h at 4 °C with constant agitation. The beads were precipitated by centrifugation at 500 × g and washed once with ice-cold Buffer A (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM sodium

pyrophosphate, and 1% Triton X-100 and protease inhibitors), two times with Buffer B composed of Buffer A supplemented with 500 mM NaCl, and once again with Buffer A. After the final wash, the beads were resuspended in 25 µl of sample buffer and subjected to SDS-PAGE.

Whole-cell COS-7 Cell and Hippocampal Slice Metabolic ^{32}P Labeling

COS-7 cells were transfected and incubated as described above. Cells were initially incubated in 2 ml of phosphate-free DMEM (Invitrogen) for 30 min at 37 °C. Following this incubation, cells were labeled with 0.5 mCi/ml [^{32}P]orthophosphoric acid for 4 h in phosphate-free DMEM. Hippocampal slices were prepared as described above. Slices were individually transferred to polypropylene tubes containing 2 ml of fresh ACSF; gassed with a mixture of 95% O_2, 5% CO_2; and maintained in a 30 °C water bath. Labeling was performed by adding 0.5 mCi/ml [^{32}P]orthophosphoric acid for 1 h. For both COS-7 cells and hippocampal slices. Samples were treated with drugs after the labeling period, followed by the cell lysis and immunoprecipitation procedure described above. Results were attained by SDS-PAGE followed by autoradiography.

Phosphopeptide Mapping and Phosphoamino Acid Analysis

To perform phosphopeptide mapping, gel slices from ^{32}P labeling experiments were excised from SDS-polyacrylamide gels and washed and digested with 0.1mg/ml trypsin and subjected to two-dimensional mapping,

first by electrophoresis and then by thin layer chromatography (TLC). The resulting plate was then visualized by autoradiography (McDonald and Moss 1997). For phosphoamino acid analysis, phosphoproteins from gel slices were hydrolyzed using 6 N HCl. The resulting phosphoamino acids, along with phosphoamino acid standards, were separated by TLC and visualized by autoradiography (McDonald and Moss 1997).

Metabolic [^{35}S]Methionine Labeling

Transfected COS-7 cells were incubated in methionine-free DMEM for 20 min and then pulsed with 0.5 mCi/ml [^{35}S]methionine (PerkinElmer Life Sciences) for 30 min. Cells were washed and incubated in complete DMEM/F-12 with an excess amount of unlabeled methionine for the indicated time periods (chase). Cells were lysed and subjected to immunoprecipitation as described above.

COS-7 Cell and Hippocampal Slice Cell Surface Biotinylation Assay

For transfected COS-7 cells, cultures were washed once with ice-cold PBS and then incubated in 2 ml of ice-cold PBS containing 1 mg/ml NHS-SS-biotin (Pierce) for 20 min in order to label surface proteins with biotin. After labeling, the biotin was quenched by incubating cells in PBS containing 25 mM glycine and 10 mg/ml bovine serum albumin (BSA) (Kittler et al. 2004, Fairfax et al. 2004). Cells were then lysed in lysis buffer and sonicated. For hippocampal slice experiments, slices were incubated

in ACSF described above at 30 °C for 1 h for recovery before experimentation. Slices were then placed on ice and incubated for 30 min with 1 mg/ml NHS-SS-biotin. Excess biotin was removed by washing slices three times in ice-cold ACSF and lysed as described above (Terunuma et al. 2004). For both COS-7 cells and hippocampal slices, insoluble material was removed by centrifugation. The supernatant lysates were incubated with NeutrAvidin beads (Pierce) for 18–24 h at 4 °C. Bound material was eluted with sample buffer and subjected to SDS-PAGE and then immunoblotted with indicated antibodies. Blots were then quantified using the CCD-based FujiFilm LAS 300 system.

Fluorescent BBS Cell Membrane Insertion Assay

COS-7 cells were transfected with $^{RFP\text{-}BBS}\alpha4$ or $^{RFP\text{-}BBS}\alpha4^{S443A}$ and the β3 subunit. All surface proteins expressing the BBS were blocked with 10 μg/ml unlabeled α-Bgt for 15 min at 18 °C. The cells were then washed extensively to remove unbound α-Bgt. Newly inserted $^{RFP\text{-}BBS}\alpha4$ or $^{RFP\text{-}BBS}\alpha4^{S443A}$ was labeled with 2 μg/ml Alexa 647-conjugated α-Bgt and fixed immediately with 4% paraformaldehyde after the indicated time points (Bogdanov et al. 2006). Confocal images of fluorescently labeled COS-7 cells were collected using a ×60 objective, acquired with Nikon acquisition software, and analyzed with MetaMorph.

Patch Clamp Electrophysiology

Cells were superfused, at a rate of 2 ml/min, with an extracellular solution containing 140 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES, 11 mM glucose and adjusted to pH 7.4 with NaOH. Borosilicate glass patch pipettes (resistance 2–5 megaohms) were filled with an internal solution containing 140 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1.1 mM EGTA, 10 mM HEPES, 2 mM ATP (Mg^{2+} salt), adjusted to pH 7.4 with KOH. GABA was applied once every 120 s via a fast step perfusion system (Warner Instruments, Hamden, CT). All experiments were carried out at 32–33 °C using a recording chamber and in-line perfusion heaters (Warner Instruments). Phorbol esters were applied to the cell either internally via the electrode solution or superfused into the recording chamber.

Data Acquisition and Analysis

For biochemical and immunofluorescent experiments, data are presented as means ± S.E. Statistical analysis was performed by using Student's t test where a p value of <0.5 is considered significant. For electrophysiological experiments, currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz and digitized at 20 kHz with a Digidata 1320A data acquisition system (Molecular Devices), and analyzed using either Clampfit (pClamp, Molecular Devices) or GraphPad Prism version 4 software (GraphPad Software, Inc., San Diego, CA). Statistical analysis was performed by

using one-way ANOVA with a Bonferroni post-test with statistical significance set at $p < 0.05$. All data are expressed as mean ± S.E.

RESULTS

Basal Phosphorylation of the $GABA_A$ Receptor α4 Subunit Is Enhanced by PKC When Expressed in COS-7 Cells

Immunoprecipitation was used to examine the phosphorylation of $GABA_A$ receptors in COS-7 cells transiently transected with the $GABA_A$ receptor α4 and β3 subunits. Immunoprecipitation with anti-α4 from transfected COS-7 cells that had been prelabeled with [^{32}P]orthophosphoric acid under basal conditions yielded a major phosphoprotein at ~64 kDa, demonstrating that the recombinant α4 subunit is basally phosphorylated (Figure 1A). A corresponding band was not observed in untransfected COS-7 cells. Previous studies have shown that $GABA_A$ receptor subunits are the target of PKC (Brandon et al. 2000, Krishek et al. 1997, McDonald et al. 1998). To determine whether the α4 subunit is a substrate of PKC, specific kinase activators and inhibitors were utilized. Activation of PKC with 500 nM phorbol 12,13-dibutyrate (PDBu) for 10 min produced a significant increase ($p < 0.05$) in α4 subunit phosphorylation of 223.7 ± 25.17% ($n = 3$) compared with control cells treated with DMSO for 10 min (Figure 1A). An inhibitor of PKC, GF 109203X (GFX) (10 μM for 20 min), had little effect on the basal phosphorylation of the α4 subunit (74.39 ±

15.38% of control, n = 3). However, treating transfected COS-7 cells with GFX 10 min prior to PDBu treatment prevented the increase in α4 subunit phosphorylation (81.1 ± 26.98% of control, n = 3) observed with PDBu treatment alone (Figure 1A). Peptide mapping and phosphoamino acid analysis revealed that the PKC-dependent phosphorylation of the α4 subunit primarily occurs on serine residues (Figure 1C) within one major phosphopeptide (Figure 1B, circled). Together these results strongly suggest that PKC enhances basal levels of phosphorylation on the α4 subunit.

Serine 443 Is a Major Site for PKC-dependent Phosphorylation of the α4 Subunit When Expressed in COS-7 Cells

To further analyze α4 subunit phosphorylation, site-directed mutagenesis was utilized to convert candidate serine residues within the α4 subunit intracellular domain to alanines. Based on the consensus PKC motif of $(R/K)X_{1-4}(S/T)X_{1-3}(R/K)$ (Kennelly and Krebs 1991, Senawongse et al. 2005), a mutant version of the α4 subunit was produced in which serine 443 was changed to an alanine ($\alpha4^{S443A}$ subunit) (Figure 2A). COS-7 cells transfected with wild-type α4 or $\alpha4^{S443A}$ and β3 subunits were subjected to [^{32}P]orthophosphoric acid labeling and treated with either DMSO or PDBu. PDBu significantly enhanced ($p < 0.05$) levels of phosphorylation of wild-type α4 336 ± 50.03% of control Figure 2B). In contrast to wild type, PDBu did not significantly enhance the phosphorylation of the $\alpha4^{S443A}$ subunit

(Figure 2B). These results strongly suggest that the major site for PKC phosphorylation within the intracellular domain of the α4 subunit is serine 443.

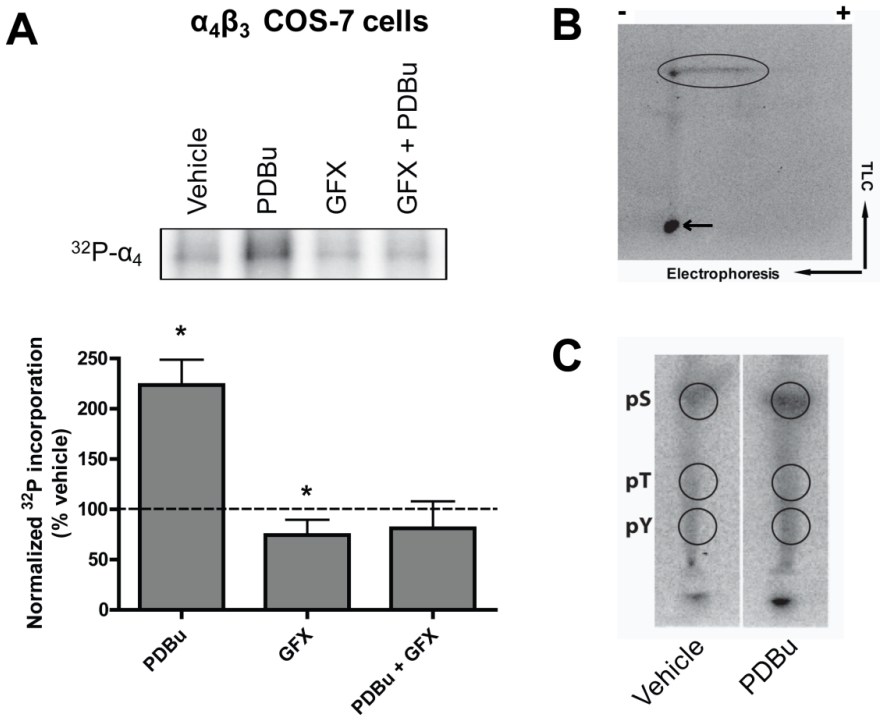


Figure 1: α4 subunit is phosphorylated by Protein Kinase C at serine 443. (***A)*** *COS-7 cells transfected with $GABA_AR$ α4 and β3 subunits were labeled with 0.5 mCi/ml ^{32}P-orthophosphoric acid and then treated with either PDBu (500 nM for 10 mins) alone or following pre-treatment with GF109203X (GFX; 1 μM for 10 mins), a PKC inhibitor. The α4 subunit was immunoprecipitated, subjected to SDS-PAGE and visualized with autoradiography (upper panel). The level of phosphorylation was normalized to the amount observed in vehicle treated samples (lower panel; dashed line represents vehicle set at 100%, $p < 0.05$).* ***(B)***

Phosphopeptide map of the α4 subunit. ^{32}*P-α4 immunopurified from transfected COS7 cells were digested with trypsin, and the resulting phosphopeptides were blotted on to TLC plates and subjected to electrophoresis followed by ascending chromotography. Small arrow indicates the origin.* ***(C)*** *The α4 subunit was subjected to phosphoamino acid analysis followed by autoradiography. The migration of phosphoserine (pS), -threonine (pT), and –tyrosine (pY) standards is indicated.*

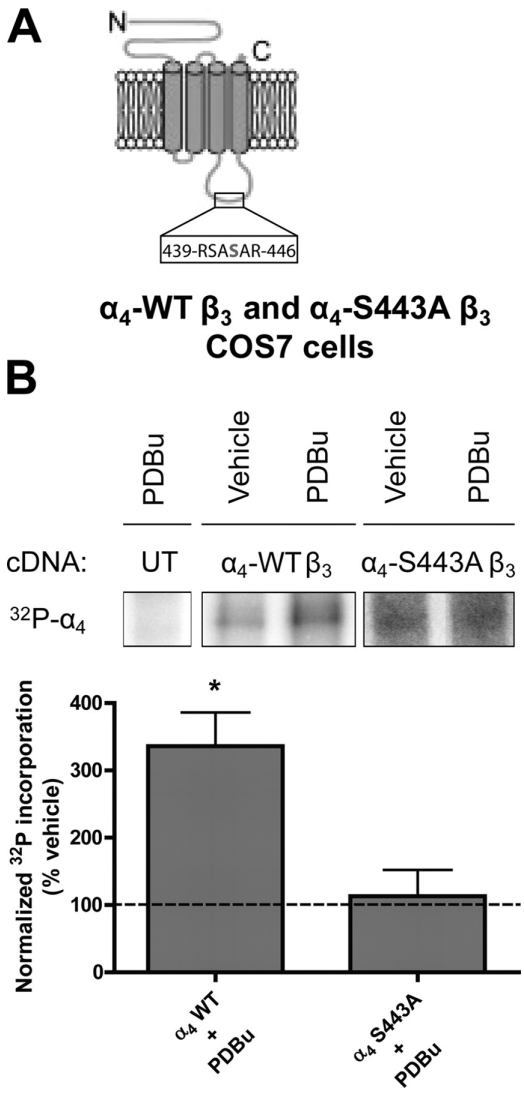


Figure 2. PKC-dependent phosphorylation of the α4 subunit occurs within a PKC consensus motif. (A) *schematic depicting the protein structure of the α4 subunit. Examination of the intracellular domain (ICD) between transmembrane domains 3 and 4 (TM3 and TM4) reveals a serine that is located in a PKC consensus motif.* ***(B)*** *untransfected COS-7 cells (UT) or COS-7 cells co-transfected with either wild-type (α4-WT) or S443A mutant (α4-S443A) $GABA_AR$ α4 and β3 subunits were labeled with [32P]orthophosphoric acid and treated with PDBu (500 nm for 10 min).*

Detergent-soluble extracts were immunoprecipitated with anti-α4, resolved by SDS-PAGE, and then visualized by autoradiography (top). The histogram is presented as ^{32}P incorporation expressed as a percentage of vehicle-treated control (bottom) (dashed line represents vehicle set at 100%, $p < 0.05$). Error bars, S.E.

PKC-dependent Phosphorylation of serine 443 Enhances α4 Subunit Cell Surface Expression Levels in COS-7 Cells

Previous studies have shown that $GABA_AR$ subunit phosphorylation dynamically regulates $GABA_AR$ cell surface expression (Brandon et al. 1999, Kittler et al. 2004, Kittler and Moss 2003). To address the functional consequences of α4 subunit phosphorylation, we first measured the effect PKC activation has on α4 subunit cell surface expression in transfected COS-7 cells using a biotinylation assay (Kittler et al. 2004, Fairfax et al. 2004). This revealed that a 10-min treatment with 500 nM PDBu significantly increased ($p < 0.05$) the cell surface levels of the α4 subunit by 144.9 ± 21.78%, but the total levels of protein were not altered (Figure 3A). Treatment with GFX alone had no significant effect, and co-treatment with GFX and PDBu resulted in a modest increase in α4 subunit cell surface levels (Figure 3A). Insignificant amounts of actin protein were pulled down in our biotinylated samples, ensuring that only cell surface proteins were being collected (Figure 3A). In contrast to these results, PDBu treatment did not significantly increase ($p < 0.05$) the cell surface

expression level of $\alpha4^{S443A}$ (Figure 3B). Together, these biochemical experiments indicate that the PKC-dependent phosphorylation of the α4 subunit on serine 443 increases receptor cell surface expression.

Analyzing the Phosphorylation of the β3 and δ Subunits

In the brain, the α4 subunit assembles with the β2/3 subunit with or without the δ subunit (Brunig et al. 2002, Sun et al. 2004, Jia et al. 2005, Bencsits et al. 1999, Mortensen and Smart 2006, Pirker et al. 2000). Thus, we examined if these subunits are also subject to PKC-dependent phosphorylation. To do so, the α4, β3, and δ subunits were co-expressed in COS-7 cells and then labeled with [^{32}P]orthophosphoric acid. After treatment with 500 nM PDBu for 10 min, the δ subunit was isolated via immunoprecipitation after denaturing lysis. Under these conditions, minimal levels of phosphorylation of the δ subunit were seen under basal conditions or after the activation of PKC (Figure 4B). However, robust immunoprecipitation of the δ subunit was seen as measured via immunoblotting (Figure 4B).

Previous studies have revealed that the β3 subunit is predominantly phosphorylated on serines 408/409 in neurons upon activation of PKC (Jovanovic et al. 2004, Jacob et al. 2009). To examine if these residues are phosphorylated in COS-7 cells, lysates were immunoblotted with a phosphospecific antibody against these residues, phosphoserines 408/409. PDBu treatment produced a robust enhancement of serine

408/409 phosphorylation as measured via immunoblotting with Ser(P)$^{408/409}$ antibody (Figure 5A). Thus, this experiment suggests that the δ subunit is not a PKC substrate at least when expressed in COS-7 cells and suggests that the principle sites for PKC phosphorylation within $GABA_AR$ subtypes that mediate tonic inhibition are serine 443 in the α4 subunit and serine 408/409 in β3.

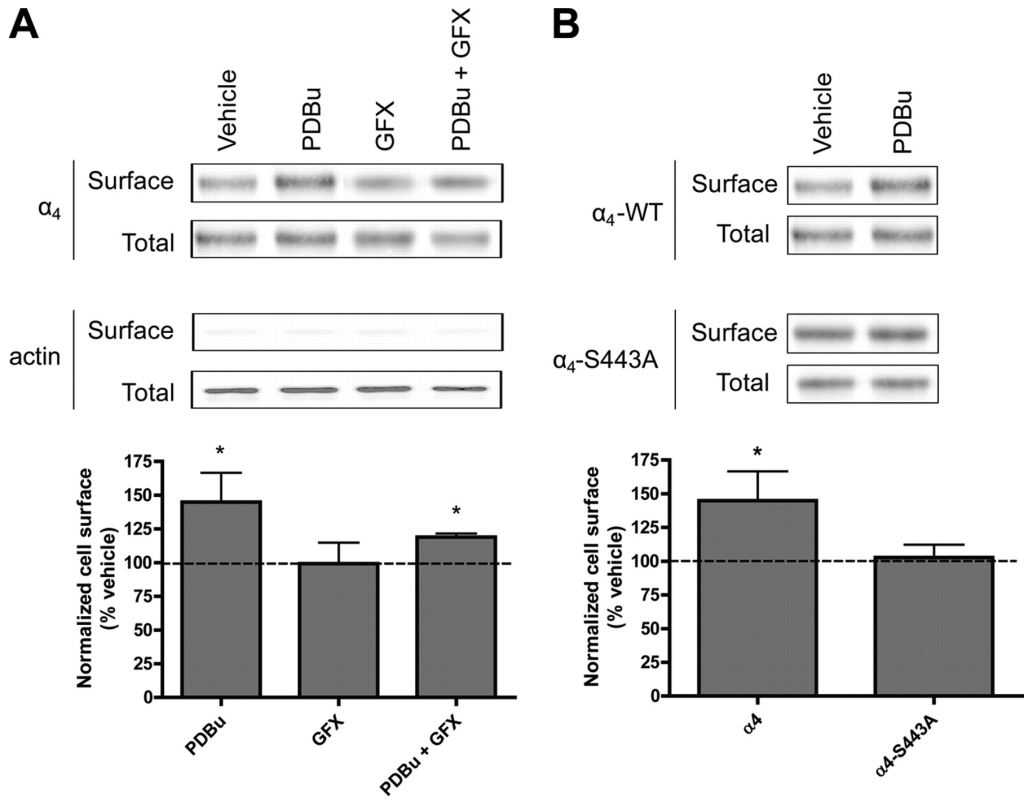


Figure 3: PKC-dependent phosphorylation on Ser443 regulates the cell surface expression of the α4 subunit in COS-7 cells. *A, COS-7 cells transfected with $GABA_AR$ α4 and β3 subunits were treated with either PDBu (500 nm for 10 min) alone or following pretreatment with GFX (1 μm for 10 min) and then labeled with NHS-SS-biotin. Detergent-soluble*

extracts were then purified on NeutrAvidin. The purified cell surface (Surface) and 10% of the total fraction (Total) were immunoblotted with α4 antibodies (top). Surface and total fractions were also blotted with actin to ensure the integrity of the cell surface assay. The amount of α4 subunit on the cell surface was then measured for each condition and normalized to the amount observed in vehicle-treated samples (lower panel) (dashed line represents vehicle set at 100%, $p < 0.05$). ***(B)*** *COS-7 cells co-transfected with either wild-type (α4-WT) or S443A mutant (α4-S443A) GABAA receptor α4 and β3 subunits were treated with either vehicle or PDBu (500 nm for 10 min) and then subjected to biotinylation. Histograms show the proportion of cell surface α4 protein expressed as a percentage of vehicle-treated controls (dashed line represents vehicle set at 100%; $p < 0.05$). Error bars, S.E.*

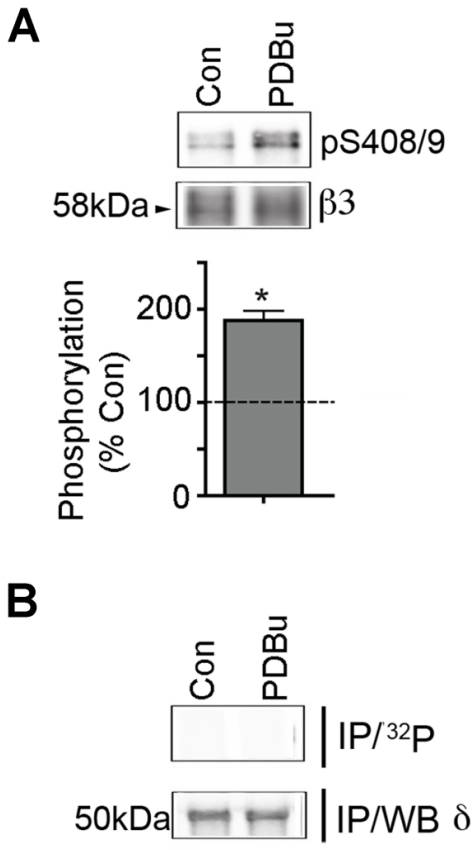


Figure 4. Analyzing PKC phosphorylation of $GABA_AR$ subunits that mediate tonic inhibition. A*. COS7 cells expressing the α4 and β3 subunits were treated with 500 nM PDBu for 10 min and then immunoblotted with pS408/9A or β3 antibodies and the ratio of pS408/9 β3 immunoreactivity was determined and normalized to control (dashed line represents vehicle set at 100%; p < 0.05)* ***B.*** *COS7 cells expressing α4, β3, and δ subunits were labeled with 0.5 mCi/ml ^{32}P-orthophosphoric acid and treated with 500 nM PDBu for 10 min. The a δ subunit was isolated by denaturing immunoprecipitation followed by SDS-PAGE*

(IP/^{32}P). Parrallel cultures were immunoprecipitated and immunoblotted with δ antibodies (IP/WB δ).

Mutation of serine 443 Increases the Rate of Insertion of the α4 Subunit on the Cell Membrane

To further evaluate the mechanism underlying PKC-dependent modulation of the α4 subunit cell surface stability, we determined what effect mutating the PKC site on the α4 subunit has on the level of insertion of α4 subunit-containing receptors. To do so, we utilized a Bgt binding assay that has previously been used to measure the rates of insertion of various receptor types (Bogdanov et al. 2006,Sekine-Aizawa and 2004). To analyze the insertion of the α4 subunit, we engineered the α4 subunit with the BBS peptide, WRYYESSLEPYPD. The BBS is derived from the α subunit of the muscle nicotinic receptor and has been established to bind Bgt with an affinity of ~3 nM (Scherf et al. 2001, Katchalski-Katzir et al. 2003). The BBS together with a red fluorescent protein reporter were added to the N-terminal region of both the α4 and α4^{S443A} subunit.

Before conducting our insertion assay, we verified that the BBS-tagged versions of our α4 constructs were capable of forming a functional $GABA_A$ R. To do so, we expressed $^{RFP-BBS}$α4 and non-tagged α4 separately with the β3 subunit in HEK293 cells and measured the current responses to 1 μM and 1 mM GABA. Utilizing this method, we demonstrated that the $^{RFP-BBS}$α4β3 $GABA_AR$ subtype forms a functional receptor and has GABA-

mediated currents similar to those of the α4β3 $GABA_AR$ subtype (Figure 5).

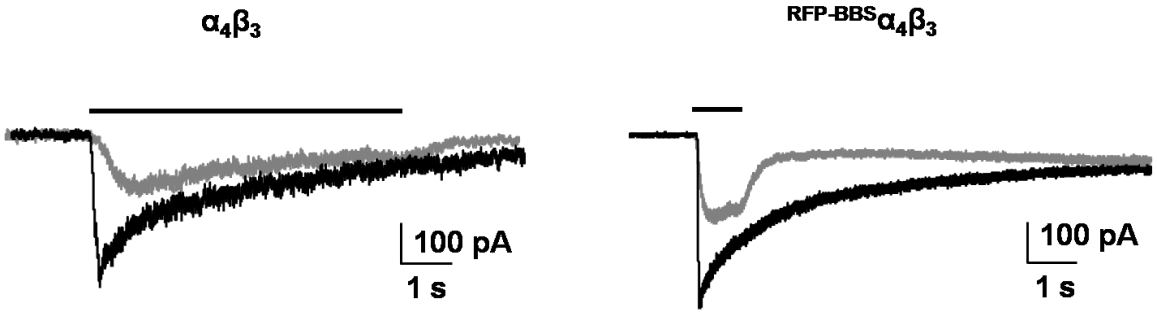


Figure 5. The $^{RFP\text{-}BBS}$α4 subunit forms a functional channel when expressed with the β3 subunit. *Examples of GABA-mediated currents recorded from HEK293 cells expressing wild-type α4β3 and $^{RFP\text{-}}$BBSα4β3 $GABA_ARs$. Solid line above the trace represents the application of either 1 mM (black line) or 1 μM (grey line) GABA.*

To measure the role that serine 443 plays in regulating the cell surface accumulation of α4, $^{RFP\text{-}BBS}$α4 and $^{RFP\text{-}BBS}$α4^{S443A} cDNAs were separately transfected into COS-7 cells along with the $GABA_A$ β3 subunit and subjected to the BBS insertion assay. To perform this assay, we first masked the surface $^{RFP\text{-}BBS}$α4 and $^{RFP\text{-}BBS}$α4^{S443A} with unlabeled Bgt by incubating the transfected COS-7 cells with native Bgt at 18 °C for 15 min. Under these conditions, the unlabeled Bgt completely blocked the existing cell surface population of $^{RFP\text{-}BBS}$α4 and $^{RFP\text{-}}$

$^{BBS}\alpha4^{S443A}$ subunits. Next, the cells were incubated at 37 °C with Alexa 647-Bgt in order to fluorescently label newly inserted $^{RFP\text{-}BBS}\alpha4$ and $^{RFP\text{-}BBS}\alpha4^{S443A}$. Visually, it is apparent that there is a higher amount of $^{RFP\text{-}BBS}\alpha4^{S443A}$ inserted after 10 min compared with $^{RFP\text{-}BBS}\alpha4$ (Figure 6A). To quantify these results, we calculated the ratio of the level of Alexa 647-Bgt staining to the level of RFP fluorescence. This ratio was significantly higher ($p < 0.05$) in COS-7 cells transfected with $^{RFP\text{-}BBS}\alpha4^{S443A}$ than in cells transfected with $^{RFP\text{-}BBS}\alpha4$ after 10 min of labeling (1.35 ± 0.33 versus 0.78 ± 0.16, n = 3; Figure 6B). We also tested the rate of endocytosis of the $^{RFP\text{-}BBS}\alpha4$ and $^{RFP\text{-}BBS}\alpha4^{S443A}$ in a similar BBS assay and found no significant difference between the wild-type and mutant α4 subunit (data not shown). Together, these results strongly suggest that phosphorylation of serine 443, the major site of PKC-dependent phosphorylation within the α4 subunit, regulates the rate of insertion of the α4 subunit in COS-7 cells.

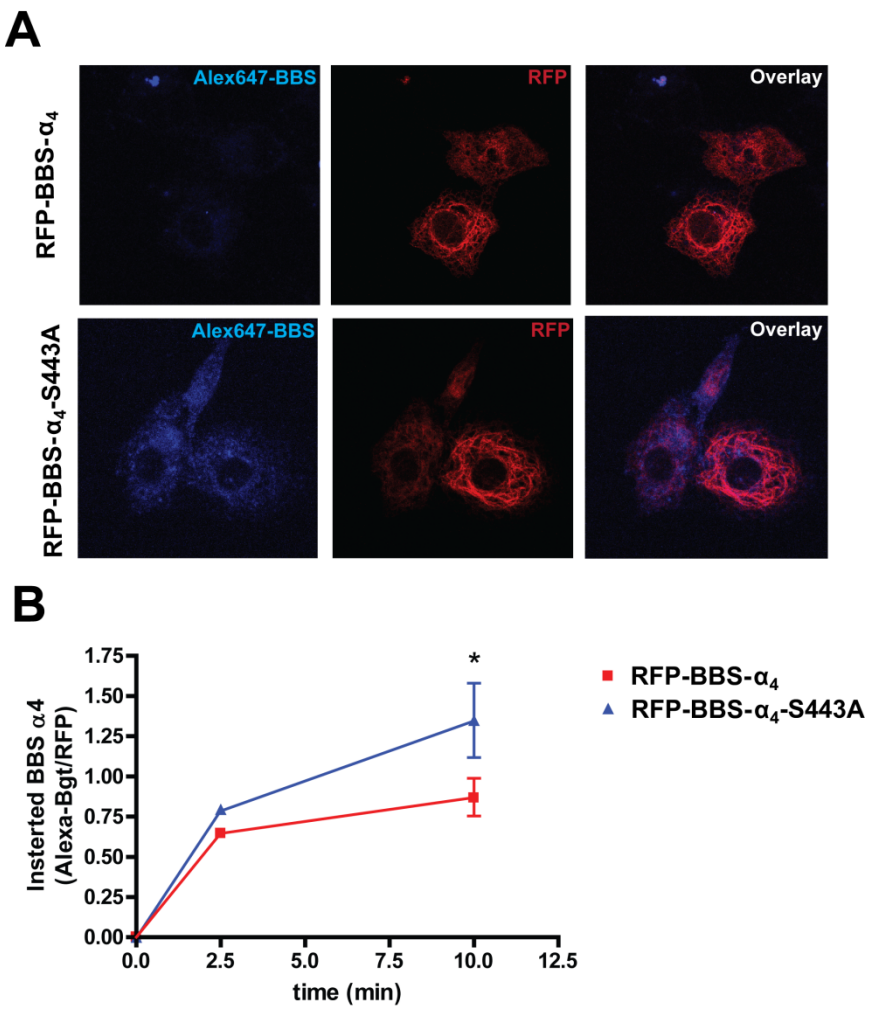


Figure 6. S443A point mutation increases the rate of insertion of the α_4 subunit into the cell membrane. (A) *α4-WT and α4-S443A DNA constructs were made containing a red fluorescent protein (RFP) tag as well as a bungarotoxin (Bgt) binding sie (BBS). COS-7 cells were then co-transfected with either wild-type (α4-WT-BBS) or S443A mutant (α4-S443A-BBS) $GABA_A$ receptor α4 and β4 subunits were incubated with unlabeled Bgt (10 nM for 10 mins) at 12°C to block insertion. Cells were then incubated with Alexa-conjugated Bgt (10 nM for 10mins) at 37°C .*

Cells were then fixed and the level of newly inserted Alexa-tagged α was determined using confocal microscopy and quantified using MetaMorph. ***(B)*** *the graph is presented as a ratio of Alexa fluorescent intensity (newly inserted protein) to RFP fluorescence (total protein) over specific time periods. Ratios for α4-WT and α4-S472A were then compared to one another ($p < 0.05$).*

Mutation of serine 443 Increases the Protein Stability of the α4 Subunit

To determine the role phosphorylation plays in the production and stability of the α4 subunit, COS-7 cells transfected with α4 or $\alpha4^{S443A}$ alone were subjected to a [^{35}S]methionine pulse-chase assay. Transfected COS-7 cells were labeled with 100 μCi/ml [^{35}S]methionine for 30 min and chased for 0 and 4 h with excess cold methionine. Cell lysates were then prepared and subjected to immunoprecipitation with anti-α4 and resolved on SDS-polyacrylamide gels and quantified on a Bio-Rad isotope imager (Figure 7A). Data at 4 h are presented as a percentage of [^{35}S]methionine-labeled protein existing at time 0. α4 subunit protein does not reach the cell surface unless a β3 subunit is also present. Therefore, under these conditions, we are measuring the stability of protein that is retained in the endoplasmic reticulum. Using this technique, we determined that 47.40 ± 8.18% of newly synthesized α4 subunit protein remained after 4 h (Figure 7). Interestingly, this reduction was markedly less robust for the $\alpha4^{S443A}$

subunit protein, reducing to 80.20 ± 7.00% of newly synthesized protein after 4 h (Figure 7). Therefore, the $\alpha4^{S443A}$ subunit is more stable than the wild-type α4 subunit, suggesting that PKC phosphorylation of the serine 443 site regulates the α4 subunit protein half-life.

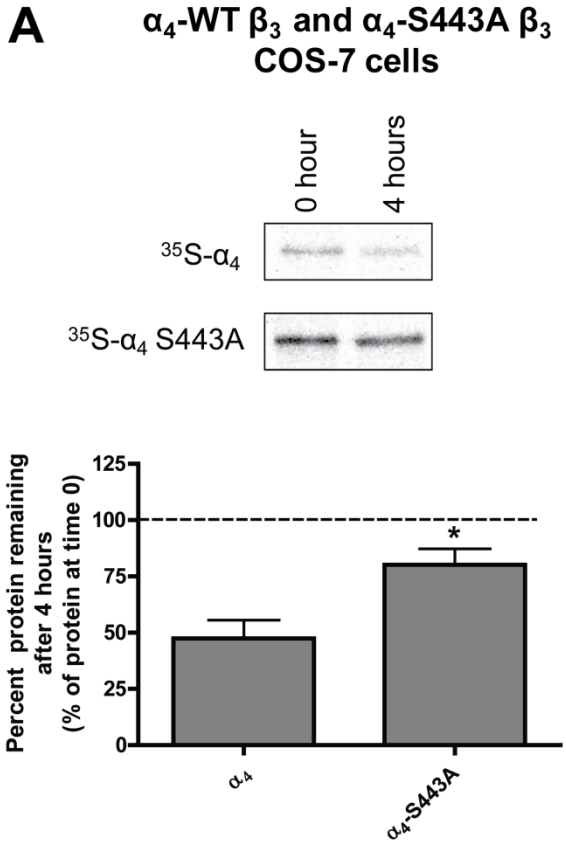


Figure 7. S443A point mutation reduces turnover of the α4 subunit in transfected COS7 cells. *COS7 transfected with either wild-type (α4-WT) or S443A mutant (α4-S443A) $GABA_A$ receptor subunits subjected to a pulse chase with ^{35}S-methionine. Cells were lysed and immunoprecipitated with anti-α4 subunit antibody and then subjected to SDS-PAGE. Bands were then analyzed by autoradiography (upper*

panel). Turnover levels are presented as a percentage of levels at time 0 (lower panel, $p < 0.05$).

Protein Kinase C Activation Reverses Current Run-down

The functional effects of protein *kinase* C activation were determined by whole-cell patch clamp recording of HEK293 cells. Transient expression of wild-type α4β3 receptors and mutant $\alpha4^{S443A}\beta3$ receptors resulted in functional channels that had GABA-evoked EC_{50} values of 1.7 ± 0.7 and 2.6 ± 0.9 μM (n = 3–10), respectively, with both Hill coefficients of 0.8 ± 0.2 (Figure 8). To examine the run-down of GABA-mediated currents, 1 μM GABA was applied to the cells once every 2 min. In the absence of PKC activation, the GABA-mediated current amplitude decreased over time but appeared to plateau after 16 min of recording. After 20 min of recording, the GABA-mediated current was 37 ± 12% (n = 5) of the initial response. Inclusion of 100 nM PDBu in the recording pipette solution prevented the GABA-mediated current amplitude run-down. At 20 min after the start of the experiment, the GABA-mediated current amplitude was 97 ± 9% (n = 3) of the initial GABA-mediated response. Similarly, external application of 100 nM PDBu also reverses GABA-mediated current amplitude run-down with current amplitude being 107 ± 12% (n = 4) compared with that at the start of the experiment (Figure 9).

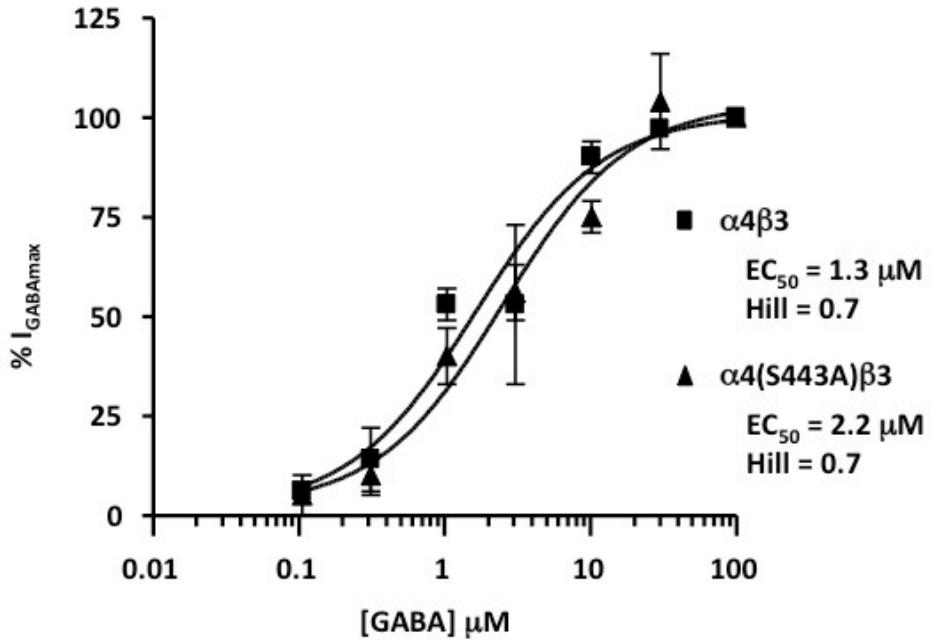


Figure 8: $GABA_AR$ composed of α4β3 and α4 S443Aβ3 subunits have similar sensitivity to GABA. *Whole cell recordings of HEK-293 cells transfected with either $\alpha 4\beta 3$ or $\alpha 4(S443A)\beta 3$ $GABA_AR$ subunits, were exposed to GABA at indicated concentrations at 120 second intervals. GABA Concentration-response curves of HEK-293 cells expressing $\alpha 4\beta 3$ or $\alpha 4(S443A)\beta 3$ $GABA_AR$ have similar EC_{50} values.*

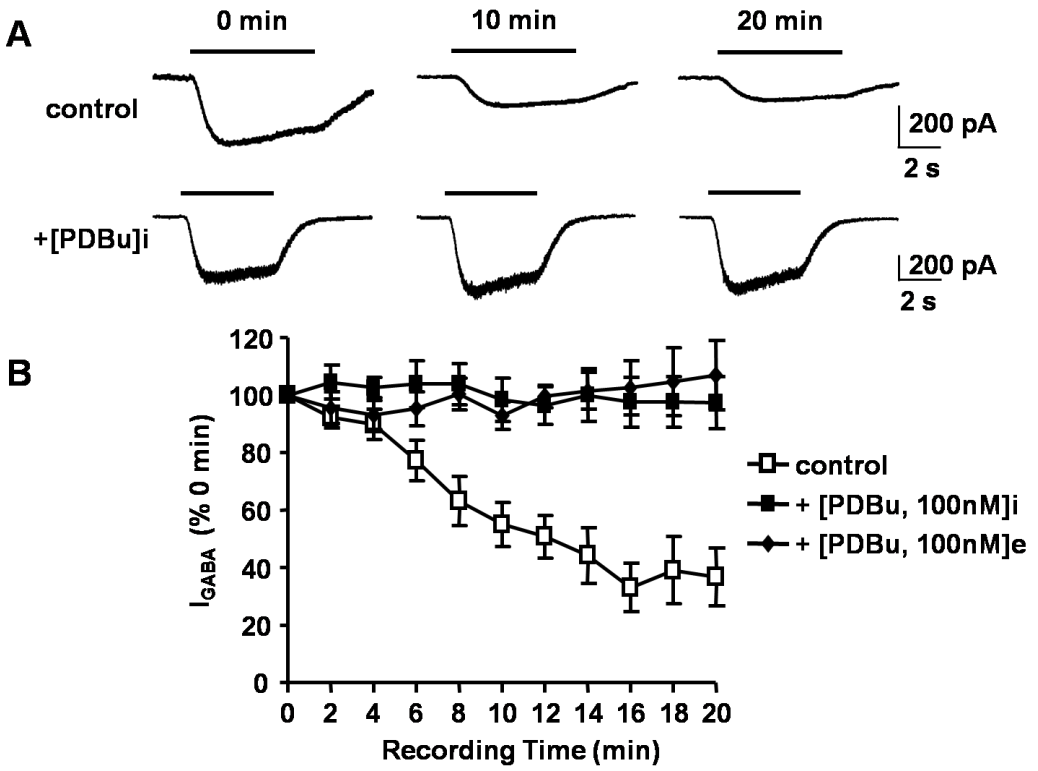


Figure 9: Run-down of $GABA_A$ receptor α4β3-mediated responses are prevented with protein kinase C activation.

(A) *1 μM GABA-activated currents recorded at 0, 10 and 20 minutes after the start of the experiment (defined as t = 0 min and 100%), recorded 3-5 minutes after achieving the whole-cell configuration. Whole-cell currents were recorded from HEK-293 cells expressing α4 and β3 subunits in the absence (control, upper currents) and presence (+[PDBu]i, lower currents) of 100 nM internal PDBu. Holding potential was -60 mV at 32 °C.* ***(B)*** *Time-dependence relationship for 1 μM GABA-activated currents recorded from α4β3 receptors without (open squares), with (solid squares) 100 nM PDBu internally perfused or with (solid diamonds) 100 nM PDBU externally perfused. All data points are mean ± SEM.*

When the PKC-inactive phorbol ester, 4-α-phorbol 12,13-didecanoate (100 nM), was included in the intracellular solution of the recording pipette, the GABA-mediated current amplitude run-down was no different from control. Cells rarely remained healthy past 16 min of recording. After 16 min of recording, the GABA-evoked currents were 17 ± 6% (n = 3) of the first current in the presence of 100 nM 4-α-phorbol 12,13-didecanoate compared with 33 ± 10% (n = 7) in control (Figure 10). At this time point, minimal run-down was observed in cells internally perfused with 100 nM PDBu (98 ± 9% of control, n = 3).

The $\alpha4^{S443A}$ Subunit Mutation Prevents Run-down

GABA-mediated currents in cells expressing $\alpha4^{S443A}\beta3$ receptors did not show the typical time-dependent run-down phenomena. Unlike that observed with wild-type α4-containing receptors in control conditions, after 20 min of recording, the GABA-mediated current from $\alpha4^{S443A}$ containing receptors was 84 ± 13% (n = 4) of the initial GABA-mediated response. In the presence of internal 100 nM PDBu, GABA-mediated current was 98 ± 9% (n = 4) of the initial response after 20 min of recording (Figure 11).

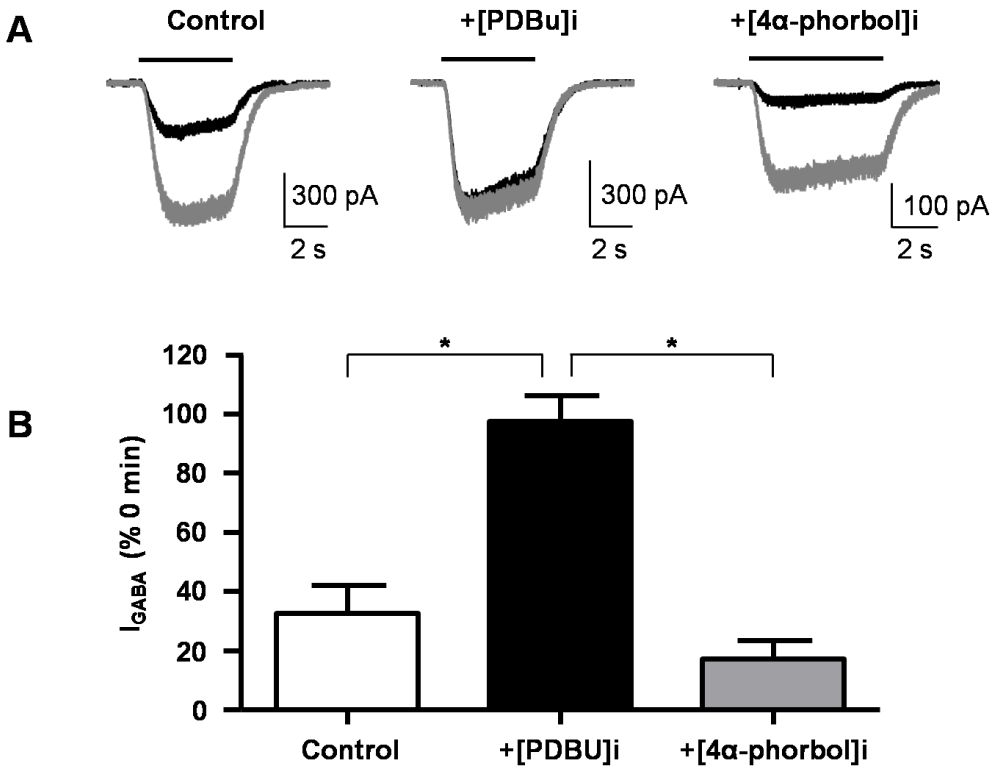


Figure 10: Inclusion of the inactive phorbol ester, 4-α-Phorbol 12,13-didecanoate, does not prevent run-down of $GABA_AR$ α4β3-mediated responses. ***(A)*** *Overlaid GABA-evoked currents from HEK-293 cells expressing α4β3 receptors recorded at t=0 (grey) and t=16 (black) minutes after the start of the experiment. Significant run-down of current amplitude at t=16 compared to t=0 is observed in control and in the presence of 4α-phorbol. In comparison, the current at t=16 minutes in the presence of internal 100 nM PDBU was not different compared to that at t=0 minute.* ***(B)*** *Bar graph of the relative current at t=16 minutes compared to current at t=0 minutes for cells in control conditions (n=8), perfused internally with*

100 nM PDBU (n=3) or 100 nM 4α-phorbol (n=3). Values are mean ± SEM.

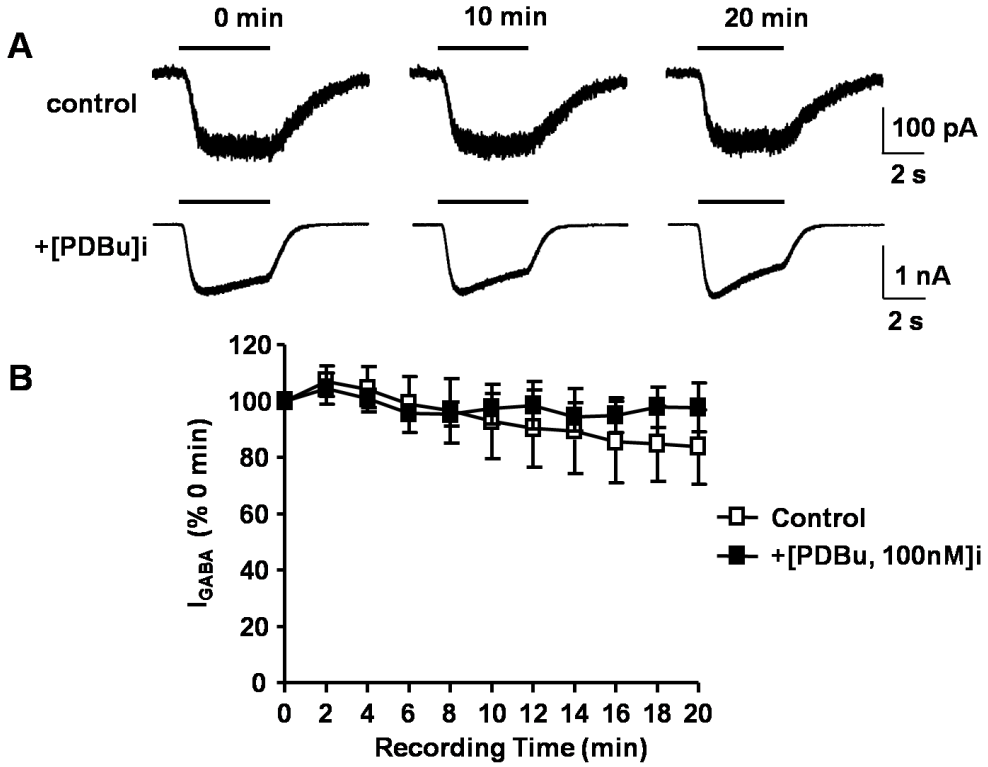


Figure 11: Run-down is prevented with the inclusion of the α4 S443A mutation. ***(A)*** *Whole-cell currents recorded from HEK-293 cells expressing α4 (S443A) β3 receptors. Currents mediated by 1 μM GABA recorded at 0, 10 and 20 minutes after the start of the experiment, recorded 3-5 minutes after achieving the whole-cell configuration (defined as t = 0 min and 100%). Current recorded in the absence (control, upper currents) and presence of 100 nM PDBu (lower currents).* ***(B)*** *Time-dependence relationship for 1 μM GABA-activated currents recorded from*

α4β3 receptors without (open squares), or with (solid squares) 100 nM PDBu internally perfused. All data points are mean ± SEM.

PKC Activity Modulates the Phosphorylation and Cell Surface Stability of Endogenous α4 Subunit in Hippocampal Slices

To determine the relevance of our recombinant studies, we examined the phosphorylation of the endogenous α4 subunit and its effects on cell surface stability of this protein in hippocampal slices. We began by examining the PKC-dependent phosphorylation of the α4 subunit. Hippocampal slices were cut from brains dissected from 10–11-week-old C57BL/6 mice. Slices were labeled with [^{32}P]orthophosphoric acid for 4 h in ACSF continuously being bubbled with a 95% O_2, 5% CO_2 gas mixture. Toward the end of the 4 h, control slices were treated with DMSO for 10 min, whereas PKC slices were treated with 500 nM PDBu for 10 min. Afterward, slices were lysed and subjected to immunoprecipitation with anti-α4. Under control conditions, a very faint band at ~64 kDa was detected, representing the basal level of α4 subunit phosphorylation (Figure 12A). Treatment of slices with PDBu significantly increased ($p < 0.05$) α4 subunit phosphorylation to 2016 ± 1260% of control (Figure 12A)

We further evaluated phosphorylation of the α4 subunit in hippocampal slices by performing phosphoamino acid analysis. We determined that PKC activation with PDBu resulted in the phosphorylation of the α4 subunit principally on serine resides (Figure 12B), similar to what was

observed in transfected COS-7 cells (Figure 1C).

We also examined the effects of PKC activation on the cell surface stability of the α4 subunit in hippocampal slices using biotinylation. This revealed that activation with PDBu produced a significant increase ($p < 0.05$) in the amount of α4 subunit protein on the surface of cells in the hippocampal slice to 293.7 ± 90.70% of DMSO-treated slices (Figure 12C). Similar to what was observed in our recombinant studies, these results demonstrate that the α4 subunit, in its native environment, is phosphorylated in a PKC-dependent fashion and that this post-translational modification increases the targeting of the α4 subunit to the cell surface.

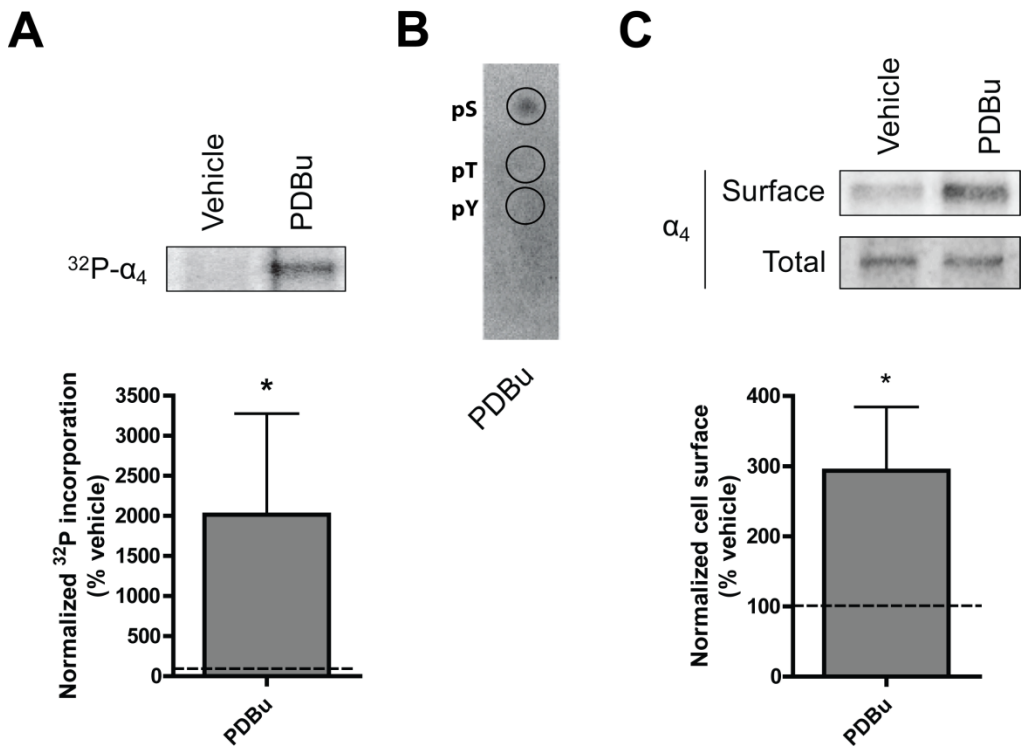


Figure 12: PKC increases the level of phosphorylation and cell surface expression of the α_4 subunit in hippocampal slices. *A*, Hippocampal slices from 10 week old C57BL/6 male mice were labeled with ^{32}P-orthophosphoric acid and treated with either vehicle or PDBu (500nM for 10 mins). Detergent-soluble extracts were immunoprecipitated with anti-α_4, resolved by SDS-PAGE and then visualized by phospho-imaging (*upper panel*). Histograms are presented as ^{32}P incorporation expressed as a percentage of vehicle treated control (*lower panel;* dashed line, $p<0.05$). *B*, The immunopreciptiated α_4 subunit from ^{32}P-orthophosphoric acid treated hippocampal slices were subjected to phosphoamino acid analysis followed by autoradiography. The migration of phosphoserine (pS), -threonine (pT), and –tyrosine (pY) standards is indicated. *C*, Hippocampal slices from 10 week old C57BL/6 male mice treated with either vehicle or PDBu (500nM for 10 mins) were labeled with NHS-Biotin and detergent-soluble extracts were purified on NeutrAvidin. Cell surface (Surface) and 10% of total fractions (Total) were analyzed by immunoblotting with anti-α_4 (*upper panel*). Histograms show the proportion of cell surface α_4 protein expressed as a percentage of vehicle-treated controls (*lower panel*; dashed line represents vehicle set at 100%, $p<0.05$).

DISCUSSION

The hippocampus is responsible for the formation of various types of

memory in mammals. Pivotal to this role, the dentate gyrus of the hippocampus acts as an information-processing center between afferent connections from the entorhinal cortex and efferent connections to cornu ammonis region 3 of the hippocampus (Carlton and Coulter 2008). Specifically, the dentate gyrus is responsible for filtering out stimulus-related high frequency firing from the entorhinal cortex and organizing it into a coherent signal that the rest of the hippocampus can utilize (Behr et al. 1998). Compromising filtering function of the dentate gyrus leads to epileptiform activity in downstream hippocampal structures (Heinemann et al. 1992). The filter-like function of the dentate gyrus is due to the intrinsic low excitability of granule cells residing in this region. This low excitability is a result of the high levels of protein expression of the $GABA_AR$ α4 subunit, creating a significant degree of tonic inhibition (Jia et al. 2005). Therefore, understanding the cellular mechanisms that control the activity of the α4 subunit is crucial for better comprehending the complex mechanisms of memory formation as well as other higher brain functions where α4 subunit-mediated tonic inhibition has been shown to play a role.

Here we have begun to analyze the endogenous mechanisms that neurons utilize to regulate the efficacy of tonic inhibition, focusing on the possible role that direct phosphorylation may play in these processes. For these studies, we used receptors composed of α4β3 and α4β3δ subunits because both of these combinations have been demonstrated to be

present within the dentate gyrus of the hippocampus as measured using biochemical and electrophysiological experiments. We initiated these studies by determining if the α4 subunit is phosphorylated by expressing this protein in COS-7 cells and measuring the amount of radiolabeled phosphate that covalently bonds to the α4 subunit. This revealed that the α4 subunit is phosphorylated basally, and activation of PKC enhances subunit phosphorylation. Peptide mapping and phosphoamino acid analysis showed that PKC-dependent phosphorylation of the α4 subunit occurs on a serine residue in one distinct phosphopeptide region. Using site-directed mutagenesis, we determined that the α4 subunit is phosphorylated at serine 443, an amino acid located in the major intracellular domain between TM3 and TM4. In addition to our recombinant studies, we also showed that PKC leads to high levels of α4 subunit serine phosphorylation in hippocampal slices from adult male mice. We also analyzed the phosphorylation of the δ and β3 subunits in our study. Consistent with studies on $GABA_AR$ subtypes that mediate phasic inhibition, serines 408/409 in the β3 subunit were phosphorylated by PKC activity when expressed with α4. However, at least in COS-7 cells, only low levels of δ subunit phosphorylation were seen. Thus, these results suggest that the primary PKC substrates with $GABA_AR$ subtypes that mediate tonic inhibition are serine 443 in the α4 subunit and serine 408/409 in β3.

To begin ascertaining the functional consequences of phosphorylation on tonic inhibition, we looked at the effect PKC activation had on the cell surface stability of the α4 subunit. The activation of PKC leads to a dramatic increase in the amount of α4 subunit protein at the cell surface in both transfected COS-7 cells and hippocampal slices, as measured by biotinylation. The serine 443 phosphorylation site plays a crucial role in this enhancement because mutation of this residue did not result in elevated levels of α4 subunit protein at the cell surface of COS-7 cells. At this point, it was clear that serine 443 is essential in mediating the effects of PKC activity on α4 subunit cell surface accumulation. To begin to address the underlying mechanism, we measured the rate of insertion of the α4 subunit into the cell membrane using a BBS fluorescent insertion assay. Here we discovered that over a 10-min period, more mutant α4 was being inserted into the COS-7 cell membrane than wild type α4 subunit. This increased rate of insertion was also paralleled with an increase in stability of newly translated $\alpha4^{S433A}$ subunit compared with wild type α4 when expressed alone in COS-7 cells. Given that the α4 subunit is retained within the endoplasmic reticulum in homomeric expression, this result suggests that phosphorylation of serine 443 acts to regulate the stability of the α4 subunit in this intracellular compartment, which would be predicted to increase receptor assembly, leading to increased insertion into the plasma membrane.

To investigate this possibility, we measured the amount of protein degradation using an [^{35}S]methionine pulse-chase assay and found that the α4 subunit serine 443 mutant was more stable in the endoplasmic reticulum over a 4-h period. Taking these results together, we see a situation in which the mutant version of the α4 subunit is not only degraded less but is inserted faster into the cell membrane. At first glance, this seems at odds with our results showing that serine 443 is a critical residue for the PKC-dependent phosphorylation of the α4 subunit that leads to higher levels of this protein on the cell surface. How is it then that ablating this phosphorylation site prevents α4 from being phosphorylated, but still causes the mutant protein to be inserted at a faster rate? One answer to this question is that mutation of serine 443 to an alanine results in phosphorylation mimic of the α4 subunit. That is to say that masking the hydroxyl group that is normally found in a serine residue by removing it, which is what we do when we replace this residue with an alanine, is tantamount to masking it by covalently attaching a phosphate group. Both situations may lead to similar protein conformational changes that result in the α4 subunit being more stable and therefore being inserted at a faster rate. We can further draw this conclusion from our electrophysiological studies, which suggest a similar occurrence. In these studies, we found that PKC activation reverses the rundown that normally occurs in non-treated HEK-293 cells expressing the wild-type α4 subunit, as is expected from our biotinylation studies. Interestingly, HEK-293 cells expressing the

α4 phospho-mutant do not exhibit any rundown, neither in the presence nor in the absence of PKC activation. That is, that the mutant by itself is protected by the rundown effect that is normally observed in the wild-type α4 subunit. If this is the case, it is logical to conclude that the reason PKC does not exert an effect on the mutant α4 subunit is that the protein is already acting as if it is constitutively phosphorylated and is being inserted into the membrane at a maximal rate.

Here we have for the first time a description of a PKC-dependent mechanism that regulates the activity of a $GABA_AR$ subunit that is primarily expressed in extrasynaptic sites. Our laboratory has shown in the past that synaptic $GABA_ARs$ are highly regulated by kinase and phosphatase activity (Tretter and Moss 2008). Due to the plethora of kinases and phosphatases that exert an effect on synaptic $GABA_ARs$ and the different brain regions and cell types in which this activity has been observed, phosphorylation regulates synaptic inhibition in a multitude of ways. However, one common facet of phosphoregulation of synaptic $GABA_ARs$, with respect to cell surface stability, is that it modulates the endocytosis of the receptor. In contrast, we have shown in this study that kinase activity affects the insertion of extrasynaptic $GABA_AR$ subtypes. Synaptic and extrasynaptic inhibition are two fundamentally different ways a neuron can regulate its excitability; therefore, it is important that the neuron be able to regulate each form by modulating different cellular

mechanisms.

In summary, our studies demonstrate that the α4 subunit, a protein critical for tonic inhibition in the dentate gyrus of the hippocampus, is phosphorylated by PKC on serine 443. This phosphorylation leads to an increase in the functional expression of the α4 subunit-containing $GABA_AR$ by increasing its stability and enhancing the rate at which this receptor is inserted into the plasma membrane. Therefore, PKC-dependent phosphorylation of the α4 subunit may have profound effects on the efficacy of tonic inhibition mediated by $GABA_ARs$.

The α4 subunit expression shows a significant degree of plasticity in the brain. Expression of the α4 subunit is increased in mouse models of status epilepticus (Terunuma et al. 2007), following withdrawal from chronic ethanol treatment (Cagetti et al. 2003) and following rapid withdrawal from neurosteroids (Smith et al. 1998). It is possible that PKC phopshorylation of α4 serine 443 mediates these changes in the cell surface expression of α4-containing $GABA_ARs$. Furthermore, changes in the levels of the α4 subunit have been associated with anxiety (Chandra et al. 2006, Gulinello et al. 2003), Pre-menstrual dysmorphic disorder (Smith et al. 1998) and epilepsy (Reddy et al. 2001, 2010). Increased hippocampal expression of the α4 subunit is associated with increased anxiety (assessed by the elevated plus maze behavioral paradigm) and cognitive deficits (Smith et

al. 1998, Guilnello et al. 2001) whereas in catamenial epilepsy, a reduction in the cell surface levels of the α4 subunit is strongly associated with increased seizure susceptibility (Reddy et al. 2001).

Our findings provide a novel mechanism for the dynamic regulation of α4 $GABA_AR$ subunit expression and might aid in the identification of novel pharmacological agents to modulate the cell surface stability of this subunit (and hence the tonic inhibition mediated by α4-containing $GABA_ARs$) for the treatment of disorders in which tonic inhibition is altered.

CHAPTER 3

NEUROSTEROIDS SELECTIVELY MODULATE $GABA_AR$ SUBTYPES THAT MEDIATE TONIC INHIBITION BY PROMOTING A PHOSPHO-DEPENDANT INCREASE IN α4 SUBUNIT CELL MEMBRANE INSERTION

Manuscript to be submitted

Tufts University Patent pending

CONTRIBUTIONS TO THIS PAPER

I conducted the majority of the experiments presented in this chapter (All figures presented here are part of a larger manuscript). I developed experimental procedures, contributed all the electrophysiological experiments, TIRF microscopy imaging and writing. Radiolabeling and biotinylation experiments were conducted by post-doctoral fellow Mathew Abramian PhD.

ABSTRACT

Gamma-aminobutyric acid type A receptors ($GABA_A$Rs) are the principal mediators of inhibitory transmission in the mammalian central nervous system. $GABA_A$Rs can be localized at postsynaptic inhibitory specializations or at extrasynaptic sites. While synaptic $GABA_A$Rs are activated transiently following the release of GABA from presynaptic vesicles, extrasynaptic $GABA_A$Rs are activated continuously by resting GABA concentrations and thus mediate tonic inhibition. The tonic inhibitory currents mediated by extrasynaptic $GABA_A$Rs control neuronal excitability and the strength of synaptic transmission. In the hippocampus and thalamus, extrasynaptic $GABA_A$Rs are predominantly composed of α4 β2/3 and δ subunits.

Steroid metabolites of progesterone and deoxycortisone (known as neurosteroids) have been shown to be potent positive allosteric

modulators of extrasynaptic $GABA_A$Rs. However, the exact mechanisms by which neurosteroids alter extrasynaptic $GABA_A$Rs function are not well understood. We show that the deoxycortisone metabolite, Tetrahydrodeoxycorticosterone (THDOC) induces the phosphorylation of the α4 $GABA_A$R subunit on serine 443 (S443) dependent upon the ability of this agent to directly activate PKC. We analyzed the functional significance of THDOC induced phosphorylation of the α4 subunit using patch-clamp recording and TIRF microscopy. Collectively these approaches revealed that THDOC increases the cell surface stability of α4-containing $GABA_A$Rs by promoting their insertion into the plasma membrane, a phenomenon critically dependent on S443 in the α4 subunit. Significantly, the ability of THDOC to increase the expression levels of $GABA_A$Rs was independent of the delta subunit and glutamine 246 (Q246) in the α4 subunit. However, Q246 was essential for allosteric potentiation of receptor activity by THDOC. Collectively we have identified a novel mechanism by which neurosteroids can induce long-term changes in the expression levels of $GABA_A$Rs via PKC dependent phosphorylation of the α4 subunit.

INTRODUCTION

Fast synaptic inhibition in the central nervous system is mediated largely by $GABA_A$Rs. $GABA_A$R are heteropentamers that can be assembled from 19 $GABA_A$R subunits divided into eight classes based on sequence homology; α1–6, β1–3, γ1–3, δ, ε, θ, ρ(1-3), and π. The heterogeneity of

$GABA_AR$ subunit composition allows for the assembly of $GABA_ARs$ with different physiological and pharmacological properties, sub-cellular distribution, as well as differential expression throughout the brain (Connolly et al. 1996, Rudolph et al. 2001, Sieghart and Sperk 2002).

$GABA_ARs$ of different sub-cellular distribution are activated in a different manner, display dissimilar channel kinetics and mediate distinct forms of neuronal inhibition (Farrant and Nusser 2005). $GABA_ARs$ incorporating the α1–3, β1–3 and γ2 subunits are located predominantly at synaptic sites (Rudolph and Mohler 2006) and are activated in a transient manner after brief exposure to GABA released from the presynaptic membrane. This transient activation of synaptic $GABA_ARs$ results in phasic inhibition. Whereas $GABA_ARs$ composed of the α4/6, β1–3 and δ subunits are localized primarily at peri- or extrasynaptic sites (Farrant and Nusser 2005, Chandra et al. 2006, Zheleznova et al. 2009) and are activated in a persistent, less temporally restricted manner by low ambient concentrations of GABA that either escapes from the synapse into the extracellular space or is released from non-synaptic sites (such as neurogliaform cells and astrocytes; Kozlov et al. 2006, Olah et al 2009). When activated, extrasynaptic $GABA_ARs$ generate an uninterrupted form of conductance that is referred to as tonic inhibition.

Extrasynaptic $GABA_AR$ are targets for various endogenous and exogenous molecules including anticonvulsants, anesthetics and steroids synthesized in the brain known as neurosteroids (Belleli et al. 2002, Bianchi and MacDonald 2003, Stell et al .2003). At low physiological concentrations (10-100nM), neurosteroids selectively enhance the tonic conductance mediated by extrasynaptic $GABA_ARs$ containing the α4-6 and δ subunits while having little effect on the phasic conductance mediated by synaptic $GABA_ARs$ (Stell et al. 2003, Ferrant and Nusser 2005).

Neurosteroids have been shown to bind $GABA_ARs$ at an allosteric site distinct from that of GABA, Benzodiazepines and barbiturates. Work by Hosie et al. identified residues located within the transmembrane domain of $GABA_AR$ α and β subunits as critical for the direct binding (αThreonine 236, βTyrosine 284) and potentiating effect (αAsparagine 407, and αGlutamine 246) of neurosteroids (Hosie et al. 2006, 2007). For α4β3δ $GABA_AR$, which are predominantly expressed at extrasynaptic sites, co-expression of the β3 and δ subunits along with an α4 where a critical residue for the neurosteroid modulatory site was mutated (glutamine 246-$\alpha4^{Q246L}$), significantly decreased or abolished THDOC potentiation of the receptor (Hosie et al. 2009).

Neurosteroids can also regulate changes in $GABA_AR$ subunit cell surface

expression (Maguire and Mody 2007, 2009, Gulinello et al. 2001, Hsu et al. 2003). However, the molecular mechanisms by which neurosteroids alter $GABA_AR$ subunit expression and function are not well understood.

In this chapter we present the novel finding that the stress hormone metabolite THDOC can regulate the phosphorylation and cell surface insertion rate of the α4 $GABA_AR$ subunit in a PKC-dependant manner. Functionally, this increase in the cell surface accumulation of the α4 subunit, results in an abolishment of the rundown of GABA evoked currents mediated by α4-containing $GABA_ARs$ as well as an increase in tonic inhibition in hippocampal neurons. Furthermore, we show that THDOC modulation of the α4 cell surface insertion is likely independent from the potentiating actions of the steroid, since the mutation of a critical glutamine 246 known to abolish potentiation (Hosie et al. 2009) did not block the THDOC effects on the rundown of GABA-evoked currents.

EXPERIMENTAL PROCEDURES

Antibodies and Expression Constructs.

Polyclonal rabbit anti-α4 and anti-δ antibodies were provided to us by Dr. Verena Tretter and Dr. Werner Sieghart from Medical University Vienna. Peroxidase-conjugated IgG secondary antibody was from Jackson ImmunoResearch Laboratories. Fluorescently labeled α-bungarotoxin (α-Bgt) was purchased from Invitrogen. α1, α4, β3 δand cDNAs were cloned into the mammalian cytomegalovirus (CMV) promoter vector PRK5 for

transgene expression. dsRed monomer fluorescent protein (RFP) was introduced after the 4th amino acid of the mature α4 subunit followed by the BBS sequence (WRYYESSLEPYPD). Likewise, pHluorin was introduced between amino acids 4 and 5 of the α1 and δ subunits, and all mutations were verified by DNA sequencing. Plasmids were transfected into COS-7 or HEK293 cells using electroporation (Bio-rad) with 2 μg of plasmid DNA per construct. Nuclofection (AMAXA systems) was used to introduce the respective plasmids into freshly dissociated hippocampal neurons that were used at Div10-14.

Cell culture.

cell lines: COS-7 and Human embryonic kidney (HEK 293) cells are co-transfected with selected $GABA_AR$ subunits via electropermeabilization (Gene Pulser MXcell Electroporation System, Bio-Rad). Cells are maintained in the appropriate media for culture according to manufacturer. For HEK 293 cells, DMEM/F12 media (GIBCO) supplemented with 5% Fetal bovine serum and 1% Pen-Strep is used. For some experiments, media is supplemented with 30mM HEPES buffer (Invitrogen). Imaging experiments are conducted 48-72 hours after transfection.

Primary hippocampal neurons: Rat embryonic day 18 (E18) hippocampal neurons are nucleofected at plating with selected $GABA_AR$ subunits via electropermeabilization (AMAXA systems). Neurons are maintained in

Neurobasal media (GIBCO) supplemented with B-52, glutamax (Invitrogen) and glucose (Sigma Aldrich, St. Louis MO). 150 thousand neurons are plated in each Poly-L-Lysine coated 35mm dish. Experiments are conducted at DIV 14-18.

Whole-cell COS-7 Cell and Hippocampal Slice Metabolic ^{32}P Labeling

COS-7 cells were transfected and incubated as described above. Cells were initially incubated in 2 ml of phosphate-free DMEM for 30 min at 37 °C. Following this incubation, cells were labeled with 0.5 mCi/ml [^{32}P]orthophosphoric acid for 4 h in phosphate-free DMEM. Hippocampal slices were prepared as described above. Slices were individually transferred to polypropylene tubes containing 2 ml of fresh ACSF; gassed with a mixture of 95% O_2, 5% CO_2; and maintained in a 30 °C water bath. Labeling was performed by adding 0.5 mCi/ml [^{32}P]orthophosphoric acid for 1 h. For both COS-7 cells and hippocampal slices, samples were treated with drugs the labeling period, followed by the cell lysis and immunoprecipitation procedure described above. Results were attained by SDS-PAGE followed by autoradiography.

Biotinylation

Cultures were washed once with ice-cold PBS and then incubated in 2 ml of ice-cold PBS containing 1 mg/ml NHS-SS-biotin (Pierce) for 20 min in order to label surface proteins with biotin. After labeling, the biotin was

quenched by incubating cells in PBS containing 25 mM glycine and 10mg/ml bovine serum albumin (BSA). Cells were then lysed in lysis buffer and sonicated. For hippocampal slice experiments, slices were incubated in ACSF described above at 30 °C for 1 h for recovery before experimentation. Slices were then placed on ice and incubated for 30 min with 1 mg/ml NHS-SS-biotin. Excess biotin was removed by washing slices three times in ice-cold ACSF and lysed. For both COS-7 cells and hippocampal slices, insoluble material was removed by centrifugation. The supernatant lysates were incubated with NeutrAvidin beads (Pierce) for 18–24 h at 4 °C. Bound material was eluted with sample buffer and subjected to SDS-PAGE and then immunoblotted with indicated antibodies. Blots were then quantified using the CCD-based FujiFilm LAS 300 system.

Cell Lysis and Immunoprecipitation

Samples collected from either COS-7 cell cultures or hippocampal slices were lysed in lysis buffer containing the following: 20 mM Tris-HCl (pH 8.0), 150 mMNaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM sodium pyrophosphate, 1% Triton X-100, and 0.1% SDS. In addition, the following protease inhibitors were added: 250µg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; 10 µg/ml leupeptin, 1 µg/ml pepstatin, and 10 µg/ml antipain. Samples were then sonicated and spun at 16,000 × *g*. The supernatant was collected and then subjected to a

protein assay using a standard Bradford protocol. 100–200 µg of protein were loaded per immunoprecipitation sample along with 3 µg of indicated antibody and 40 µl of protein A-Sepharose beads (1:1 slurry) (GE Healthcare). Samples were allowed to conjugate for 18–24 h at 4 °C with constant agitation. The beads were precipitated by centrifugation at 500 × *g* and washed once with ice-cold Buffer A (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM sodium pyrophosphate, and 1% Triton X-100 and protease inhibitors), two times with Buffer B composed of Buffer A supplemented with 500 mM NaCl, and once again with Buffer.

Patch-Clamp Electrophysiology.

Human embryonic kidney cells (HEK 293) or DIV 14-18 hippocampal neurons expressing fluorescent-tagged $GABA_AR$ subunits were superfused, at a rate of 2 ml/min, with an extracellular solution containing (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, 11 glucose and adjusted to pH 7.4 with NaOH. Borosilicate glass patch pipettes (resistance 2-5 MΩ) will contain (in mM) 140 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, 2 ATP (Mg^{2+} salt) and adjusted to pH 7.4 with KOH. GABA was applied once every 120 s via a fast-step perfusion system (Warner Inst., Hamden, CT). All experiments were carried out at 32-33 °C using recording chamber and in-line perfusion heaters (Warner Inst.). Phorbol esters, THDOC (Tocris Bioscience, Minneapolis MN) and PKC

Inhibitor peptide 19-36 (Promega, Madison WI) were applied to the cell either via passive intracellular delivery in the patch pipette or superfused into the recording chamber. Currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz and digitized at 20 kHz with a Digidata 1320A (Molecular Devices) and analyzed using Clampfit (pClamp, Molecular Devices).

Total Internal Reflection Microscopy.

HEK 293 or DIV 14-18 hippocampal neurons were transfected/nuclefected with fluorescent-tagged $GABA_AR$ subunits and plated in Poly-L-lysine (1mg/ml-Sigma Aldrich, St. Louis MO) glass-bottom plates (FluoroDish sterile culture dishes, World Precision Intruments, Sarasota. FL). Live TIRF imaging microscopy was conducted with a Nikon Eclipse Ti Inverted TIRF Microscope (Nikon Intruments, Melville NY) equipped with Perfect focus mechanism to avoid focus drift and a stage top incubator with CO2 and temperature control. All experiments were conducted at 32°C. Cells were allowed to equilibrate in the stage top incubator for 10 minutes before conducting experiments, Samples were viewed through a plan Apo TIRF 60X high-resolution (1.45NA) oil immersion objective. Fluorescent images were collected with an iXion EMCCD camera (Andor Technology, South Windsor CT) interfaced to a PC running Nikon NIS-Elements software. Images were collected with a 200ms exposure time before and immediately after addition of THDOC. For Botulinum Neurotoxin A (BotNT-

SIGMA) experiments, 50uM of activated toxin was added 10 minutes prior THDOC addition. Total insertion events per minute were marked and taken as insertion frequency (Lin et al. 2009). GraphPad Prism Software was used for data analysis.

Data Acquisition and Analysis.

For biochemical and immunofluorescent experiments, data are presented as means ± S.E. Statistical analysis was performed by using Student's *t* test where a*p* value of <0.5 is considered significant. For electrophysiological experiments, currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz and digitized at 20 kHz with a Digidata 1320A data acquisition system (Molecular Devices), and analyzed using either Clampfit (pClamp, Molecular Devices) or GraphPad Prism version 4 software (GraphPad Software, Inc., San Diego, CA). Statistical analysis was performed by using one-way ANOVA with a Bonferroni post-test with statistical significance set at $p < 0.05$. All data are expressed as mean ± S.E.

RESULTS

THDOC selectively regulates the PKC-dependent phosphorylation and cell surface expression of the $GABA_AR$ α4 subunit. Previously published data has suggested that the actions of neurosteroids on tonic inhibition in the hippocampus are dependent upon the activity of protein

kinase C (PKC) (Harney et al., 2003; Hodge et al., 2002). Consistent with this, we have established that the α4 subunit is phosphorylated by PKC on Serine 443 (S443) (Chapter 2). To determine whether THDOC modulates phosphorylation of the α4 subunit in a PKC- dependent manner, we compared levels of phosphorylation of the α4 subunit in transfected COS7 cells in the presence and absence of THDOC and PKC inhibitors. Immunoprecipitation was used to examine the phosphorylation of $GABA_AR$s in COS-7 cells transiently transected with the $GABA_AR$ α4 and β3 subunits. Immunoprecipitation with anti-α4 from transfected COS-7 cells that had been prelabeled with [^{32}P]-orthophosphoric acid under basal conditions yielded a major phosphoprotein at approximately 64 kDa (Figure 1A). Treating COS-7 cells with 100 nM THDOC for 10 min produced a significant increase in α4 subunit phosphorylation compared to control cells treated with dimethyl sulfoxide (DMSO) for 10 min (Figure 1A). Pre-treatment with GF 109203X (GFX-10 μM for 20 min), a PKC inhibitor, significantly reduced the level of phosphorylation of the α4 subunit compared to vehicle treated controls (Figure 1A). In addition, treating transfected COS-7 cells with GFX 10 min prior to THDOC treatment not only prevented the increase in α4 subunit phosphorylation observed with THDOC treatment alone, but also led to a significant reduction in the level of phosphorylation compared to vehicle-treated controls (Figure 1A).

We have shown that PKC activation leads to an increase in α4 subunit cell surface protein expression (Chapter 2). We next examined the effect of THDOC on α4 subunit cell surface expression in transfected COS-7 cells using a biotinylation assay (Fairfax et al. 2004, Kittler et al. 2004). This revealed that a 10 min treatment with 100 nM THDOC significantly increased the cell surface levels of the α4 subunit, but the total levels of protein were not altered (Figure 1B). Treatment with 10 μM GFX for 10 min had little effect on the basal cell surface levels of the α4 subunit. In contrast, treating transfected COS-7 cells with GFX 10 min prior to THDOC treatment prevented the increase in cell surface expression of the α4 subunit observed with THDOC treatment alone (Fig 1B). These results indicate that the THDOC-dependent phosphorylation of the α4 subunit increases receptor cell surface expression.

In our published studies (Chapter 2), we identified serine 443 as the major site for PKC phosphorylation in the α4 subunit. A mutant version of the α4 subunit in which serine 443 was exchanged for an alanine residue, abolished the PKC-dependant phosphorylation of the α4 subunit (Chapter 2). We examined whether the neurosteroid mediated enhancement of α4 phosphorylation and cell surface expression is dependant on serine 443. COS-7 cells transfected with wild-type α4 or $\alpha4^{S443A}$ and β3 subunits were subjected to [^{32}P]-orthophosphoric acid labeling and treated with either DMSO or THDOC. THDOC significantly enhanced levels of

phosphorylation of wild-type α4 (Figure 1C). In contrast to wild-type α4, THDOC did not significantly enhance the phosphorylation of the $\alpha4^{S443A}$ subunit (Figure 1C). Together, these results strongly suggest that THDOC enhances basal levels of phosphorylation and increases the cell surface protein levels of the α4 subunit. Furthermore, this enhancement is dependant on α4 serine 443 and requires that PKC be in an active form.

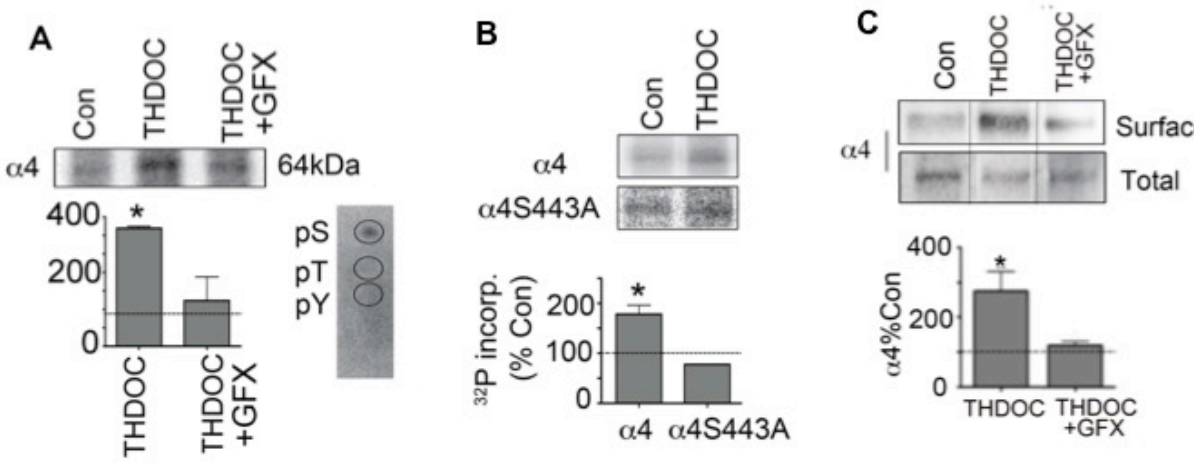


Figure 1: THDOC selectively regulates the PKC-dependent phosphorylation and cell surface expression of the $GABA_AR$ α4 subunit. *(**A**) COS-7 cells expressing $\alpha4\beta3$ subunits were treated with either vehicle labeled with 1 mCi/ml ^{32}P-orthosphosphoric acid, then treated for 10 min with either vehicle control (DMSO), 100 nM THDOC or pre-treatment with PKC inhibitor GF109203X (GFX) followed by 100nM THDOC application. The $\alpha4$ subunit was isolated using immunoprecipitation. Phosphorylation was normalized to vehicle-treated*

*samples. ^{32}P-α4 was subject to phospho-amino acid analysis. The migration of phospho-serine (pS), phospho-threonine (pT), and phospho-tyrosine are shown (pY). (B) Transfected cells were treated with either vehicle DMSO or THDOC (100nM for 10mins) and were labeled with NHS-Biotin and detergent-soluble extracts were purified on NeutrAvidin. Cell surface and 10% of total fractions were analyzed by immunoblotting with anti-α4. Histograms show the proportion of cell surface α_4 protein expressed as a percentage of vehicle-treated controls (dashed line; p<0.05). In all panels * = significantly different from control.*

Internally applied THDOC selectively modulates the function of α4–containing $GABA_AR$ subtypes. Our published studies have shown that in fibroblasts GABA-activated currents for receptors composed of α4β3 receptors recorded in the whole-cell conformation show significant rundown, a phenomenon that could be alleviated via the activation of PKC dependent upon S443 in the α4 subunit (chapter 2). Rundown is believed to reflect a loss in the number of functional cell surface $GABA_ARs$ due to deficits in their insertion into the plasma membrane. This result can be explained by the insertion of the α4 subunit containing $GABA_ARs$ into the plasma membrane following PKC-mediated phosphorylation of S443 (Chapter 2).

To determine the functional significance of our biochemical experiments we examined the effects of internally applied THDOC (100nM; via the patch pipette) on $\alpha4\beta3$ $GABA_AR$-mediated current rundown. While extracellular application of neurosteroids enhances GABA-evoked currents, intracellular application has been reported to have no effect (Lambert et al., 1990). HEK293 cells were transfected via electroporation with $\alpha4$ and $\beta3$ subunits DNA plasmids as well as with a Green Fluorescent Protein (GFP) construct to allow for visualization of transfected cells. Rundown was examined by applying ~ EC_{50} concentrations of GABA every 2 minutes. Under control conditions (Internally applied DMSO), we observed significant rundown in the magnitude of $\alpha4\beta3$-mediated GABA-evoked currents (Figure 2A,B). At 20 minutes after the beginning of the experiment, the GABA-evoked current amplitude was $35\pm10\%$ of the initial response (n=6). Inclusion of THDOC in the patch pipette significantly reduced rundown compared to control (DMSO). After 20 minutes of recording, GABA-mediated current amplitude was $86\pm12\%$ of the initial response (n=5) (Figure 2A,B). Furthermore, the ability of THDOC to prevent current rundown was critically dependent upon the activation of PKC. In cells exposed to both THDOC and PKC inhibitory peptide 19-36 via the patch pipette, GABA-activated currents exhibited rundown to a similar degree as during control conditions (Figure 2A,B).

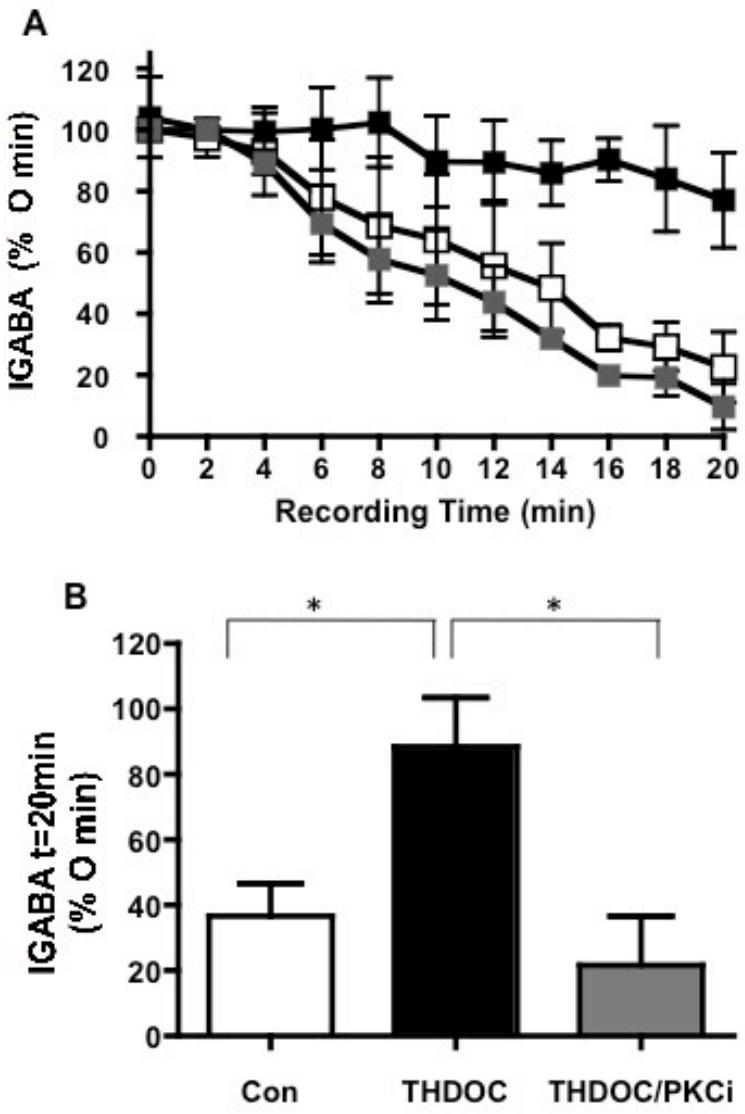


Figure 2:Internally applied THDOC selectively modulates the function of α4 –containing $GABA_AR$ subtypes. *HEK293 cells were transfected via electroporation with a α4 and β3 subunit DNA constructs as well as a GFP construct to allow for visualization. Whole-cell currents were recorded 24-72 hours after transfection. For all experiments, 1 μM GABA (~EC_{50}) was added in the bath solution every 2 minutes via a fast-step perfusion.* GABA-activated currents were recorded 3-5 min after achieving

whole-cell configuration. ***(A)****Time-dependence relationship for 1 μM GABA-activated currents recorded in the presence of either internally applied vehicle control (DMSO)* (□) *100nM THDOC* (■) or *100nM THDOC and 200nM PKC Inhibitor Peptide 19-36* (■). ***(B)*** *Bar graph of the relative 1 μM GABA current at t=20 minutes compared to current at t=0 minutes for α4β3 receptors in control conditions (□), perfused internally with 100 nM THDOC (■) or 100nM THDOC along with 200nM PKC Inhibitor Peptide 19-36 (■). *=Significantly different from control DMSO and PKC Inhibitor (T-test p<0.05;n=4-6).*

The ability of the neurosteroid to prevent the rundown of $\alpha4\beta3$-mediated currents is not a general phenomenon for $GABA_A$Rs allosteric modulators, since passive intracellular delivery of the anesthetic propofol (3μM in the patch pipette) did not prevent the rundown of GABA-activated $\alpha4\beta3$-mediated currents (Figure 3A,B).

Next, we explored whether the ability of THDOC to prevent the rundown of GABA-currents is mediated via the ability of this agent to modify the phosphorylation of the $\alpha4$ subunit. We examined the rundown of GABA evoked currents for receptors expressing the $\alpha4^{S443A}$ subunit. HEK293 cells were transiently transfected with either wild-type ($\alpha4\beta3$) or mutant ($\alpha4^{S443A}\beta3$) receptors. GABA-activated currents in cells expressing $\alpha4^{S443A}\beta3$ receptors exhibited minimal current rundown under control

conditions (Internal DMSO application). After 20 minutes of recording, the $\alpha4^{S443A}\beta3$-mediated GABA currents were approximately 84% (n=5) of the GABA-evoked current amplitude at the beginning of the experiment (Fig 4A,B). Furthermore, the presence of 100nM THDOC in the patch pipette had no significant effect in the rundown of GABA-evoked currents when compared to control (Figure 4A,B). Collectively, these results reveal that THDOC selective modulates the function of $\alpha4$-containing $GABA_AR$ in a mechanism dependent upon serine 443 and PKC activity.

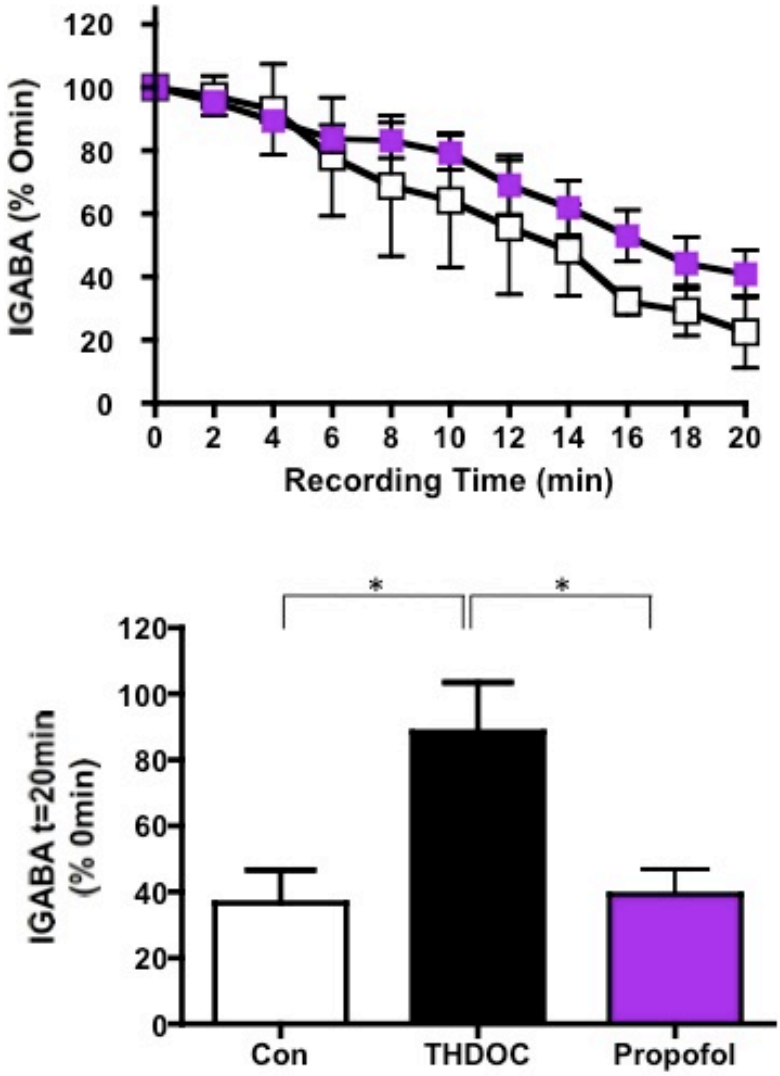


Figure 3: Internal application of the intravenous anesthetic Propofol does not prevent the rundown of α4β3-mediated currents. GABA-evoked currents from HEK293 cells transiently transfected *with α4 and β3 subunits, were recorded 3-5 min after achieving* whole-cell configuration. ***(A)*** *Time-dependence relationship for 1 μM GABA-activated currents recorded in the presence of either internally applied vehicle control (DMSO)* (□) *or 3μM Propofol* (■). ***(B)*** *Bar graph of the relative 1 μM*

*GABA current at t=20 minutes compared to current at t=0 minutes for α4β3 receptors in control conditions (□), perfused internally with 100 nM THDOC (■) or with 3μM Propofol (■). *=Significantly different from control DMSO and Propofol (T-test p<0.05;n=4-7).*

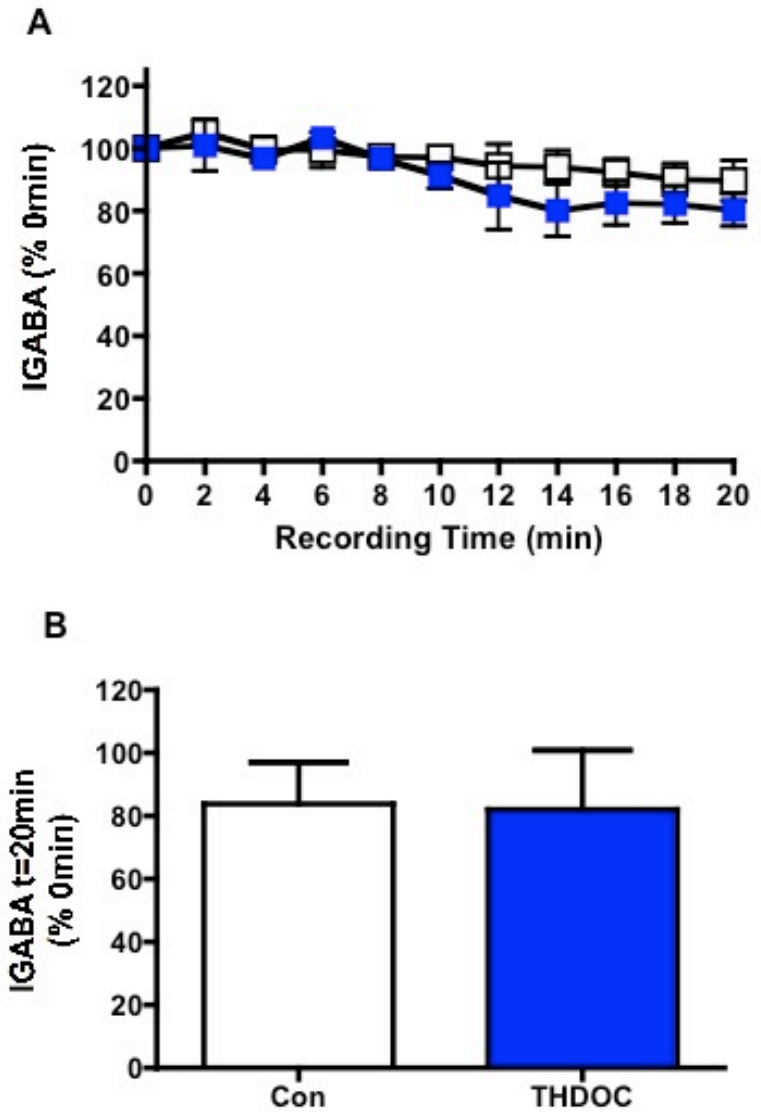


Figure 4: THDOC modulation of α4β3-mediated current rundown is dependant on α4 serine 443. *HEK293 were transiently transfected with*

α4^{S443A} and β3 subunits along with GFP to allow for visualization. GABA-evoked currents were recorded 3-5 min after achieving whole-cell configuration. ***(A)*** *Time-dependence relationship for 1 μM GABA-activated currents recorded in the presence of either internally applied vehicle control (DMSO)* (□) *100nM THDOC* (■). ***(B)*** *Bar graph of the relative 1 μM GABA current at t=20 minutes compared to current at t=0 minutes for α4β3 receptors in control conditions (□), perfused internally with 100 nM THDOC (■).*

THDOC treatment does not modulate the cell surface expression of GABA$_A$Rs subunits responsible for fast synaptic Inhibition

For synaptic GABA$_A$Rs, phosphorylation has been shown to be a critical mechanism for the regulation of GABA$_A$R trafficking (Arancibia-Cárcamo and Kittler 2008, Vithlani and Moss 2009). Furthermore, phosphorylation has been associated with the actions of various GABA$_A$Rs allosteric modulators (Leidenheimer et al. 1997,Leidenheimer and Chapell 1997). We wished to determine whether the effects of THDOC treatment were specific for the α4 subunit, or whether it could also enhance the cell surface stability of GABA$_A$R subunits that mediate phasic inhibition. To answer this question, we evaluated the effect of THDOC treatment on the cell surface stability of the GABA$_A$R α1 subunit in transfected COS7 cells. The α1 subunit is the main synaptic α GABA$_A$R subunit in the cerebellum, hippocampus and globus pallidus (Craig et al. 1994; Nusser et al. 1995;

Somogyi et al. 1996; Brunig et al. 2002). Cells transfected with both α1 and β3 subunit DNA plasmids were treated with THDOC and cell surface levels were measured using a biotinylation assay. Using this technique we determined that treatment with 100 nM THDOC for 10 min had no significant effect on the cell surface stability of the α1 subunit compared to vehicle-treated controls (Figure 5A). We then confirmed the functional significance of this finding by examining the rundown of α1β3-mediated GABA-activated currents. HEK293 were transiently transfected with α1 and β3 DNA constructs and exposed to EC_{50} concentrations of GABA every two minutes via fast perfusion system. Infusion of 100nM THDOC did not prevent the rundown of GABA-evoked α1β3 currents (Figure 5C,D). Similar to control, when THDOC was applied in the patch pipette, currents after 20 minutes of recording were approximately 28% of the initial response (Figure 5C,D).

Collectively, these results suggest that THDOC selectively regulates the cell surface stability of $GABA_AR$s known to be exclusively extrasynaptic and largely responsible for tonic inhibition in specific brain regions.

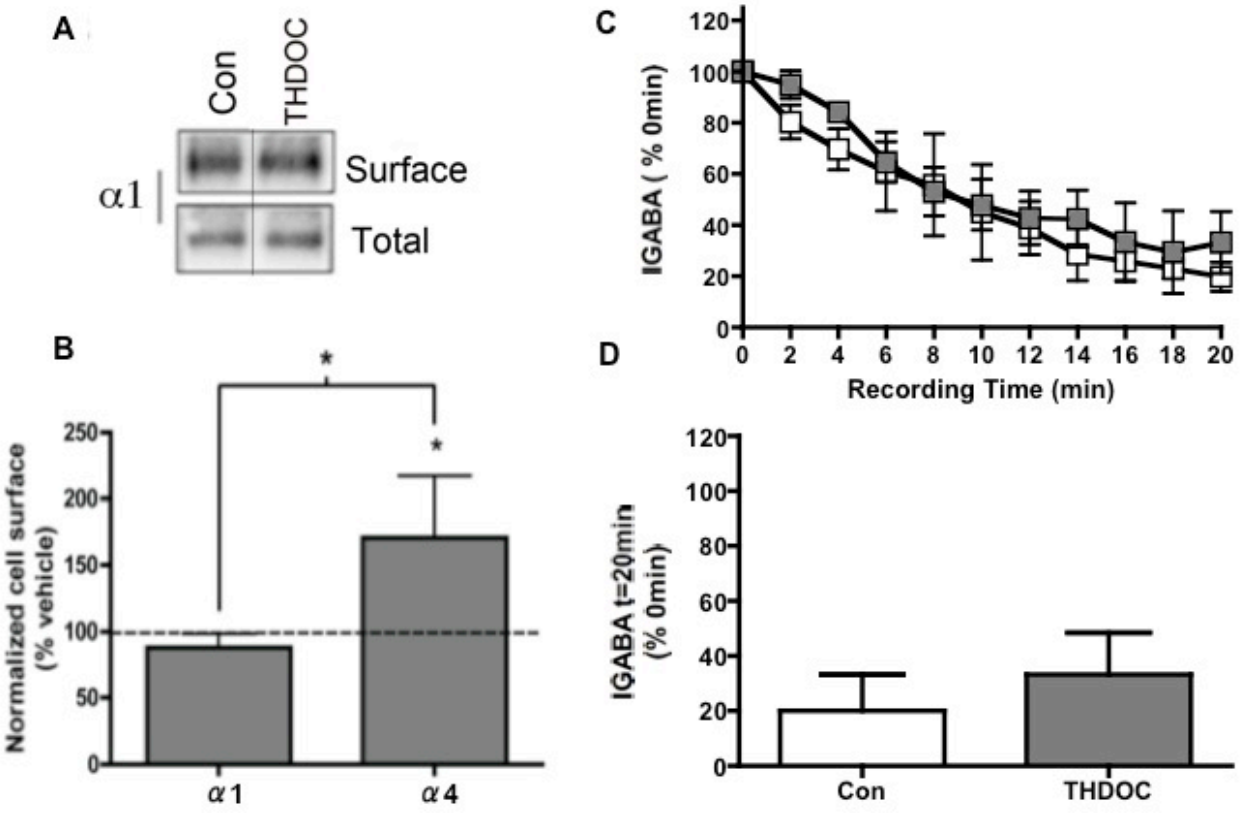


Figure 5: THDOC treatment does not modulate the cell surface expression of $GABA_A$Rs subunits responsible for fast synaptic Inhibition

C-D.*HEK293 were transiently transfected via electropermeabilization with a α1 subunit tagged with a* pH sensitive form of green fluorescent protein (*^{pHlourin}α1) and a β3 subunits.* GABA-evoked currents were recorded 3-5 min after achieving whole-cell configuration. ***(C)*** *Time-dependence relationship for 1 μM GABA-activated currents recorded in the presence of either internally applied vehicle control (DMSO)* (□) *or 100nM THDOC* (■). ***(D)*** *Bar graph of the relative 1 μM GABA current at t=20 minutes compared to current at t=0 minutes for α1β3 receptors in control*

conditions (□) or perfused internally with 100 nM THDOC (■).=Significantly different from control (T-test p<0.05;n=4-6).*

The $GABA_AR$ neurosteroid binding site does not contribute to the THDOC-mediated enhancement of the α4 subunit functional stability.

Neurosteroids are capable of selectively enhancing $GABA_AR$ activity by directly binding to a specific site on the α subunit. Work by Hosie et al. identified a specific glutamine residue on the α1 subunit as critical for the neurosteroid binding site (Hosie et. al., 2006). This site is universally conserved in all the α subunits (Hosie et al., 2009). In our current study, we observe that THDOC treatment results in increased levels of the α4 subunit at the cell surface and in this way prevents the rundown of currents mediated by α4-containing $GABA_AR$s. We hypothesize that this enhancement is independent of the neurosteroid binding site and therefore occurs through a novel mechanism. To test this hypothesis, we constructed a α4 subunit plasmid in which glutamine 246, a critical residue of the endogenous neurosteroid binding site, has been mutated to a leucine ($\alpha4^{Q246L}$). This mutation has been shown to abolish THDOC-mediated potentiation of GABA-evoked currents (Hosie et al. 2009). We examined the rundown $\alpha4^{Q246L}\beta3$-mediated currents when exposed to internally applied THDOC. We first examined whether agonist potency was altered by the mutation. $\alpha4^{Q246L}\beta3$ subunits formed functional channels when co-expressed in HEK293 cells. Similar to wild-type

receptors, $\alpha4^{Q246L}\beta3$ channels had a GABA-evoked EC_{50} value of 1.66μM±0.9 with a hill coefficient of 0.6±0.3 (Data not shown).

Whereas during control conditions (internal DMSO application), $\alpha4^{Q246L}\beta3$-mediated GABA-activated currents exhibited rundown over time (Figure 6C,D), application of 100nM THDOC via the patch pipette prevented the rundown GABA-evoked currents (Figure 6C,D). When THDOC was internally applied, after 20 minutes of the experiment the GABA-evoked currents were 71±17% of the original response.

These results strongly suggests that, in addition to the classic kinetic regulation of $GABA_AR$ activity, THDOC is capable of modulating the trafficking of $GABA_ARs$ via a mechanism independent from the previously identified neurosteroid binding site.

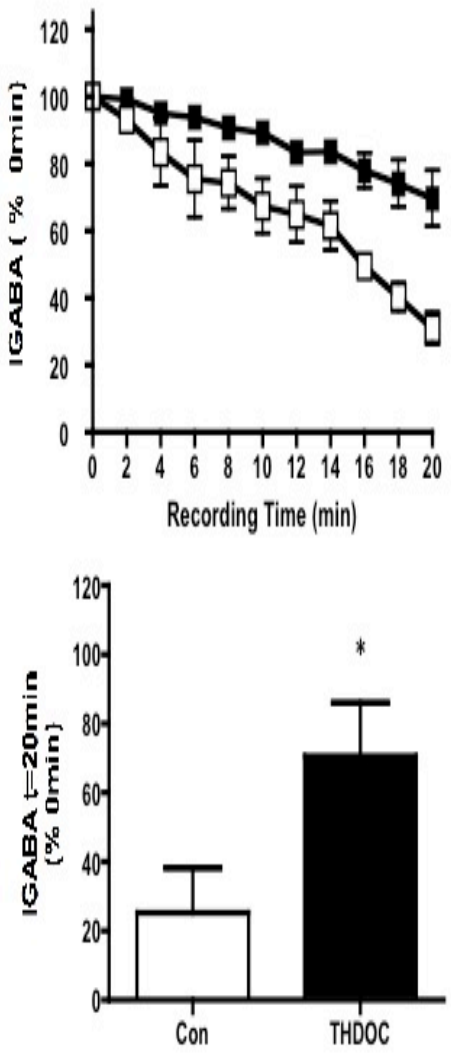


Figure 6:The $GABA_AR$ neurosteroid binding site does not contribute to the THDOC-mediated enhancement of the α4 subunit functional stability.

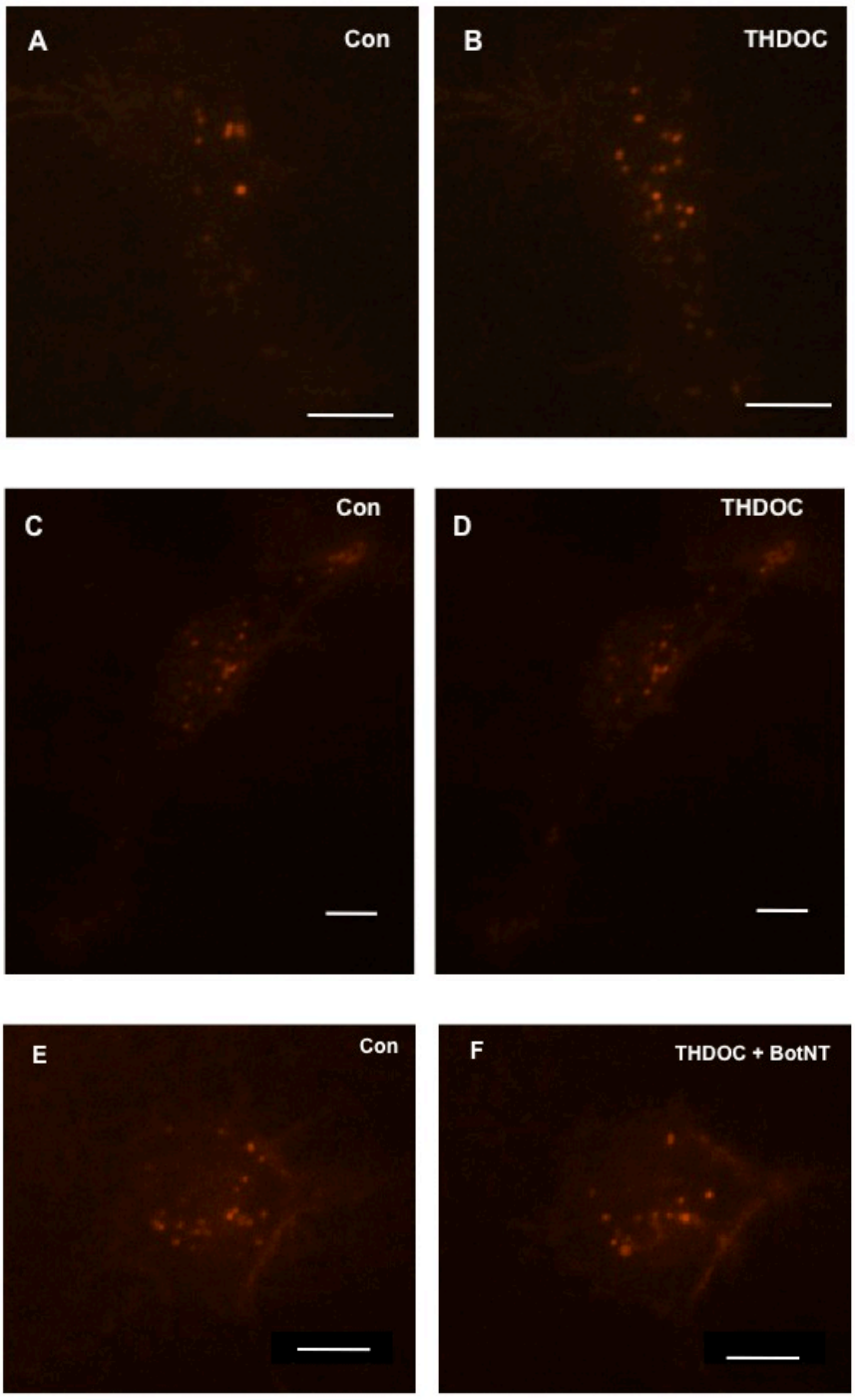
GABA-evoked currents from HEK293 cells transiently transfected with $\alpha4^{Q246L}$ and β3 subunits, were recorded 3-5 min after achieving whole-cell configuration. ***(C)*** *Time-dependence relationship for 10 μM GABA-activated currents recorded in the presence of either internally applied vehicle control (DMSO)* (□) *or 100nM THDOC* (■). ***(D)*** *Bar graph of the*

relative 10 μM GABA current at t=20 minutes compared to current at t=0 minutes for $\alpha4^{Q246L}\beta3$ *receptors in control conditions (□) or perfused internally with 100 nM THDOC (■).*=Significantly different from control (T-test p<0.05;n=3-6).*

THDOC selectively enhances the membrane insertion of $GABA_AR$ subtypes containing α4 subunits in HEK293 cells. In order to visualize the effects of THDOC on the plasma membrane accumulation of $GABA_ARs$, we modified the N-terminal extracellular domains of the individual receptor subunits between amino acids 4 and 5 with fluorescent reporters. Specifically we introduced Red Fluorescent protein into wild-type and phospho-mutant α4 subunit (^{RFP}α4, ^{RFP}α4^{S443A}), and pHluorin (pH sensitive green fluorescent protein) into the α1 subunit (^{pHlourin}α1). The respective subunits along with non-tagged β3 subunits, were then expressed in HEK293 cells via electropermeabilization. Live insertion events in the plasma membrane were examined with total internal reflection microscopy (TIRF). TIRF measures fluorescent signals within 100 nm of the cell surface, thus making this method ideal for measuring the dynamics of membrane proteins. Fluorescent images were collected before and after 100nM THDOC application. Neurosteroid application dramatically increased frequency of insertion of the α4 subunit (Figure 7A,C). This effect was critically dependent upon S443 as the insertion frequency for receptors in which this residue had been mutated (^{RFP}α4^{S443A}) were insensitive to THDOC treatment (Figure 7C-D,I). We next explored whether the neurosteroid-mediated increase in α_4 cell surface stability is dependant on vesicle-mediated trafficking. To assess this we utilized Botulinum Neurotoxin A (BotNT), a potent blocker of vesicle-dependent trafficking. Pre-treatment with 50uM BotNT prevented the THDOC-mediated increase in ^{RFP}α4 frequency of insertion (Figure 7 E-F,I). Furthermore, THDOC did not increase the insertion frequency of ^{pH}α1-expressing $GABA_ARs$ (Figure

7G-H,I). Collectively, these results suggest that THDOC modulated the cell surface stability of α4-containing $GABA_A$Rs by increasing the cell surface insertion rate of the α4 subunit. This is dependent on serine 443 and on vesicle-mediated trafficking and is selective for this $GABA_A$R subunit that mediates tonic inhibition.

THDOC enhances the cell surface insertion rate of α4-containing $GABA_A$Rs in hippocampal neurons. We confirmed our heterologous cell line studies in hippocampal neurons. E18 rat hippocampal neurons were transfected at plating via nuclefection with the ^{RFP}α4 $GABA_A$Rs subunits. At DIV 12-18, neurons were imaged before treatment (control), after incubation with 100nM THDOC. Live TIRF microscopy was conducted using a temperature and CO2 controlled chamber. Using this technique, puncta of RFP fluorescence were evident on the cell bodies and neuronal process of neuclofected neurons (Figure 8A-B,E). 3-dimensional projection images (Nikon elements software) were used to visualize the insertion of receptors throughout neuclofected neurons (Figure 8C-D). We compared the rate of insertion of $GABA_A$Rs containing ^{RFP}α4 under control conditions and after 20 min incubation with 100 nM THDOC (Figure 8E). THDOC increased the frequency of insertion of the ^{RFP}α4 subunit (Figure 8E), which appear at existing sites of receptor expression and also in regions devoid of existing ^{RFP}α4 subunits (Figure 8A-B).


A
Con
B
THDOC
C
Con
D
THDOC
E
Con
F
THDOC + BotNT

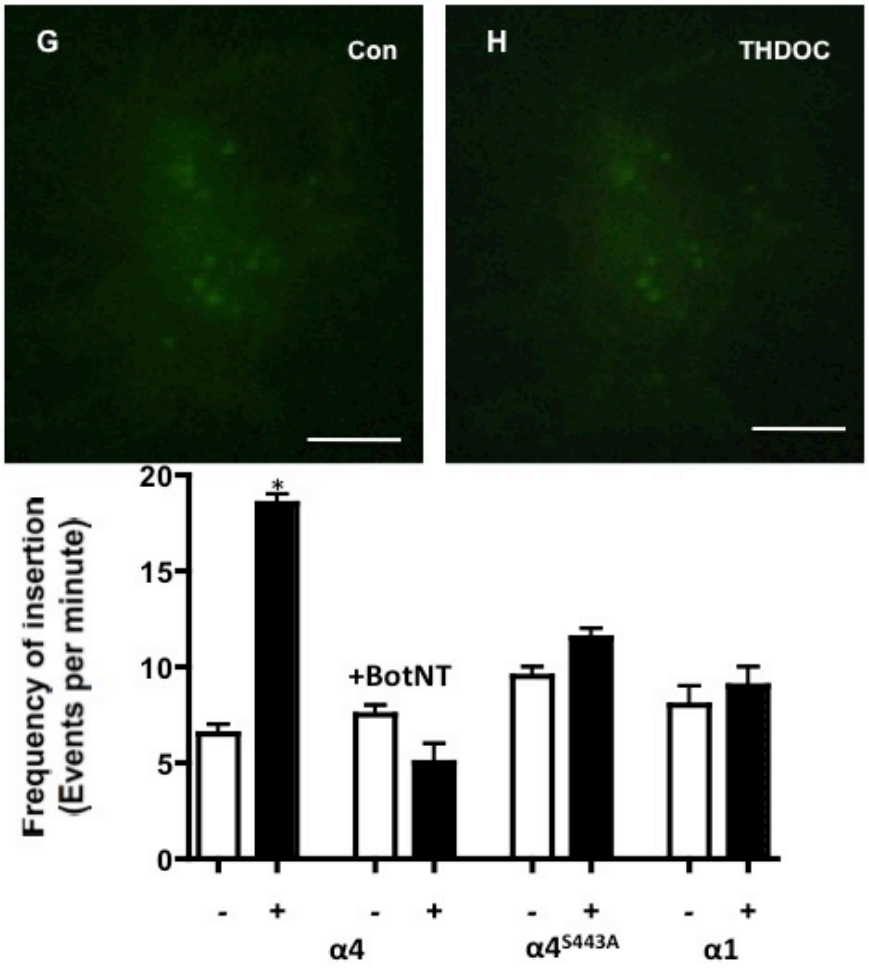
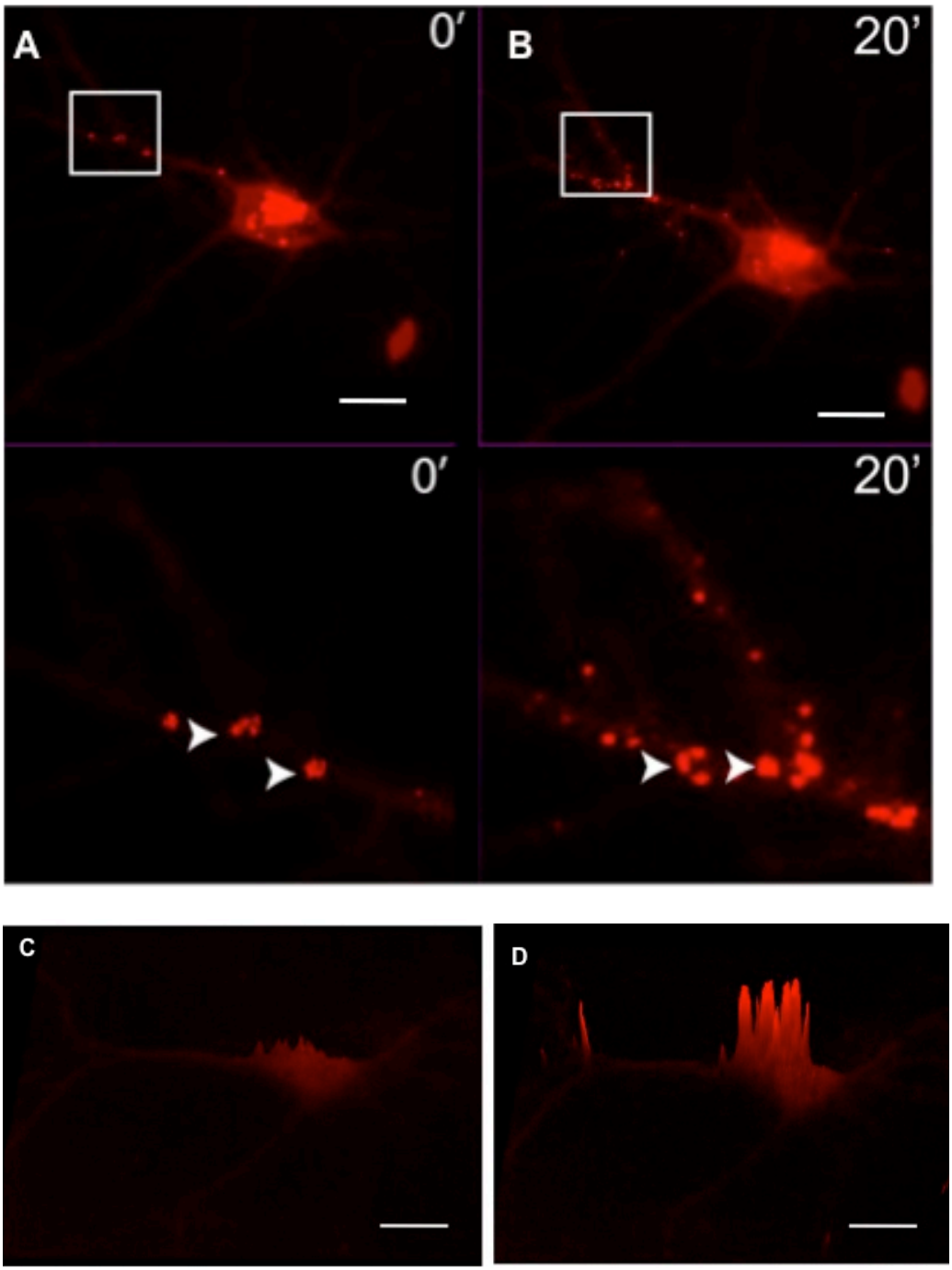


Figure 7: THDOC selectively enhances the membrane insertion of $GABA_AR$ subtypes containing α4 subunits in HEK293 cells. *HEK293 cells were transfected via electroporation with wild-type, S443A mutant α4 constructs tagged with red fluorescent protein (^{RFP}α4 and ^{RFP}α4^{S443A} respectively) or with a pH sensitive form of green fluorescent protein α1 (^{pHlourin}α1) subunits, along with* β3 and δ *$GABA_AR$ subunits. 24-72 hours after transfection, cells were imaged for 5 minutes and where then exposed to 100nM THDOC for 20 minutes.* ***(A-H)*** *Representative images collected before and 20 min after THDOC application for* ***(A-B)*** *Wild-type* ^{RFP}α4β3δ, ***(C-D)*** ^{RFP}α4^{S443A}β3δ, **(E-F)** ^{RFP}α4β3δ following pre-incubation

with BotNT and **(G-H)** *^{pHlourin}α1-containing receptors. Total insertion events per minute of live imaging were marked and taken as frequency of insertion **(I)**. White bar represents 10μm. *=Significantly different from control (T-test p<0.05;n=5-7).*



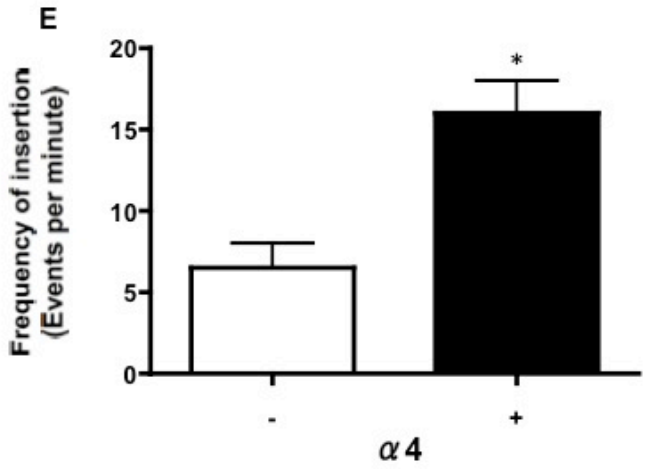


Figure 8: THDOC enhances the cell surface insertion rate of α4-containing GABA$_A$Rs in hippocampal neurons. *Hippocampal neurons were nucleofected with $^{RFP}\alpha 4$ and subject to TIRF imaging. Images were collected immediately* ***(A)*** *prior and after 100nM THDOC application.* ***(B)*** *Representative image of 20 min after exposure to THDOC. The arrowheads indicate puncta of newly inserted GABA$_A$Rs containing $^{RFP}\alpha 4$ subunits.* ***(C-D)*** *Representative 3-D Fluorescent Intensity Projection Images before* ***(A)*** *and after* ***(B)*** *THDOC application. Total insertion events per minute of live imaging were marked and taken as frequency of insertion* ***(E).*** **=Significantly different from control. Scale bar = 10 μm*

THDOC-mediated increase in α4-containing $GABA_A$Rs cell surface functional expression is independent of the δ subunit. The α4 subunit can be expressed at extrasynaptic sites along with β2/3 with or without a δ subunit. Binary αβ have been proposed to mediate certain forms of tonic inhibition in cultured hippocampal neurons (Mortensen and Smart, 2006). Interestingly, biochemical data suggest that 50% of α4 subunit-containing $GABA_A$Rs do not contain γ or δ subunits (Bencsits et al. 1999). It is also likely that the tonic currents seen in δ knock out mice could be mediated, at least in part, by αβ receptor subtypes (Herd et al. 2008). The δ subunit does not appear to be subject to phosphorylation (Chapter 2) and it does not seem to form part of the Neurosteroid binding site (Hosie et al. 2009). However, incorporation of the δ subunit into functional $GABA_A$Rs has been shown to increase the potency of the receptor to GABA, when compared to those composed of only αβ subunits (Meera et al. 2009). The high potency δ-$GABA_A$Rs, allows for receptor activation at low nanomolar GABA concentrations, which is a critical feature of receptors that mediate tonic inhibition. We wished to examine whether the co-expression with the δ subunit had any effect on the THDOC- mediated enhancement of α4-containing $GABA_A$Rs functional expression. To test this, we studied the rundown of α4β3δ-mediated currents, under control conditions (Figure

9A,B) or with internal application of 100nM THDOC (Figure 9A,B). In order to ascertain that currents observed were indeed from α4β3δ receptors and not α4β3, we utilized a δ subunit tagged with a pH sensitive form of green fluorescent protein (pHluorin- ^{pHlourin}δ). In addition, experiments were conducted with 1μM Zn^{2+} in the bath solution, which blocks αβ-mediated currents while having little effect on $GABA_AR$ containing the δ subunit (Meera et al. 2009, Zheleznova et al. 2008). Under control conditions α4β3^{pHlourin}δ-mediated currents exhibited the typical decreased current amplitude over time in response to EC_{50} GABA every 2 minutes (0.5uM GABA for α4β3δ as shown by Meera et al. 2009). Inclusion of THDOC in the patch pipette prevented the rundown of α4β3^{pHlourin}δ-mediated currents to a similar degree as observed for α4β3 receptors (Figure 9A,B). These results suggest that THDOC enhancement of the α4 subunit functional cell surface expression is independent and unaltered by the expression of δ subunit.

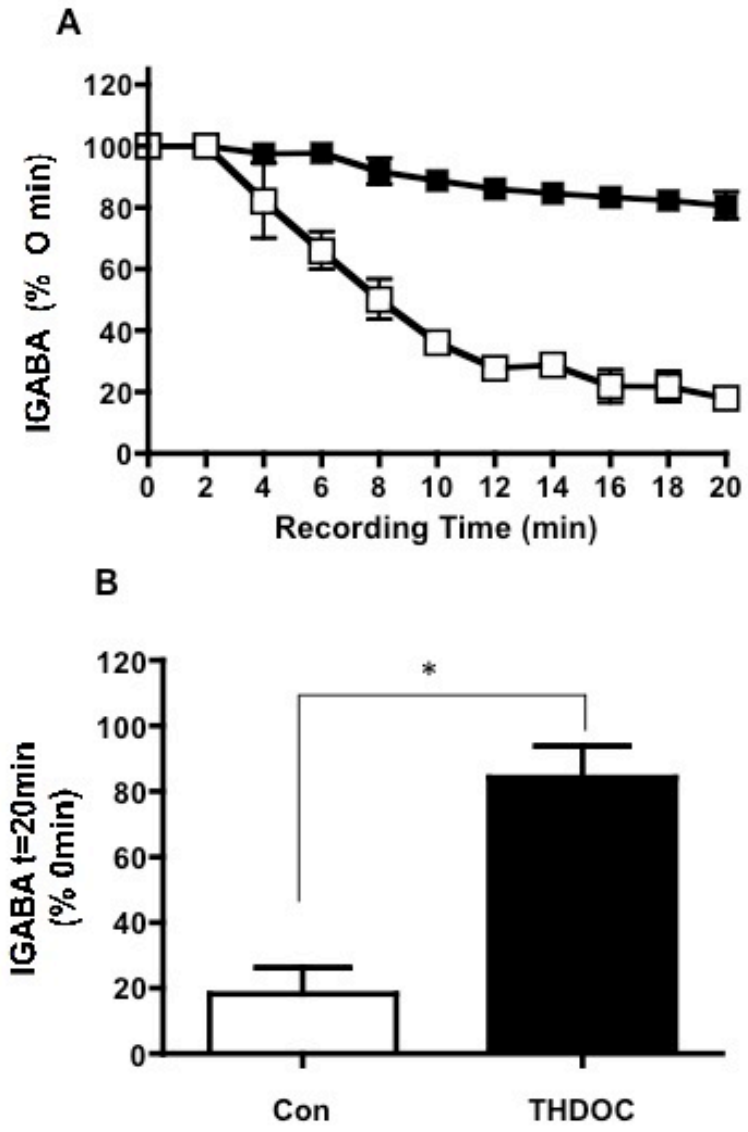


Figure 9: THDOC-mediated increase in $GABA_ARs$ cell surface insertion is independent of the delta subunit. *GABA-evoked currents from HEK293 cells transiently transfected with α4, β3 and ^{pHlourin}δ subunits, were recorded 3-5 min after achieving whole-cell configuration. α4β3-mediated currents were blocked by the addition of 1μM Zn^{2+} to bath solution.* ***(A)*** *Time-dependence relationship for 0.5 μM GABA-activated currents recorded in the presence of either internally applied vehicle*

*control (DMSO) (□) or 100nM THDOC (■). **(B)** Bar graph of the relative 10 μM GABA current at t=20 minutes compared to current at t=0 minutes for α4β3^{pHlourin}δ receptors in control conditions (□) or perfused internally with 100 nM THDOC (■).*=Significantly different from control DMSO (T-test p<0.05;n=4-6).*

DISCUSSION

Neuroactive steroids are potent endogenous allosteric modulators of $GABA_A$Rs (Belelli and Lambert et al. 2005, Gunn et al. 2011). Neurosteroids allosterically enhance $GABA_A$Rs function and through this enhancement exert anesthetic, anxiolytic, anticonvulsant, antidepressant, hypnotic and sedative actions (Belelli et and Lambert 2005, Gunn et al. 2011). Deficits in neurosteroid action are believed to contribute to addiction, autism, anxiety, depression, epilepsy, schizophrenia and traumatic brain injury (Belelli et al., 2009, Brickley and Mody, 2012).

Extrasynaptic $GABA_A$Rs are exquisitely sensitive to neurosteroids. Low physiological neurosteroid concentrations can greatly and selectively enhance the tonic conductance mediated by extrasynaptic $GABA_A$R subtypes (Reviewed by Belelli and Lambert 2005). Neurosteroids have been suggested to exert long-term effects on neuronal excitation by dynamically regulating the expression levels of extrasynaptic $GABA_A$Rs (Lambert et al. 2009, Harney et al. 2003). Furthermore, extrasynaptic

$GABA_AR$ subunits have been shown to undergo marked changes in expression in response to fluctuating steroid levels (Maguire and Mody 2007 2009,Gulinello et al. 2001, Hsu et al. 2003). However, the molecular mechanisms by which neurosteroids alter $GABA_ARs$ subunit expression were not known.

Here we reveal a novel mechanism by which the stress hormone derived neurosteroid THDOC, selectively enhances the cell membrane stability of $GABA_ARs$ that mediate tonic inhibition. This unique mechanism of action is dependent on the ability of the neurosteroid to enhance the phosphorylation of the α4 subunit on serine 443. Interestingly, this THDOC-mediated enhancement is independent of the neurosteroid binding site and of $GABA_AR$ δ subunit.

For synaptic $GABA_ARs$, phosphorylation of residues within the intracellular domain of subunits is critical for the regulation of synaptic $GABA_ARs$ trafficking and cell surface stability (Jacob et al. 2008, Arancibia-Cárcamo 2009). Phosphorylation has also been shown to alter the actions of various allosteric modulators on $GABA_ARs$ (Leidenhemer et al. 1993, Leidenheiheimer and Chapell 1997). Furthermore, several studies have suggested that PKC activity is required for neuroteroid modulation of $GABA_ARs$ (Brussaard and Koksma 2003, Harney et al. 2003, Choi et al. 2008)

We have previously identified serine 443, located within the intracellular loop of the α4 subunit, as a critical PKC phosphorylation site on a $GABA_AR$ subtype that mediates tonic inhibition (Chapter 2). We first examined whether THDOC could alter the phosphorylation state of the α4 subunit. To study this, we measured the amount of radiolabeled phosphate bound to the α4 subunit after treatment with the neurosteroid THDOC. THDOC treatment significantly increased the α4 subunit phosphorylation. Furthermore, biotinylation experiments revealed a dramatic increase in the cell surface expression of the α4 subunit after THDOC (Figure 1). We then examined the functional effects of the THDOC-mediated increase in α4 phosphorylation and cell surface expression. We have shown that $\alpha4\beta3$-mediated currents evoked by ~EC_{50} concentrations of GABA exhibit a decrease in the current amplitude over time or current rundown. Current rundown was prevented by the internal application a phorbol ester PKC activator (Chapter 2). Here we examined whole cell patch-clamp recordings of $\alpha4\beta3$-mediated currents with internal application of THDOC in the patch pipette. Internally applied THDOC prevented the rundown of $\alpha4\beta3$-mediated currents. This effect was abolished by inclusion of a PKC inhibitor peptide along with the neurosteroid suggesting that PKC activation is required for this THDOC enhancement.

$\alpha4$ subunit serine 443 is critical for neurosteroid enhancement of the $\alpha4$ subunit cell surface expression, since mutation of this serine to an alanine

abolishes THDOC enhancement of phosphorylation and cell surface expression (Figure 1). In addition, internal application of THDOC did not have any significant effects on the rundown of $\alpha4^{S443A}\beta3$-mediated currents (Figure 3).

Neurosteroids allosterically potentiate $GABA_AR$ receptor activity and this potentiation is dependant upon conserved amino acid residues within the second transmembrane domains of all α subunits (Hosie et al 2006, Hosie et al 2009). We utilized an α4 construct in which glutamine 246, a critical residue for neurosteroid binding, has been mutated to a leucine ($\alpha4^{Q246L}$). Internal THDOC application prevented the rundown GABA-evoked currents (Figure 6), suggesting that there are two independent mechanisms for neurosteroid action on extrasynaptic $GABA_ARs$; allosteric enhancement of the receptor for which glutamine 246 is critical, and enhancement of the cell surface stability of α4-containing receptors via an increase in PKC phosphorylation at serine 443.

Here we show that neurosteroid modulation of plasma membrane insertion is specific for the extrasynaptic $GABA_ARs$ receptor α4 subunit, since THDOC had no effect on α1 cell surface expression or rundown of $\alpha1\beta3$-mediated currents. Interestingly, while both synaptic and extrasynaptic $GABA_ARs$ can be subject to allosteric enhancement by

THDOC, THDOC-mediated enhancement of cell surface accumulation seems to be specific for extrasynaptic $GABA_A$Rs subtypes.

The α4 subunit can be expressed along with β2/3 and with or without a δ subunit. The δ increases the potency for GABA and appears to be critical for extrasynaptic $GABA_A$Rs sensitivity to low neurosteroid concentrations (Meera et al. 2009). We found that for α4β3δ receptors, internal application of THDOC resulted in abolishment of current rundown compared to control, to a similar degree to what we see in α4β3-mediated currents, suggesting that the THDOC mediated effects that we observe are independent of the δ subunit.

We used TIRF microscopy to more closely examine THDOC mediated changes in cell surface dynamics of α4-containing receptors of transiently transfected HEK293 cells. Fluorescently tagged subunits were used to measure the cell surface insertion rate of cells transfected via electropermeabilization with ^{RFP}α4 along with β3 and δ subunits. Treatment with 100nM THDOC dramatically increased the cell surface insertion rate of the α4 subunit. This effect was blocked when exposed to Botulinum Neurotoxin, a potent blocker of vesicle-mediated trafficking. THDOC application did not increase the cell surface insertion of α1-containing $GABA_A$Rs. Furthermore, hippocampal neurons chronically expressing the ^{RFP}α4 subunit also showed an increased cell surface insertion ratio when exposed to THDOC.

Collectively our results show a novel mechanism by which nerosteroids exert selective effects on the cell surface stability of $GABA_AR$s that mediate tonic inhibition. We demonstrate that the stress metabolite THDOC selectively increases the cell surface stability of α4-$GABA_AR$s by increasing the rate of insertion in the plasma membrane in a manner dependent on PKC phosphorylation of serine 443 and via vesicle-mediated trafficking.

The exact mechanism of interaction between THDOC and PKC is still unknown. THDOC could either: (i) directly activate PKC, (ii) mediate a change in conformation of the α4 subunit that facilitates PKC phosphorylation, (iii) or interact with some unknown molecule that then facilitates PKC activation. Preliminary studies suggest that THDOC does not directly interact with PKC (data not shown). Some neurosteroids have been shown to interact with PKC anchoring molecules, which mediate the stability of active PKC forms (Racchi et al. 2001). It is therefore likely that THDOC increases the phospho-dependent cell surface accumulation of the α4 subunit by interacting with an unknown PKC anchoring molecule that activates PKC.

The levels of steroid hormones can fluctuate in a wide range of physiological states including stress, puberty, pregnancy and the menstrual cycle. Steroid-induced fluctuations in $GABA_AR$ subunit expression result in alterations in neuronal excitability and they are

implicated in syndromes such as premenstrual syndrome, pubertal mood swings and epilepsy. Notably, decreased α4 subunit cell surface expression results in a marked increase in seizure susceptibility in animal models of catamenial epilepsy (Reddy et al. 2001, 2010), a neuroendocrine disorder that is characterized by seizure exacerbations linked to the reduction in neurosteroids levels during the perimenstrual period. Therefore, neurosteroids have been suggested to be critical regulators of seizure frequency via the modulation of extrasynaptic $GABA_AR$ subunit expression. The novel phospho-dependant mechanism for THDOC regulation of α4 subunit cell surface accumulation that we describe here might mediate these changes associated with seizure susceptibility.

The entorhinal cortex-subiculum-hippocampal circuitry forms a critical pathway in epileptogenesis. The high frequency firing entorhinal cortex neurons are connected via the perforant path to low-excitability dentate gyrus neurons that function as a filter for the high frequency stimulus from the entorhinal cortex and then send the filtered signal to the CA3 region via mossy fibers (Amaral and Witter 1989, Behr et al. 1998). Tonic inhibition mediated by α4-containing extrasynaptic $GABA_ARs$ is believed to be critical for maintaining the low intrinsic excitability of dentate gyrus granule cells. Alterations in the tonic inhibition mediated by α4-containing $GABA_ARs$ in the dentate gyrus, is associated with a reduction in the

threshold for seizures and with cognitive deficits (Shen and Smith 2009).

The observation that neurosteroid can have an impact on $GABA_AR$ stabilization via PKC phosphorylation, provides further insight into neurosteroid mediated changes in neuronal excitability and could lead to new pharmacological targets to modulate the surface expression of extrasynaptic $GABA_AR$ for treatment of postpartum depression, epilepsy and anxiety disorders.

CHAPTER 4

THE PHOSPHORYLATION STATE OF THE $GABA_AR$ β3 SUBUNIT REGULATES THE PHOSPHO-DEPENDANT THDOC MODULATION OF α4 SUBUNIT CELL SURFACE INSERTION

CONTRIBUTIONS THIS CHAPTER

I performed the majority of the experiments presented in this chapter. I conducted all the TIRF microscopy imaging, electrophysiological recordings and writing. ^{32}P experiments and biotinylation experiments (Figure 1), were performed by Matthew Abramian PhD, a postdoctoral fellow in the Moss Laboratory.

ABSTRACT

$GABA_A$Rs are the principal mediators of inhibitory transmission in the mammalian central nervous system. $GABA_A$Rs can be localized at postsynaptic inhibitory specializations or at extrasynaptic sites. Synaptic $GABA_A$Rs are activated in a transient or phasic manner following the release of GABA from presynaptic vesicles. Extrasynaptic $GABA_A$Rs are primarily composed of α4-6, β2/3 and δ $GABA_A$Rs subunits and they are activated in a persistent manner by low concentrations of GABA that escapes from the synapse into the extracellular space. The continuous or tonic inhibitory currents mediated by extrasynaptic receptors control neuronal excitability and the strength of synaptic transmission (Farrant and Nusser 2005). Extrasynaptic $GABA_A$Rs are uniquely sensitive to low physiological concentrations of neurosteroid that can potentiate the response to GABA and have significant effects tonic inhibition (Stell et al. 2003, Farrant and Nusser 2005). Furthermore, fluctuations in the levels of neurosteroids are associated with changes in the cell surface expression

of extrasynaptic $GABA_AR$ subunits. In the previous chapters, we have shown that the α4 subunit cell surface accumulation is modulated by the stress hormone metabolite THDOC in a PKC-dependant manner. The α4 subunit can be expressed at extrasynaptic sites along with β2/3 and δ subunits. Although extrasynaptic receptors can include β2 or β3 subunits, the majority of extrasynaptic $GABA_ARs$ in the hippocampus and cerebellum include the β3 subunit.

Previous experiments from our laboratory have identified serines 408/409 within the extracellular loop of β3 subunits as a critical site for PKC/PKA phosphorylation and subsequent modulation of cell surface stability. Here we show that the phospho-dependant neurosteroid modulation of α4 cell surface insertion is dependant on the phosphorylation state of the β3 subunit when co-expressed. The co-expression of the α4 $GABA_AR$ subunit along with a β3 phospho-mutant (serines 408/409 to alanines), abolishes the neurosteroid-mediated effects on the rundown of GABA activated currents and prevents the THDOC-mediated increase in cell surface insertion rate.

INTRODUCTION

Gamma-aminobutyric acid type A receptors ($GABA_ARs$) are chloride ion channels that belong to the cys-loop ligand gated ion channel superfamily. $GABA_ARs$ are assembled as hetero-oligomers from eight subunit classes

(Sieghart and Sperk 2002), with some of these subunit groups having multiple members. Subunits assemble to form a central pore that constitutes a chloride permeable channel (Sieghart et al. 1999). Variability in the composition and sub-cellular localization of $GABA_A$Rs allows for a functional heterogeneity of these receptors in the CNS (Lushner et al 2011).

$GABA_A$Rs localized at synaptic contacts are primarily composed of α1–3, β1–3 and γ2 subunits and they are activated in a transient manner by high concentrations of GABA released form pre-synaptic vesicles (Jacob et al. 2008). They therefore mediate a short-lived hyperpolarization that transiently reduces the probability for an action potential to occur. $GABA_A$Rs located at peri- or extrasynaptic sites are primarily composed of α4-6, β2/3 and δ subunits and they are activated in a persistent manner by low concentrations of GABA that either escapes from the synapse to the extracellular space or is released from non-neuronal cells (Farrant and Nusser, 2005, Zheleznova et al., 2009).

Extrasynaptic $GABA_A$Rs mediate an uninterrupted or tonic inhibition that increases membrane conductance and persistently reduces the probability for an action potential to occur (Farrant and Nusser, 2005; Hass and McDonald, 1999).

The $GABA_A$Rs subtypes that mediate tonic inhibition are important selective targets for a variety of endogenous and exogenous compounds

including alcohol and neurosteroids (Stell et al. 2003, Nie et al. 2011, Olsen 2011). However, the mechanisms by which neurons control the properties of $GABA_AR$ subtypes that mediate tonic inhibition remain largely unknown.

For synaptic $GABA_ARs$, modulating the $GABA_AR$ abundance at the cell surface is a critical mechanism for determining the strength of synaptic inhibition (Jacob et al. 2008). The membrane trafficking and channel kinetics of synaptic $GABA_ARs$ are subject to dynamic modulation via phosphorylation of key residues within the intracellular domains of individual receptor subunits (Jacob et al. 2008, Arancibia-Cárcamo and Kittler 2008). Conserved residues for phosphorylation by cAMP-dependent protein kinase A (PKA), Ca^{2+}/calmodulin-dependent protein kinase (CamKII) and protein kinase C (PKC) within the intracellular domains of the β subunit isoforms (In the case of the β3 subunit serines 408 and 409) are of critical importance for this modulation (Jacob et al. 2008, Terunuma et al. 2008). These residues are located within a basic patch-binding motif for the Clathin adaptor protein (AP2). Phosphorylation of Serines 408/409 enhances the cell surface stability of β3-containing synaptic $GABA_ARs$ by limiting their endocytosis, leading to long-lasting changes in both dendritic morphology and neuronal excitability (Jacob et al. 2009). Furthermore, sites within the intracellular loop of the β3 subunit are key for interactions

with molecules of the secretory pathway and scaffolding molecules (Lushner et al. 2011).

The β3 subunit is required for normal neuronal function, proper neurodevelopment and for the action of agents such as alcohol and anesthetics (Laurie et al. 1992, Jurd et al. 2002, Wallner et al. 2003, Zeller et al. 2007). Mutations and alterations in the expression of the β3 $GABA_AR$ subunit are highly correlated with many neurological disorders including epilepsy and autism spectrum disorders (Ma et al. 2005 Terunuma et al. 2008, Tanaka et al. 2008). For example, Status Epilepticus (SE), defined as prolonged rapidly repeating or unremitting seizures (Lowenstein and Alldredge 1998), results in a selective decrease in the PKC-dependant phosphorylation of serines 408 and 409 in the β3 $GABA_AR$ subunit and therefore mediating a subsequent reduction in β3 cell surface levels (Terunuma et al. 2008). Furthermore, the β3 subunit plays a crucial role in the formation and coordination of complex neuronal networks in the thalamus (Huntsman et al. 1999), the olfactory bulb (Nusser et al. 2001) and the Hippocampus (Hentschke et al. 2008) as revealed by studies in β3 null allele mutant and conditional knock-out mice (Homanics et al 1997, Ferguson et al. 2007).

The β3 subunit can also be assembled into $GABA_ARs$ subtypes located at extrasynaptic sites (Sperk et al. 1997, Caraiscos et al. 2004, Cheng et al.

2005). α4β3δ $GABA_A$Rs mediate tonic inhibition in hippocampal dentate granule cells and tonic inhibition mediated by this receptor subtype is likely critical for the learning and memory processes mediated by brain region (Sperk et al. 1997, Mody and Pierce 2004). However, the significance of these β3 phosphorylation sites for $GABA_A$Rs subtypes that mediate tonic inhibition has not been addressed.

We have begun to elucidate mechanisms by which extrasynaptic $GABA_A$Rs receptors are regulated. We have shown that the α4 subunit is phosphorylated on Ser 443 within the intracellular loop between transmembrane domains 3 and 4 (TM3 and TM4) in a PKC-dependent manner (Chapter 2). Furthermore, both activation of PKC and treatment with THDOC, dramatically increase α4 subunit cell surface expression, stability and function (Chapters 2 and 3).

In this chapter, we examined the role of β3 subunit serines 408 and 409 for the functional regulation of extrasynaptic $GABA_A$R subtypes. We utilized a β3 mutant contruct in which serines 408 and 409 were replaced for alanines (Jacob et al. 2009). We examined how these point mutations in the PKC phosphorylation sites within the β3 subunits alter the neurosteroid-mediated changes in the phosphorylation, cell surface expression and the magnitude of GABAergic currents mediated by extrasynaptic α4β3-containing $GABA_A$Rs.

Here we demonstrate that PKC phosphorylation of serines 408/409 is critical for the THDOC-mediated effects on the cell surface accumulation and function of α4-containing $GABA_ARs$. Expression of the α4 subunit along with the $\beta3^{S408/409A}$ mutant, prevents the PKC-dependant increase in α4 subunit phosphorylation, cell surface expression, and insertion rate.

In contrast with wild-type receptors, THDOC infusion did not prevent the rundown of GABA-evoked currents mediated by $\alpha4\beta3^{S408/409A}$ $GABA_ARs$.

For synaptic $GABA_ARs$, expression of the S408/409A mutation limited receptor endocytosis and increased the number and size of inhibitory synapses (Jacob et al. 2009). The results presented here represent a novel subtype-specific role for residues 408 and 409 in the modulation of extrasynaptic $GABA_AR$.

EXPERIMENTAL PROCEDURES

Antibodies and Expression Constructs.

Polyclonal rabbit anti-α4 was graciously provided to us by Dr. Verena Tretter and Dr. Werner Sieghart from Medical University Vienna. Peroxidase-conjugated IgG secondary antibody was from Jackson ImmunoResearch Laboratories. Fluorescently labeled α-bungarotoxin (α-Bgt) was purchased from Invitrogen.

α4, β3 and $\beta3^{S408/409A}$ cDNAs were cloned into the mammalian cytomegalovirus (CMV) promoter vector PRK5 for transgene expression. dsRed monomer fluorescent protein (RFP) was introduced after the 4th

amino acid of the mature α4 subunit followed by the BBS sequence (WRYYESSLEPYPD). Likewise a pH sensitive form of green fluorescent protein (pHluorin) was introduced between amino acids 4 and 5 of the β3 and $\beta3^{S408/409A}$ subunits. Mutations were verified by DNA sequencing. Plasmids were transfected into COS-7 or HEK293 cells using electroporation (Bio-rad) with 2 μg of plasmid DNA per construct. Nuclofection (AMAXA systems) was used to introduce the respective plasmids into freshly dissociated hippocampal neurons which were used at 10-14 DiV.

Cell culture.

cell lines: Human embryonic kidney (HEK-293) or COS-7 cells were co-transfected with selected $GABA_AR$ subunits via electropermeabilization (Gene Pulser MXcell Electroporation System, Bio-Rad). Cells were maintained in the appropriate media for culture according to manufacturer. For HEK 293 cells, DMEM/F12 media (GIBCO) supplemented with 5% Fetal bovine serum and 1% Pen-Strep is used. For some experiments, media is supplemented with 30mM HEPES buffer (Invitrogen). Imaging experiments are conducted 48-72 hours after transfection.

Whole-cell COS-7 ^{32}P Labeling

COS-7 cells were transfected and incubated as described above. Cells were initially incubated in 2 ml of phosphate-free DMEM for 30 min at 37

°C. Following this incubation, cells were labeled with 0.5 mCi/ml [^{32}P]orthophosphoric acid for 4 h in phosphate-free DMEM. Hippocampal slices were prepared as described above. Slices were individually transferred to polypropylene tubes containing 2 ml of fresh ACSF; gassed with a mixture of 95% O_2, 5% CO_2; and maintained in a 30 °C water bath. Labeling was performed by adding 0.5 mCi/ml [^{32}P]orthophosphoric acid for 1 h. For both COS-7 cells, samples were treated with drugs where indicated after the labeling period, followed by the cell lysis and immunoprecipitation procedure described above. Results were attained by SDS-PAGE followed by autoradiography.

Biotinylation

Cultures were washed once with ice-cold PBS and then incubated in 2 ml of ice-cold PBS containing 1 mg/ml NHS-SS-biotin (Pierce) for 20 min in order to label surface proteins with biotin. After labeling, the biotin was quenched by incubating cells in PBS containing 25 mM glycine and 10mg/ml bovine serum albumin (BSA). Cells were then lysed in lysis buffer and sonicated. For hippocampal slice experiments, slices were incubated in ACSF described above at 30 °C for 1 h for recovery before experimentation. Slices were then placed on ice and incubated for 30 min with 1 mg/ml NHS-SS-biotin. Excess biotin was removed by washing slices three times in ice-cold ACSF and lysed. For both COS-7 cells and hippocampal slices, insoluble material was removed by centrifugation. The

supernatant lysates were incubated with NeutrAvidin beads (Pierce) for 18–24 h at 4 °C. Bound material was eluted with sample buffer and subjected to SDS-PAGE and then immunoblotted with indicated antibodies. Blots were then quantified using the CCD-based FujiFilm LAS 300 system.

Cell Lysis and Immunoprecipitation

Samples collected from either COS-7 cell cultures or hippocampal slices were lysed in lysis buffer containing the following: 20 mM Tris-HCl (pH 8.0), 150 mMNaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM sodium pyrophosphate, 1% Triton X-100, and 0.1% SDS. In addition, the following protease inhibitors were added: 250 µg/ml 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride; 10 µg/ml leupeptin, 1 µg/ml pepstatin, and 10 µg/ml antipain. Samples were then sonicated and spun at 16,000 × *g*. The supernatant was collected and then subjected to a protein assay using a standard Bradford protocol. 100–200 µg of protein were loaded per immunoprecipitation sample along with 3 µg of indicated antibody and 40 µl of protein A-Sepharose beads (1:1 slurry) (GE Healthcare). Samples were allowed to conjugate for 18–24 h at 4 °C with constant agitation. The beads were precipitated by centrifugation at 500 × *g* and washed once with ice-cold Buffer A (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 10 mM sodium pyrophosphate, and 1% Triton X-100 and protease inhibitors), two times

with Buffer B composed of Buffer A supplemented with 500 mM NaCl, and once again with Buffer.

Patch-Clamp Electrophysiology

Human embryonic kidney cells (HEK 293) or DIV 14-18 hippocampal neurons expressing fluorescent-tagged $GABA_AR$ subunits were superfused, at a rate of 2 ml/min, with an extracellular solution containing (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, 11 glucose and adjusted to pH 7.4 with NaOH. Borosilicate glass patch pipettes (resistance 2-5 MΩ) will contain (in mM) 140 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, 2 ATP (Mg^{2+} salt) and adjusted to pH 7.4 with KOH. GABA was applied once every 120 s via a fast-step perfusion system (Warner Inst., Hamden, CT). All experiments were carried out at 32-33 °C using recording chamber and in-line perfusion heaters (Warner Inst.). Phorbol esters, THDOC (Tocris Bioscience, Minneapolis MN) and PKC Inhibitor peptide 19-36 (Promega, Madison WI) were applied to the cell either internally via the electrode solution or superfused into the recording chamber. Currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz and digitized at 20 kHz with a Digidata 1320A (Molecular Devices) and analyzed using either Clampfit (pClamp, Molecular Devices).

Total Internal Reflection (TIRF) Microscopy

HEK-293 or DIV 14-18 hippocampal neurons were transfected/nuclefected with fluorescent-tagged $GABA_AR$ subunits and plated in Poly-L-lysine (1mg/ml-Sigma Aldrich, St. Louis MO) glass-bottom plates (FluoroDish sterile culture dishes, World Precision Intruments, Sarasota. FL). Live TIRF imaging microscopy was conducted with a Nikon Eclipse Ti Inverted TIRF Microscope (Nikon Intruments, Melville NY) equipped with Perfect focus mechanism to avoid focus drift and a stage top incubator with CO2 and temperature control. All experiments were conducted at 32°C. Samples were viewed through a plan Apo TIRF 60X high-resolution (1.45NA) oil immersion objective. Fluorescent images were collected with an iXion EMCCD camera (Andor Technology, South Windsor CT) interfaced to a PC running Nikon NIS-Elements software. Images were collected with a 200ms exposure time before and immediately after addition of THDOC. Total insertion events per minute were marked and taken as insertion frequency (Lin et al. 2009). GraphPad Prism Software was used for data analysis.

Data Acquisition and Analysis

For biochemical and immunofluorescent experiments, data are presented as means ± S.E. Statistical analysis was performed by using Student's *t* test where a*p* value of <0.5 is considered significant. For electrophysiological experiments, currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2

kHz and digitized at 20 kHz with a Digidata 1320A data acquisition system (Molecular Devices), and analyzed using either Clampfit (pClamp, Molecular Devices) or GraphPad Prism version 4 software (GraphPad Software, Inc., San Diego, CA). Statistical analysis was performed by using one-way ANOVA with a Bonferroni post-test with statistical significance set at $p < 0.05$. All data are expressed as mean ± S.E.

RESULTS

The phosphorylation state and the cell surface expression of the α4 subunit are not positively modulated by PKC activation or THDOC treatment when expressed with the β3 S408/409A subunit in COS7 cells.

The β3 $GABA_AR$ subunit can be expressed at both synaptic and extrasynaptic sites. We have shown that when expressed with the α4 subunit, the β3 subunit is phosphorylated by PKC at serines 408/409 (Chapter 2). The δ subunit does not appear to be phosphorylated, therefore serine 443 within the α4 subunit and serines 408/409 within the β3 subunit are the primary phosphorylation sites within this $GABA_ARs$ subtype that mediates tonic inhibition (Chapter 2). Previous work from our laboratory developed a β3 subunit mutant where serines 408 and 409 were mutated into alanines (Jacob et al. 2009). We used this construct to analyze the role of the β3 408/409 mutation for receptor primarily localized at extrasynaptic sites. Immunoprecipitation was utilized to examine the

phosphorylation of $GABA_A$Rs in COS7 cells transiently transected with the α4 and either wild-type or mutant ($\beta3^{S408/409A}$) $GABA_A$R β3 subunits. Transiently transfected COS7 cells were prelabeled with [^{32}P]-orthophosphoric acid and treated with either vehicle (DMSO), 100nM of the PKC activator PDBu or 100nM of the neurosteroid tetrahydrodeoxycorticosterone (THDOC). Cells were then subjected to immunoprecipitation with an anti-α4 antibody. As presented in previous chapters, when expressed with wild-type β3, both PDBu and THDOC increased the phosphorylation of the α4 subunit. In contrast, when the α4 subunit was co-expressed with the $\beta3^{S408/409A}$ mutant, neither PDBu nor THDOC increased the phosphorylation of the α4 at serine 443 (Figure 1A). Next we explored whether the THDOC-mediated changes in α4 cell surface expression were dependant on phosphorylation of the β3 subunit (Figure 1B).

COS7 cells transiently transfected with α4 and either wild-type (β3) or S408/409A mutant ($\beta3^{S408/409A}$) $GABA_A$R subunits, were treated with either vehicle, PDBu or THDOC and then subjected to biotinylation. Exposure to both the PKC activator PDBu and THDOC increased the cell surface expression of the α4 subunit, whereas expression with the $\beta3^{S408/409A}$ phospho-mutant prevented the PKC-dependant THDOC mediated increase in α4 subunit cell surface expression ($p<0.05$). Collectively, we show that α4 co-expression with the $\beta3^{S408/409A}$ phospho-mutant abolishes

PKC and THDOC modulation of phosphorylation state and cell surface expression of the α4 subunit.

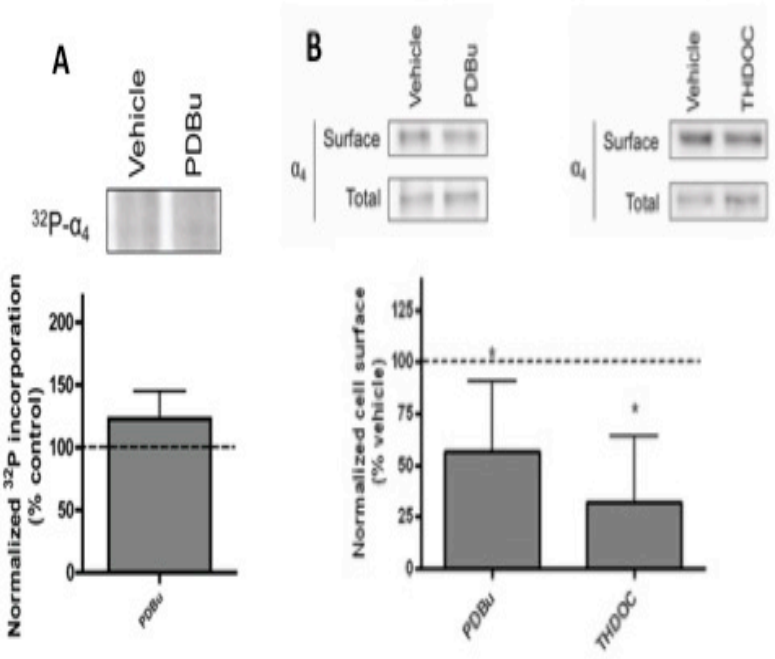


Figure 1: The phosphorylation and cell surface expression of the α4 subunit are not positively modulated by PKC activation or by THDOC treatment, when expressed with the β3$^{S408/409A}$ mutant.

COS7 cells were co-transfected via electroporation with the α4 and either wild-type β3 or the S408/409A mutant (β3$^{S408/409A}$) GABA$_A$R subunits. ***(A)*** *COS7 cells expressing α4β3 or α4β3$^{S408/409A}$ GABA$_A$Rs were labeled with 1 mCi/ml ^{32}P-orthosphosphoric acid following treatment with either with either vehicle (DMSO) or 100nM PDBu. The α4 subunit was then isolated using immunoprecipitation. Histograms are presented as ^{32}P incorporation*

expressed as a percentage of vehicle treated control (dashed line). ***(B)*** *transfected COS7 cells were exposed to either vehicle, 100nM PDBu or 100nM THDOC and then subjected to biotinylation. Histograms show the proportion of cell surface α4 protein expressed as a percentage of vehicle-treated controls (dashed line represents vehicle set at 100%; $p < 0.05$).*

The β3 S408/409A mutant prevents the PKC-dependant THDOC modulated insertion of the α4 subunit in HEK-393 cells.

In the previous chapters, we examined the changes in α4 cell surface insertion mediated by phorbol ester PKC activation and the neurosteroid THDOC. Here we examined the importance of the phosphorylation of residues 408/409 for the PKC-dependant THDOC-mediated increase in the cell surface accumulation of the α4 subunit. We used Total Internal Reflection (TIRF) microscopy in order to visualize live insertion events in HEK293 cells transiently expressing the α4 subunit, along with wild-type or mutant β3 $GABA_AR$ subunits. HEK293 cells were transiently transfected via electroporation with an α4 construct fluorescently tagged with red fluorescent protein (^{RFP}α4) and either wild-type or mutant β3 constructs that were tagged with a pH sensitive green fluorescent protein (pHluorin - ^{pHlourin}β3 and ^{pHlourin}β3$^{S408/409A}$). The ^{RFP}α4 as well as both wild-type and mutant pHluorin -^{pHlourin}β3 showed strong cell surface expression and were largely co-localized (reflected by yellow spots) (Figure 2A).

We compared the rate of insertion of $GABA_ARs$ containing $^{RFP}\alpha4^{pHlourin}\beta3$ and $^{RFP}\alpha4^{pHlourin}\beta3^{S408/409A}$ after 20 min incubation with either vehicle control (DMSO) 100nM PDBu or 100 nM THDOC. Exposure to both the phorbol ester PKC activator PDBu (Figure 2B-C,F) and the neurosteroid THDOC (Figure 2D-E,F) increased the frequency of insertion of the $^{RFP}\alpha4$ subunit when expressed with wild-type $^{pHlourin}\beta3$ (Figure 2F).

In contrast, when the $^{RFP}\alpha4$ was expressed with the $^{pHlourin}\beta3^{S408/409A}$ phospho-mutant, the $^{RFP}\alpha4$ insertion rate was not positively modulated by neither PKC (Figure 2G-H, K) activation nor THDOC treatment (Figure 2). Furthermore, we observed a small but significant decrease in the insertion of the $^{RFP}\alpha4$ when expressed with the $^{pHlourin}\beta3^{S408/409A}$ subunit under control conditions (Figure 2B,E). Our results suggest that the phospho-dependant, THDOC-mediated increase in the cell surface accumulation of the α4 subunit is dependent on the phosphorylation of the β3 subunit at serines 408 and 409.

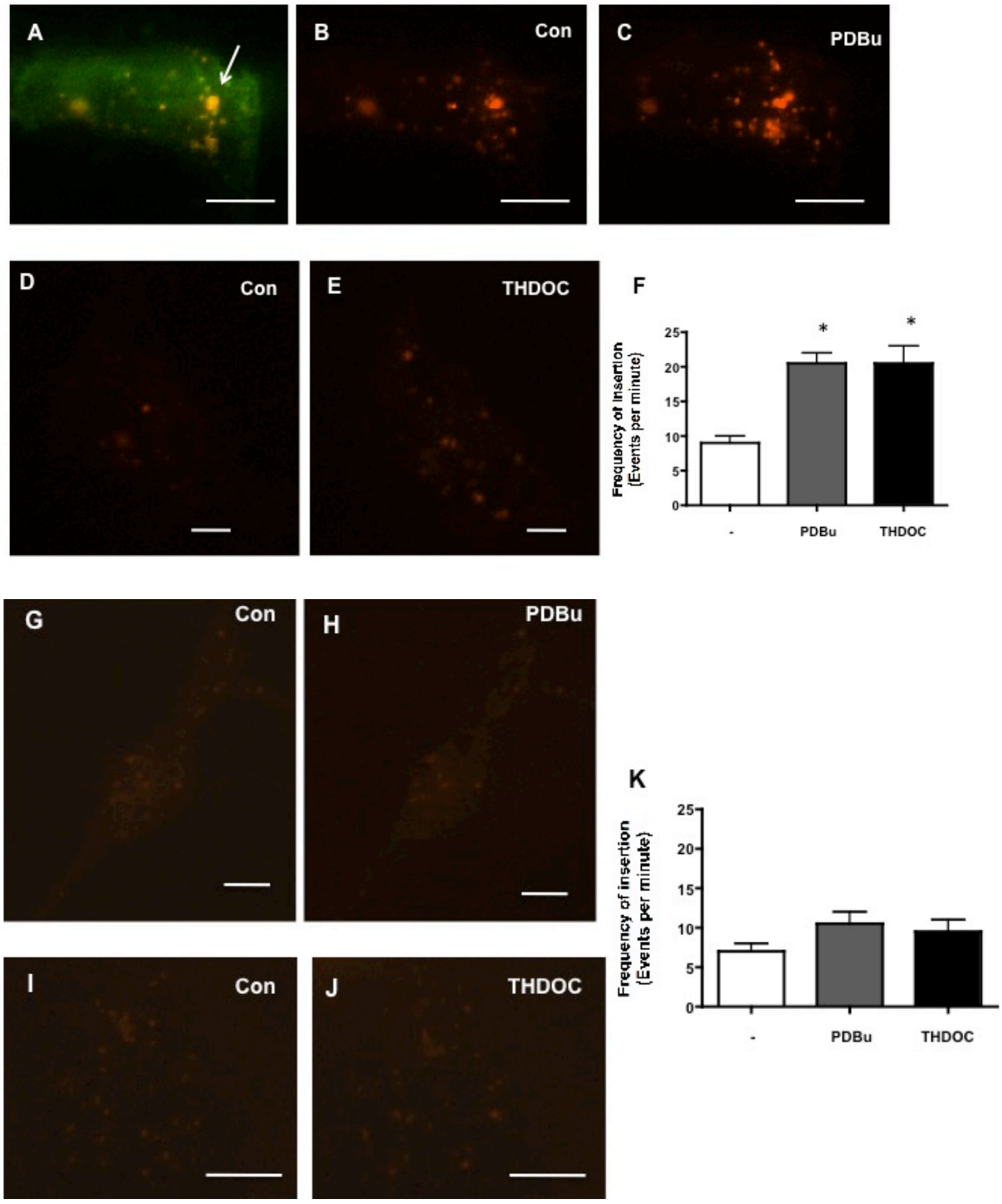


Figure 2: The β3 S408/409A mutant prevents the PKC-dependant THDOC modulated insertion of the α4 subunit in HEK-393 cells.

*HEK293 cells were transfected via electroporation with a α4 construct tagged with red fluorescent protein (^{RFP}α4) and either wild-type **(A-F)** or mutant β3 subunits **(G-K)** tagged with a pH sensitive form of green fluorescent protein (^{pHlourin}β3 and ^{pHlourin}β3$^{S408/409A}$ respectively) $GABA_AR$ subunits. 24-72 hours after transfection, cells were imaged before **(B,D,G,I)** and after exposure with **(C,H)** 100nM PDBu or **(B,E)** 100nM THDOC. Total insertion events per minute of live imaging were marked and taken as frequency of insertion **(F,K)**. *=Significantly different from control. Scale bar = 10 μm*

Mutation of serines 408 and 409 in the β3 subunit abolishes the THDOC-mediated effects on the rundown of GABA-evoked currents

Next we explored the functional significance of biochemical studies analyzing the phoshorylation and cell surface accumulation of the α4 subunit when expressed with the $^{pHlourin}\beta3^{S408/409A}$ mutant. We analyzed the rundown of GABA-evoked currents. HEK293 cells were co-transfected via electroporation with the α4 and either $^{pHlourin}\beta3$ or the $^{pHlourin}\beta3^{S408/409A}$ and whole cell patch-clamp experiments were performed 24-72 hours after transfection. 1μM (~EC_{50}) GABA was applied every two minutes via a fast step perfusion system. As seen in our previous experiments (Chapter 3), the infusion of both 100nM PDBu and 100nM THDOC in the patch-pipette prevented the rundown of α4β3- GABA-activated currents (Figure 4A,B). In contrast, neither infusion of the PKC activator PDBu, nor the internal application of THDOC could prevent the rundown of $\alpha4\beta3^{S408/409A}$-$GABA_A$Rs (Figure 4C,D).

We have shown that expression of the serine 443 to alanine α4 phospho-mutant ($\alpha4^{S443A}$) prevents the rundown of α4β3-mediated GABA-evoked currents (Chapter 2). Here we examined the rundown of $\alpha4^{S443A}$-mediated currents when co-expressed with the $\beta3^{S408/409A}$ mutant. Co-expression of $\alpha4^{S443ApHlourin}\beta3^{S408/409A}$ prevented the rundown of GABA-evoked currents. Furthermore, inclusion of the neurosteroid THDOC did not have any effect

on the rundown of GABA-activated currents (Data not shown). These results suggest that β3 serines 408 and 409 are critical for the PKC-dependent neurosteroid functional enhancement of α4 subunit cell surface levels and that this β3 modulation occurs through a mechanisms dependent on α4 serine 443, since co-expression of the α4 phospho-mimic with the $\beta3^{S408/409A}$ mutant has no effect on GABA-activated current rundown.

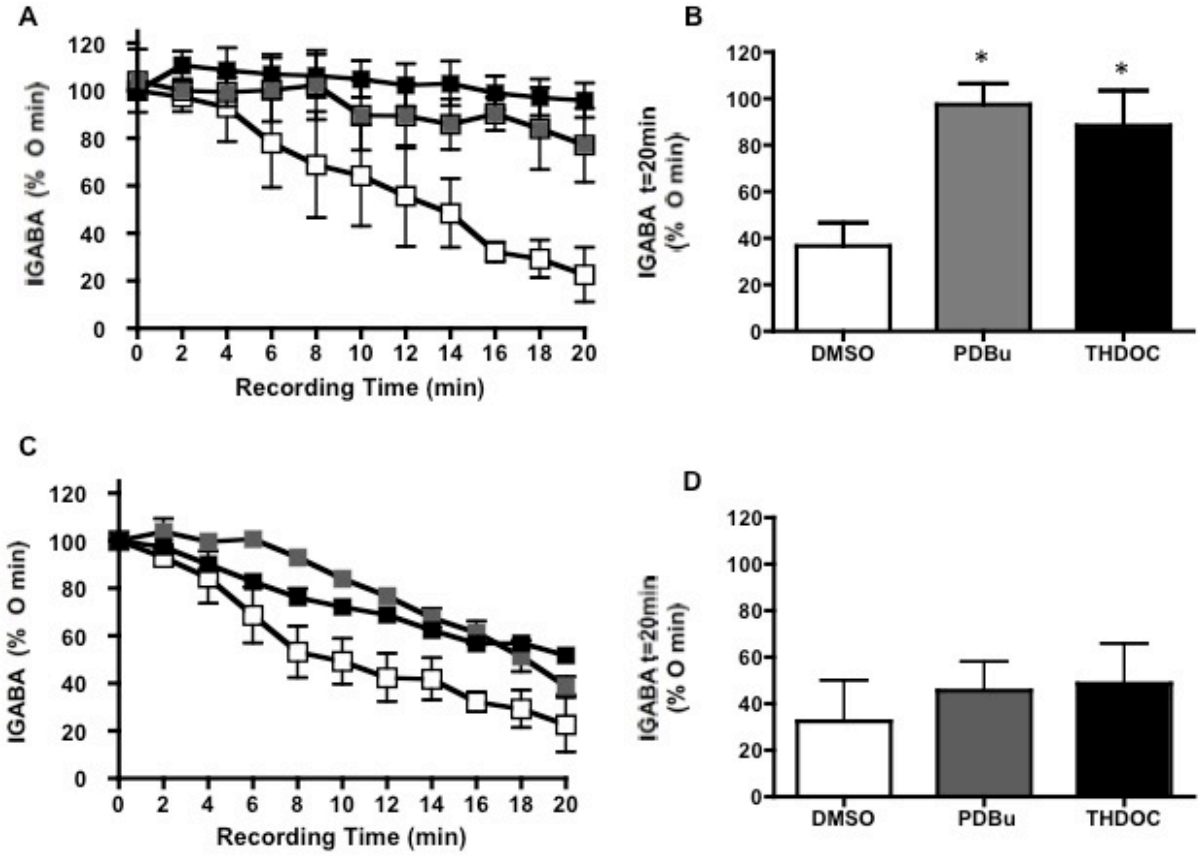


Figure 3: The $\beta3^{S408/409A}$ mutation abolishes the PKC-dependant THDOC enhancement of GABA-evoked α4β3-mediated current rundown.

HEK293 cells were transfected via electroporation with a α4 construct along with either wild-type (β3) or mutant ($\beta3^{S408/409A}$) β3 subunits $GABA_AR$ subunits. 24-72 hours after transfection, whole-cell currents were recorded from HEK293 cells expressing α4β3 or $\alpha4\beta3^{S408/409A}$ receptors. For all experiments, 1 μM GABA was added in the bath solution via a fast-step perfusion. GABA-activated currents were recorded 3-5 min after achieving whole-cell configuration. *Currents recorded in the absence (□) and presence of internally applied 100 nM PDBu* (■) *or 100nM THDOC* (■). *Time-dependence relationship for 1 μM GABA-activated currents recorded from α4β3* ***(A)*** *or* $\alpha4\beta3^{S408/409A}$ ***(C)*** *receptors with either vehicle(DMSO), with internally perfused 100 nM PDBu or internal application 100nM THDOC. Bar graph of the relative 1 μM GABA current at t=16 minutes compared to current at t=0 minutes for α4β3* ***(B)*** *or* $\alpha4\beta3^{S408/409A}$ ***(D)*** *in control conditions (□), perfused internally with 100 nM PDBu (■) or THDOC (■). *=Significantly different from control DMSO (T-test p<0.05;n=4-6)*

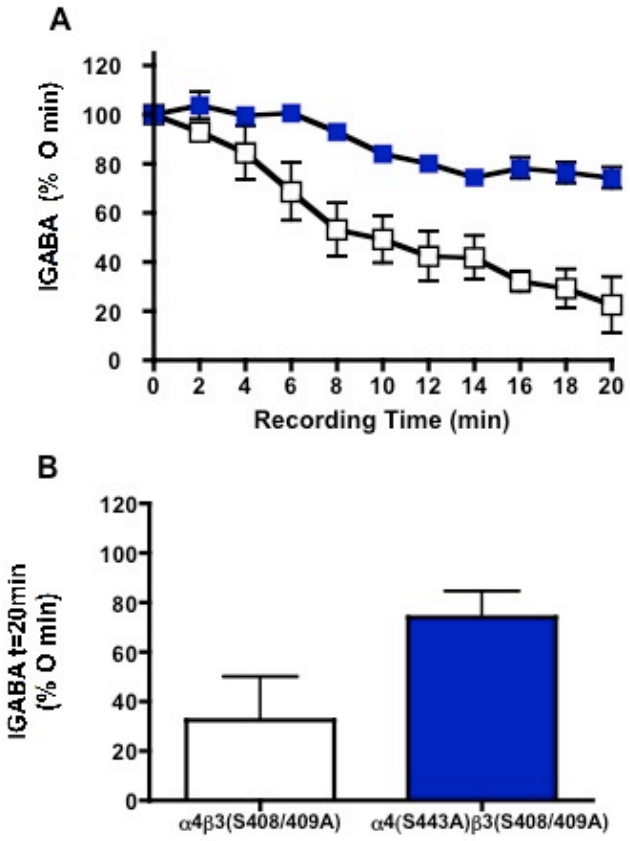


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Figure 4. The S443A α4 mutant rescues the effect of the β3$^{S408/409A}$ mutation on the rundown of α4**β3-mediated currents.**

HEK293 cells were transfected via electroporation with a β3$^{S408/409A}$ construct along with either wild-type (α4) or mutant (α4^{S443A}) subunits GABA$_A$R subunits. 24-72 hours after transfection, whole-cell currents were recorded from HEK293 cells expressing α4^{S443A}β3 or α4^{S443A}β3$^{S408/409A}$ receptors. For all experiments, 1 μM GABA was added in the bath solution via a fast-step perfusion. GABA-activated currents were recorded 3-5 min after achieving whole-cell configuration ***(A)*** *Time-dependence relationship for 1 μM GABA-activated currents recorded from α4β3$^{S408/409A}$ or*

$\alpha4^{S443A}\beta3^{S408/409A}$ receptors ***(B)*** *Bar graph of the relative 1 μM GABA current at t=20 minutes compared to current at t=0 minutes for $\alpha4\beta3^{S408/409A}$ (□) or $\alpha4^{S443A}\beta3^{S408/409A}$ (■) receptors.*

DISCUSSION

The tonic inhibition mediated by extrasynaptic $GABA_A$Rs is critical for the control of intrinsic neuronal excitability (Mody 2005). Furthermore, many studies have now implicated disturbances in tonic inhibition in a number of neurological and psychiatric disorders (Brickley and Mody 2012). Further understanding of the cellular mechanisms that modulate the trafficking of $GABA_A$R subtypes that mediate tonic inhibition will be of critical importance for the development of novel therapeutics for these disorders.

The $GABA_A$R β3 subunit is of particular interest because it has been implicated in the pathogenesis of several neurodevelopmental disorders including Rett syndrome, Angelman's syndrome, some forms of epilepsy and ASD (Samaco et al. 2005, Hogart et al. 2007). A rare mutation in the β3 subunit has been associated with a 6-fold increase in the risk for ASD (Ma et al. 2005, Delorey et al. 2008). In addition, the β3 subunit is critical for mediating the actions of exogenous and endogenous agents such as intravenous anesthetics and alcohol (Jurd et al. 2002, Wallner et al. 2003, Zeller et al. 2007).

Although the β3 subunit is widely distributed throughout the brain and spinal cord at developmental stages, in the adult brain the β3 subunit expression is primarily restricted to the olfactory bulb striatum, cerebral cortex and hippocampus (Laurie et al. 1992).

The β3 can be assembled in both synaptic and extrasynaptic $GABA_AR$ subtypes depending on regional subunit expression. In the dentate gyrus of hippocampus and in thalamic relay neurons (Brunig et al. 2002, Jia et al. 2005, Jia et al. 2007, Chandra et al. 2006), the β3 subunit can be expressed along the α4 and δ subunits at extrasynaptic sites.

Residues within the β3 subunit mediate many regulatory processes including the trafficking, assembly, targeting and cell surface stability of β3-containing $GABA_ARs$ (Jacob et al. 2008, Arancibia-Cárcamo and Kittler 2008). Previous studies have demonstrated the importance of residues 408 and 409 in regulation of the cell membrane stability of β3-containing $GABA_ARs$ (Jacob et al. 2009). We have shown that when co-expressed, serine 443 in the α4 subunit and serines 408 and 409 in the β3 are the primary PKC phosphorylation sites in this extrasynaptic $GABA_AR$ subtype (chapter 2). Yet, the role of these β3-mediated regulatory processes for the $GABA_AR$ subunits that mediate tonic inhibition had not been addressed.

Here we examined the significance of β3 subunit serines 408 and 409 for the functional regulation of extrasynaptic $GABA_AR$ subtypes. We utilized a β3 mutant contruct in which serines 408 and 409 were replaced for alanines (Jacob et al. 2009). We describe how the PKC phosphorylation of residues 408/409 within the β3 subunit can modulate the phosphorylation and cell surface accumulation of the α4 subunit as well as the magnitude of α4β3-mediated currents. Furthermore, we show that the THDOC-mediated increase in cell surface insertion and function of α4-containing $GABA_ARs$, is dependant on the phosphorylation state of the β3 subunit when they are co-expressed.

Our published work (Chapter 2) identified serine 443 within the intracellular loop of the α4 subunit, as a critical site for PKC phosphorylation. Phorbol ester activation of PKC increased the phosphorylation and cell surface insertion of the α4 subunit. PKC activation also prevented the rundown of α4β3-mediated GABAergic currents. Furthermore, the stress hormone metabolite THDOC increased the phosphorylation, cell surface accumulation of the α4 subunit and prevented α4β3-mediated current rundown in a PKC-dependant manner (Chapter 3).

We first studied how the $\beta3^{S408/409A}$ mutation altered the phosphorylation state of the α4 subunit. We measured the amount of radiolabeled

phosphate bound to the α4 subunit after treatment with either the PKC activator PDBu or the neurosteroid THDOC. Co-expression with the $\beta3^{S408/409A}$ phospho-mutant prevented the PKC-dependant increase in α4 subunit phosphorylation. Furthermore, the $\beta3^{S408/409A}$ mutant abolished the THDOC-mediated increase in the phosphorylation of the α4 subunit. We next examined the cell surface expression of the α4 subunit when co-expressed with the $\beta3^{S408/409A}$ mutant. Our biotinylation experiments revealed that the S408/409A mutation abolished the dramatic increase in the cell surface protein levels seen following PKC activation and THDOC treatment when expressed with wild-type β3.

We then used TIRF microscopy to assess the effect of the $\beta3^{S408/409A}$ in the cell surface accumulation of live transiently transfected HEK293 cells. Fluorescently tagged subunits were used to measure the cell surface insertion rate of cells transfected with $^{RFP}\alpha4$ and wild-type or mutant ($^{pHlourin}\beta3$ or $^{pHlourin}\beta3^{S408/409A}$) $GABA_AR$ subunits. PKC activation increased the α4 subunit insertion rate when expressed with wild-type β3, co-expression with the phospho-mutant abolished this enhancement. As we showed in the previous chapter, treatment with the neurosteroid THDOC increased the cell surface insertion of the α4 when co-expressed with wild-type β3, whereas expression with the $\beta3^{S408/409A}$ mutant blocked this enhancement.

We then explored the functional significance of our biochemical studies. The functional effects of the serine 408/409 to alanine mutation where determined by whole cell patch-clamp recordings of transfected HEK293 cells. Under control conditions, the amplitude of GABA-evoked currents decreases over time (Chapter 2). Enhanced $GABA_AR$ cell surface expression can be reflected at a functional level as an abolishment of the decrease in current amplitude over time or rundown. We studied the rundown of GABA-evoked currents mediated in α4β3 and $\alpha4\beta3^{S408/409A}$ $GABA_ARs$. As previously demonstrated, internal application of both the PKC activator PDBu and the neurosteroid THDOC prevented the rundown of GABA-evoked α4β3-mediated currents. However, for $\alpha4\beta3^{S408/409A}$ receptors, internal application of neither PDBu nor THDOC could prevent the rundown of GABA-evoked current amplitude.

Collectively, our results suggest that phosphorylation of serines β3 408 and 409 is required for the PKC-dependant neurosteroid increase in α4 subunit phosphorylation, cell surface accumulation and enhancement of α4β3-mediated currents.

In previous studies, mutation of β3 serines 408 and 409 to alanines resulted in increased levels of β3-containing synaptic clusters in the cell membrane (Jacob et al. 2009). Our results suggest different roles for β3

serines 408 and 409 when this subunit is assembled with extrasynaptic $GABA_AR$ subtypes.

The exact mechanism by which the phosphorylation of β3 serines 408 and 409 modulates α4 subunit phosphorylation and cell surface accumulation is still unknown. Post-translational modifications of residues within the β3 subunit are associated with proper intracellular trafficking and targeting to the neuronal membrane (Arancibia-Cárcamo and Kittler 2008, Jacob et al. 2008). It is possible that β3 phosphorylation at serines 408 and 409 triggers a conformational change that allows for α4 subunit phosphorylation, and this is therefore critical for α4 insertion in the neuronal membrane. Furthermore, β3 subunit residues 408 and 409 play an important role in the recruitment of PKC (Brandon et al. 2002). It is possible that these residues are critical for the neurosteroid-mediated recruitment of PKC for α4 subunit phosphorylation.

The α4 subunit can also be found expressed along with the β2 subunit at extrasynaptic sites in specific subsets of neurons in the thalamus and the hippocampus (Nusser and Mody 2002, Belleli et al. 2005, Jia et al. 2007). The β2 subunit can be phosphorylated at serine 410 by kinases PKC, PKA AKT and CAMKII (McDonald et al. 1998, Brandon et al. 2002, Wang et al. 2003, Reviewed by Kittler and Moss 2003). It is worthwhile exploring whether phosphorylation of the β2 subunit has the same modulatory effect

on the phosphorylation and cell surface accumulation of the α4 subunit, or whether this mechanism is exclusive to the β3 subunit. Interestingly, studies in neurons of the thalamic dorsal lateral geniculate nucleus (dLGN) show reduced enhancement of tonic inhibition after THDOC treatment when compared with dentate gyrus granule cells. Tonic inhibition in the dLGN is mediated by α4, β2 and δ subunits, whereas in dentate gyrus neurons the β3 subunit is more predominant. We speculate that these differences in β subunit expression account for the differences in the neurosteroid enhancement of tonic inhibition.

Direct phosphorylation of $GABA_AR$ subunits has emerged as a critical mechanism for the modulation of $GABA_AR$ channel kinetics and trafficking (Levitan 1999, Kittler and Moss 2006). Dynamic modulation of extrasynaptic $GABA_AR$ activity is essential for changes in neuronal function mediated by tonic inhibition.

Interestingly, in some of the neurodevelopmental disorders associated with mutations in the β3 subunit, reductions in the levels α4 cells surface expression have been also been reported (D'Hulst et al. 2006, Fatemi et al. 2010). It is possible that, in these conditions, a reduction or alteration in β3 subunit expression, might lead to a concomitant decrease in α4 subunit expression via a decrease in the PKC phosphorylation of this subunit.

Tonic conductance has been shown to be critical during pre- and early post-natal development (Ge et al. 2006, Holter et al. 2010). Tonic currents activated by ambient GABA (which is depolarizing at early developmental stages), appears to be essential for the synaptic integration of newly born dentate gyrus granule cells and for proper dendritic development (Ge et al. 2006). Tonic inhibition in the dentate gyrus is also believed to be critical for dentate granule cell maturation and may be important for the control of network excitability during the formation of the hippocampus-entorhinal cortex neuronal network (Holter et al. 2010). Disruptions in the synaptic integration and dendritic arborization of newborn neurons as well as disruptions in the network filter-like function of the dentate gyrus can lead to epileptiform activity and is also associated with some of the core symptoms of neurodevelopmental disorders such as Autism spectrum disorders and Angelman's syndrome (Heinemann et al. 1992, Tanaka et al. 2011).

Our observation that, mutation of serines 408/409 in the β3 subunit can abolish the neurosteroid mediated enhancement of α4 cell surface stability is a critical aspect to be considered when developing novel drugs for the treatment of neurodevelopmental disorders associated with disruptions or mutations of the β3 $GABA_AR$ subunit.

CHAPTER 5

DISCUSSION

SUMMARY OF RESULTS

Tonic inhibition mediated by α4-containing extrasynaptic $GABA_A$Rs is critical for the regulation of thalamo-cortical neuron firing (Cope et al. 2009) and for maintenance of the low-cell excitability of dentate gyrus granule cells (Mody 2005), and is likely to play a crucial role in the many of the brain activities mediated by the thalamus and the hippocampus. Some of these include learning and memory processes, the generation of sleep rhythms and the proper control of hippocampal network excitability (Cope et al. 2009, Mann and Mody 2010). Plasticity in α4 subunit expression has been associated with many physiological and pathophysiological conditions including alcohol exposure/withdrawal (Cagetti et al. 2003, Renawal et al. 2012), epilepsy (Brooks-Kayal et al. 1998, Benerjee et al .1998) and steroid withdrawal (Smith et al. 1998). Therefore, it is imperative to elucidate the mechanisms by which neurons control $GABA_A$R α4 subunit functional expression.

First, we show that the α4 subunit is phosphorylated by PKC at serine 443. This residue is located within a PKC consensus sequence in the intracellular loop domain of the subunit. We characterized the importance of this post-translational modification on the biochemical and functional properties of α4-containing $GABA_A$Rs. A number of studies have shown how post-translational modifications on predominantly synaptic $GABA_A$Rs subunits, can alter receptor function by either regulating the trafficking to

and from the plasma membrane or by modulating receptor properties (Jacob et al. 2008, Arancibia-Cárcamo and Kittler 2008).

We have found that PKC activation of serine 443 increases the phosphorylation and cell surface expression of α4-containing $GABA_A$Rs. Furthermore, GABA-activated currents recorded from receptors composed of $\alpha4\beta3$ subunits show significant rundown, a phenomenon that could be prevented by the activation of PKC, and is dependent upon serine 443 in the $\alpha4$ subunit. Rundown is believed to reflect a loss in the number of functional cell surface $GABA_A$Rs due to decreased insertion into the plasma membrane. Our results can be explained by the increase in the insertion of $\alpha4$-containing $GABA_A$Rs into the plasma membrane following PKC-mediated phosphorylation of serine 443. A point mutation of serine 443 into an alanine showed an increased cell surface expression. In contrast with wild-type α4-containing $GABA_A$Rs, expression of the $\alpha4^{S443A}$ mutant prevented the rundown of GABA-evoked current amplitude during control conditions and infusion with the PKC-activator PDBu did not have any further effect. Furthermore, we show that $\beta3$ serines 408 and 409 are phosphorylated by PKC. The δ subunit is not phosphorylated by PKC, suggesting that α4 serine 443 and $\beta3$ serines 408 and 409 are the primary PKC phosphorylation sites for this $GABA_A$R subtype that mediates tonic inhibition.

Phosphorylation is a key mechanism by which the endogenous and exogenous modulators can regulate the cell surface expression of extrasynaptic $GABA_AR$s (Levitan 1999). Neurosteroids have been shown to alter the expression patterns of extrasynaptic $GABA_AR$ subunits (Hen and Smith 2009, Maguire and Mody 2010). Previous studies have suggested that PKC activity is required for neurosteroid-mediated modulation of $GABA_AR$s (Brussaard and Koksma 2003, Harney et al. 2003). We hypothesized that neurosteroids mediate the phosphorylation of $GABA_AR$s subunits to alter receptor functional expression.

In order to test our hypothesis, we examined the effect of the stress hormone metabolite THDOC on the cell surface accumulation and function of α4-containing $GABA_AR$s. Here we show that neurosteroids exert selective effects on the plasma membrane stability of $GABA_AR$s subunits that mediate tonic inhibition. This novel mechanism of action is dependent on the ability of neurosteroids to enhance the phosphorylation of the α4 subunit on serine 443 (Chapter 3).

We demonstrate the ability of neurosteroids to modulate the phosphorylation of the α4 subunit on serine 443 when expressed along with the β3 subunit in heterologous cell lines as well as in hippocampal slices. THDOC significantly increased phosphorylation of the α4 subunit and this effect was blocked by the PKC inhibitor GFX (Figure 3-1).

THDOC induced PKC phosphorylation largely occurred on serine 443, as mutation of this residue to an alanine abolished THDOC dependent phosphorylation of the α4 subunit (Figure 3-1).

We then explored the effect of the THDOC-mediated increase in α4 subunit cell surface expression on the function of α4-containing $GABA_A$Rs. For this, we examined the effects of internally applied THDOC on GABA-evoked current rundown. While extracellular application of neurosteroids enhances GABA-activated currents, intracellular application has been reported to have no effect (Lambert et al., 1990). Under control conditions, significant rundown of GABA-activated current magnitude was evident for $\alpha4\beta3$ receptors. Inclusion of THDOC significantly reduced rundown compared to DMSO control (Figure 3-2). The ability of THDOC to prevent rundown was critically dependent upon the activation of PKC, because for cells exposed via intracellular application to both THDOC and PKC inhibitory peptide, the level of GABA-evoked current rundown observed were similar to control (Figure 3-2).

This phenomenon does not occur with all agents that potentiate GABA-activated currents, as internal application of the anesthetic Propofol did not prevent the rundown of $\alpha4\beta3$-mediated currents (Figure 3-3).

To further examine whether the ability of THDOC to prevent the rundown of GABA-currents is mediated via the ability of this agent to modify $\alpha4$ subunit phosphorylation, we examined the current rundown for receptors

containing $\alpha4^{S443A}$ subunits. GABA-evoked in cells expressing $\alpha4^{S443A}\beta3$ $GABA_ARs$ receptors exhibited minimal current rundown. The current at 20 minutes after the beginning of the experiment was approximately 84% of the current at the beginning of the experiment. Similar results were seen in the presence of THDOC (Figure 3-6). Although the rundown properties exhibited by the $\alpha4^{S443A}$ mutant might result surprising at first glance considering our proposed mechanism of a phosphorylation-dependent process, our results suggest that the $\alpha4^{S443A}$ mutant acts as a phospho-mimic of the $\alpha4$ subunit. Our studies indicate the requirement of the hydroxyl side chain at serine 443. That is, that removal of the hydroxyl side chain by mutation to an alanine is equivalent to masking it by phosphorylation. Interestingly, THDOC did not prevent the rundown of $GABA_ARs$ containing the α1 subunit, which is predominantly expressed at synaptic sites. This finding suggests that the THDOC-mediated increase of α4 subunit cell surface expression is specific for the α4 subunit (Figure 3-5).

Neurosteroid enhancement of GABA-mediated currents is dependent on an identified “binding pocket” conserved within the first transmembrane domain of all α subunits (Hosie et al. 2006, 2009). A glutamine residue within this pocket (glutamine 246 in the $\alpha4$ subunit) has shown to be critical for this interaction, and a point mutation of this residue into a leucine abolishes the neurosteroid-mediated potentiation of GABA-evoked

currents (Hosie et al. 2009). We explored whether the putative neurosteroid binding site contributes to its effect on the enhancement of α4-subunit cell surface expression. During control conditions, GABA-activated currents from cells expressing the $\alpha4^{Q246L}$ mutant showed similar rundown as wild-type receptors. Internal application of THDOC prevented the rundown of $\alpha4^{Q246L}\beta3$-mediated currents, comparable to what we see with α4β3-mediated currents (Figure 3-6). These results suggest that there are two distinct mechanisms of neurosteroid action on $GABA_AR$s; (i) Allosteric enhancement of $GABA_AR$s for which the glutamine 246 residue conserved in all α subunits is critical, (ii) and our newly identified long-term enhancement of α4-subunit cell surface accumulation that is dependant on PKC-phosphorylation of serine 443.

The α4 subunit can be expressed at extrasynaptic sites along with β3 and δ subunits. Previous work from our laboratory identified serines 408 and 409, located within the intracellular loop of the β3 subunit, as critical for the modulation of synaptic $GABA_AR$ trafficking (Jacob et al. 2008, 2009). Furthermore, we show that when co-expressed with the α4 subunit, β3 serines 408 and 409 are phosphorylated by PKC. We examined the significance of these residues for the neurosteroid-mediated alterations in the cell surface expression of α4-containing $GABA_AR$s (Chapter 4).

In order to study how these PKC phosphorylation sites in β3 subunits alter the neurosteroid-mediated changes in the phosphorylation, cell surface

expression and the magnitude of GABA-evoked currents mediated by α4β3-containing $GABA_ARs$, we utilized a β3 mutant construct in which serines 408 and 409 were replaced for alanines (Jacob et al. 2009).

We demonstrate that PKC phosphorylation of serines 408/409 is critical for the THDOC-mediated effects on the cell surface accumulation and function of α4-containing $GABA_ARs$. Expression of the α4 subunit along with the $\beta3^{S408/409A}$ mutant prevents the PKC-dependant increase in α4 subunit phosphorylation (Figure 4-1), cell surface expression (Figure 4-1), and insertion rate (Figure 4-2). In contrast to wild-type receptors, THDOC infusion did not prevent the rundown of GABA-evoked currents mediated by $\alpha4\beta3^{S408/409A}$ $GABA_ARs$ (Figure 4-4).

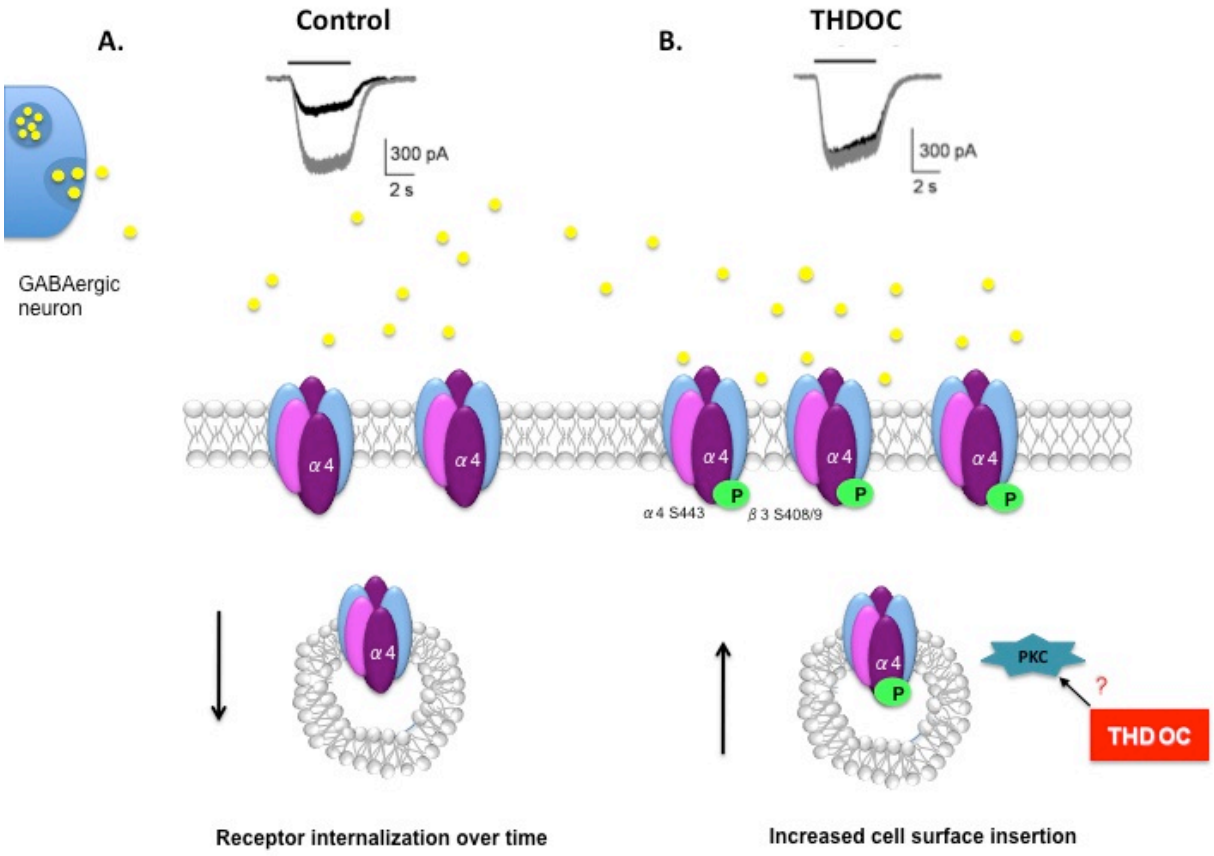

Figure 1: Neurosteroid modulation of extrasynaptic $GABA_ARs$ functional cell surface stability. *The stress hormone metabolite THDOC increases the PKC-dependent phosphorylation of the α4 subunit. This leads to enhanced cell surface accumulation of α4-containing $GABA_ARs$ which is reflected at a functional level as an abolishment of the decrease in current amplitude over time or rundown.* ***(A-B)*** *Traces represent whole cell patch-clamp electrophysiological experiments examining the rundown of currents evoked by ~EC_{50} concentrations of GABA. Currents in grey (■) are representative of currents at the start of experiments while*

currents in black (■) are representative of currents after 20min of recording.

PROPOSED MECHANISM

Collectively, our studies presented here reveal a novel mechanism by which neurosteroids enhance extrasynaptic $GABA_ARs$ function. Exposure to the stress hormone metabolite THDOC increases the PKC-dependent phosphorylation of α4 subunit serine 443 via a mechanism that also requires the phosphorylation of serines 408/409 within the β3 subunit. This THDOC-mediated increase in phosphorylation results in maintained stable cell membrane receptor number by facilitating a vesicle-mediated increase in the insertion of α4-containing $GABA_ARs$.

Neurosteroid enhancement of α4 subunit cell surface accumulation is independent of the neurosteroid binding site. These results suggest that neurosteroids can have two separate effects on $GABA_AR$ function; short-term enhancement of $GABA_AR$ potency via increase in the open probability of the channel and long-term changes in α4-containing $GABA_AR$ plasticity due to increased cell surface stability via an increase in the phosphorylation-dependant cell surface insertion of individual α4 subunits.

While allosteric potentiation can have significant effects on receptor activity, sustained receptor modulation can lead to long-term changes in the plasticity of $GABA_A$Rs subunits. For the first time, we describe a mechanism by which neurosteroids can exert long-term changes in the cell surface expression of extrasynaptic $GABA_A$R subunits. Neurosteroid mediated changes in the expression of extrasynaptic $GABA_A$Rs can have significant effects on the tonic conductance mediated by these receptors (Belleli et al. 2002, Bianchi and MacDonald 2003, Stell et al .2003).

The level of tonic inhibitory control is critical for allowing appropriate response to afferent input in neuronal circuits. The persistent activation of extrasynaptic $GABA_A$Rs, continuously decreases the membrane input resistance, and hence decreases the neuronal membrane voltage response to incoming excitatory signals. This decrease results in a persistent reduction of the probability for the neuron to fire an action potential. Individual neurons contribute to the shaping of network excitability and to the transfer of information in circuits, via the generation of action potentials. They generate output signals that reflect the strength of the excitatory inputs it receives (Mitchell and Silver 2010). This is referred to as the input-output (I-O) relationship, and it can be modified via a change in the gain modulation (slope of the I-O curve) or in the offset (threshold). Tonic inhibition has been demonstrated to modulate neuronal

excitability by altering either the gain of transmission or the threshold for firing, depending on the neuronal circuit involved.

Information stored in neuronal circuits can be represented as either changes in the current threshold for action potential firing (sparse-coded information) or in changes in the firing rate of individual neurons (rate-coded information). For neurons that process sparse-coded information such as CA1 pyramidal cells, tonic inhibition is believed have an effect on the offset of the I-O curve (Pavlov et al 2009, Silver 2010). Whereas, for those that process rate-coded information such as cerebellar granule cells, tonic inhibition is believed to modulate the gain of transmission (Mitchell and Silver 2003, Silver 2010). Tonic inhibition is therefore a powerful regulator of neuronal excitability. Neurosteroids have been shown to alter neuronal excitability via their modulation of extrasynaptic $GABA_A$Rs that mediate tonic inhibition (Stell et al. 2003, Maguire and Mody 2010).

This is of particular relevance for alterations in neuronal excitability that occur during epileptogenesis. In studies examining temporal lobe epilepsy models, the levels of synaptic $GABA_A$Rs subunits are decreased in the epileptic brain, whereas the levels of extrasynaptic $GABA_A$Rs are either maintained or increased. Specifically, the α4 subunit expression is increased in many rodent temporal lobe epilepsy models (Terunuma et al.

2008, Reviewed by Joshi et al. 2011) as well as in post-mortem samples of human patients (Joshi et al. 2011). Neurosteroid-mediated alterations in the cell surface expression of the α4 subunit are associated with increased seizure susceptibility (Reddy et al. 2001). For example, the withdrawal-like reduction in neurosteroid levels during the menstrual cycle is associated with a reduction in the expression of the α4 subunit and a concomitant decrease in the seizure threshold in catamenial epilepsy (Reddy et al. 2001, Pavlov and Walker 2012). Neurosteroid phospho-dependant modulation of α4 cell surface expression is likely the mechanism for these changes. Our newly characterized mechanism of neurosteroid action might provide a novel therapeutic strategy for the treatment of epilepsy.

Interestingly, in contrast with temporal lobe epilepsy, absence epilepsy is associated with alterations in the tonic conductance mediated by extrasynaptic $GABA_AR$s (Belelli et al. 2009, Cope et al. 2009). Absence seizures are believed to result form the generation of spike-wave discharges in thalamocortical neurons, which occur due to increased tonic inhibition. The association of tonic inhibition with absence seizures is an important aspect to consider when developing pharmacological approaches that target $GABA_AR$ subtypes that mediate tonic inhibition.

Fluctuations in the levels of neurosteroids have been associated with alterations in the levels extrasynaptic $GABA_AR$s and subsequent

alterations in the tonic inhibition mediated by these receptors (Belleli et al. 2002, Bianchi and MacDonald 2003, Stell et al .2003). However, to date no mechanism for this had been previously described. The work presented here provides further insight into the mechanisms of neurosteroid action and may aid in the development of novel drug targets for the treatment of the wide range of psychiatric and neurological conditions in which the levels of neurosteroids are perturbed.

FUTURE DIRECTIONS

Future studies will be focused on more closely examining the THDOC-PKC interaction that mediates the modulation of α4 subunit cell surface stability. It will be important to determine whether THDOC can directly activate PKC activity or whether there are other interacting partners involved that might make the receptor more accessible for PKC binding. Preliminary, studies suggest that THDOC does not directly activate PKC (Data not shown). We speculate that THDOC might interact with binding partners such as the scaffolding protein RACK-1 (Receptor for Activated C Kinase-1). RACK-1 enables the translocation of PKC isoforms and stabilizes their active forms (Mochly-Rosen and Gordon, 1998). Interestingly, RACK-1 has been shown to directly bind to the β3 $GABA_AR$ subunit at residues 395-404 (Brandon et al. 2002). Furthermore, There is some evidence suggesting that neurosteroids can increase the levels of RACK-1 (Racchi et al. 2001), making this molecule a possible mediator of

the THDOC-mediated phospho-dependant changes in α4 subunit plasticity.

PKC can be expressed as multiple isoforms or isozymes. These multiple isoforms can have different regional expression and subcellular localization and they mediate isoform-specific functions (Mochly-Rosen and Gordon, 1998). At least ten different PKC isozymes have been reported to exist and those predominantly expressed in the brain (α, βI, βII, and γ) are differentially expressed within the CNS. An important aspect to explore will be whether the neurosteroid effects on the functional expression of the α4 subunit are PKC-isoform specific. If THDOC enhancement of the α4 $GABA_AR$ subunit is PKC-isoform specific, a good candidate for this specificity would be PKCβII. PKCβII has been shown to mediate phosphorylation of serine 409 in β1 and serines 408 and 409 in the β3 $GABA_AR$ subunit (Song and Messing 2005). However, decreased expression of PKC isoforms α,β and γ has been associated with decreased β3 subunit phosphorylation in the hippocampus (Terunuma et al. 2008).

It will be important to explore whether this newly identified role for the stress hormone metabolite THDOC is also shared by other neurosteroids that exert similar allosteric actions. Our preliminary studies (Data not shown) indicate that the progesterone metabolite allopregnanolone can

increase the cell surface insertion of the α4 subunit. Determining whether steroid analogues with therapeutic potential (such as Ganaxolone) can also modulate the cell surface stability of the α4 subunit could have significant implications for the use of neurosteroids for the treatment of disorders where the expression of the α4 subunit is reduced including Fragile X syndrome and Autism (D'Hulst et al. 2006, Fatemi et al. 2010).

Finally, it will important to dissect the physiological relevance of these findings. Transgenic mouse models in which the wild-type and α4 serine 443 to alanine mutant subunits will be tagged with Red Fluorescent Protein, are currently being developed and should provide further insight into how neurosteroid modulation of α4 subunit functional stability alters the neuronal functions mediated by α4-containing extrasynaptic $GABA_A$Rs. Aspects to be assessed will include anxiety, alcohol tolerance and withdrawal, and the thresold for seizure susceptibility as studied in animal models of status epilepticus.

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