Activation of membrane progesterone receptors mediates the metabotropic effects of neurosteroids on $GABA_AR$ phosphorylation and tonic inhibition

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Abstract

Neurosteroids have been well characterized as positive allosteric modulators of the $GABA_AR$. However, previous studies in our lab have identified an *additional* role for neurosteroids in mediating phosphorylation and stability of α4-containing $GABA_ARs$. In this body of work, I show through both biochemical and electrophysiological methods that this novel role is (1) not specific to the α4 subunit; neurosteroids also mediate phosphorylation of the β3 subunit and (2) this mechanism is independent of the allosteric modulation, and instead functions through activation of membrane progesterone receptors (mPRs). I demonstrate that pregnane steroids such as allopregnanolone (3α,5α-THP), mediate phosphorylation of serines 383, 408 and 409 of the β3 subunit in the hippocampus. Allopregnanolone mediated phosphorylation of these residues is through activation of both PKC and PKA signaling mechanisms and results in enhanced tonic inhibition within the hippocampus. Allopregnanolone has previously been shown to bind membrane progesterone receptor alpha (mPRα) and activate downstream kinase signaling. To test the hypothesis that neurosteroid mediated $GABA_AR$ phosphorylation is via activation of mPRs, I use both progesterone (mPR agonist) and ORG OD 02-0 (synthetic agonist) to demonstrate an enhancement of β3 phosphorylation and of tonic current. Additionally, I show these effects are both blocked by PKC and PKA kinase inhibition. Finally, to elucidate a specific mPR subtype responsible for mediating these effects, I demonstrate that activation of mPRα significantly enhances β3 phosphorylation and β3-containing $GABA_ARs$ at the plasma membrane.

Dedication

To my parents

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List of Abbreviations

17,20βDHP: 4-pregnen-17,20b-diol-3-one
20β-S: 17α,20β,21-trihydroxy-4-pregnen-3-one
3α,5α-THP: 3α,5α-tetrahydroprogesterone (allopregnanolone)
3α-diol: 3α-androstanediol
3α-HSD: 3alpha-hydroxysteroid dehydrogenase
3β-HSD: 3 beta hydroxysteroid dehydrogenase
5-HT3: serotonin receptor
A293: Subclone A of human embryonic kidney cells
ACTH: adrenocorticotropic hormone
AKT: Protein Kinase B
AP: action potential
AP2: adaptor protein 2
ASD: Autism Spectrum Disorders
ATCC: American Type Culture Collection
ATP: adenosine triphosphate
Bay K8644: L-type Ca^{2+} channel activator
BIG2: brefeldin A-inhibited GDP/GTP exchange factor 2
BSA: bovine serum albumin
C57BL/6: most widely used inbred mouse strain created by Dr. CC Little
CA1: Cornu Amonis 1
CA3: Cornu Ammonis 3
CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II
cAMP: cyclic adenosine monophosphate
CCD: charge-coupled device
CLGIC: cys-loop ligand gated ion channel
COS-1: African green monkey kidney cells
CYP11A: side-chain cleavage enzyme A
CYP11B1: side-chain cleavage enzyme B1
CYP11B2: side-chain cleavage enzyme B2
DAG: diacylglycerol
DGGCs: dentate gyrus granule cells
DH5α: E.coli strain with recA1 and endA1 mutations
DHEAS: dehydroepiandrosterone sulfate
diH_20: deionized water
DMEM: Dulbecco's Modified Eagle Media
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
ECL: enhanced chemiluminescence
EPSPs: excitatory postsynaptic potentials
ER: endoplasmic reticulum
ERK: extracellular signal-regulated kinase

FBS: Fetal Bovine Serum
FXS: Fragile X Syndrome
G2/M: Gap 2/Mitotic phase
GABA: γ-aminobutyric acid
$GABA_AR$: γ-aminobutyric acid type A receptor
GABARAP: GABA type A receptor-associated protein
GAD65: glutamic acid decarboxylase 65 kilodalton isoform
GAD67: glutamic acid decarboxylase 67 kilodalton isoform
GDPs: Giant depolarizing potentials
GF 109203X: selective protein kinase C inhibitor
GFP: green fluorescent protein
GFX: GF 109203X
$G_{i/o}$: G_i or G_o-coupled G protein
G_i: G_i alpha subunit
GODZ: Golgi-specific DHHC zinc finger protein
G_{olf}: olfactory neuron specific G protein
GPCR: G protein-coupled receptor
GRIF/TRAK: $GABA_AR$ interacting factor/trafficking kinesin protein
GRIF1: $GABA_AR$ interacting factor-1
GRP78/BiP: 78 kilodalton glucose regulated protein, also known as BiP
G_s: G_s-coupled G protein
GST: glutathione S-transferase
GT1-7: mouse immortalized hypothalamic gonadotropin-releasing hormone neurons
GTP: guanosine triphosphate
HAM-D: Hamilton Depression Rating Scale
HAP-1: Huntingtin-associated protein-1
HEK293: human embryonic kidney cells
HEK-293T: derived from human embryonic kidney 293 cells, contains the SV40 T-antigen
HPA axis: hypothalamic pituitary adrenal axis
IPSPs: inhibitory postsynaptic potentials
Ishikawa: human endometrial adenocarcinoma cells
KCC2: neuron-specific type $2^{K+}/Cl^-$ cotransporter
kDa: kilodalton
KN-93: CaM kinase II inhibitor
KT 5720: selective protein kinase A inhibitor
LB: lysogeny broth
M11: human myometrial cells
MAPK: mitogen-activated protein kinase
MDA-MB-231: human breast adenocarcinoma cells
mIPSCs: miniature inhibitory postsynaptic currents
mPRs: membrane progesterone receptors
mPRα: membrane progesterone receptor alpha
mRNA: messenger RNA
N/A: not applicable

nACSF: normal artificial cerebrospinal fluid
NIH: National Institutes of Health
nPRs: nuclear progesterone receptors
NSF: N-ethylmaleimide-sensitive factor
ORG OD 02-0: 10-ethenyl-19-norprogesterone
P: passage
PAQR: Progestin and adipoQ receptor
PAQR5: Progestin and AdioQ Receptor Family Member 5
PAQR7: Progestin and AdipoQ Receptor Family Member 7
PAQR8: Progestin and AdipoQ Receptor Family Member 8
PAQR9: Progestin and AdipoQ Receptor Family Member 9
PBS: phosphate-buffered saline
PBS-T: phosphate-buffered saline with tween
PC12: pheochromocytoma of rat adrenal medulla cells
pCMV6: mammalian expression vector with c-terminal myc-flag tag
PDBu: phorbol 12,13-dibutyrate
pEYFP-N1: mammalian expression vector with enhanced yellow fluorescent protein tag
PFA: paraformaldehyde
PGRMC1: progesterone receptor membrane component 1
PKA: Protein Kinase A
PKC: Protein Kinase C
PKG: cGMP-dependent protein kinase G or Protein Kinase G
Plic-1: protein linking integrin-associated protein to cytoskeleton 1
PP1α: protein phosphatase 1α
PP2A: protein phosphatase 2A
PRIP1: Phospholipase C-related inactive protein type 1
pRK5: a mammalian expression vector
PXRs: pregnane X receptors
qPCR: quantitative polymerase chain reaction
RXRs: retinoid X receptors
S408/409: serines 408 and 409
SAGE-516: SAGE therapeutics compound synthetic analogue of allopregnanolone
SDS: sodium dodecyl sulfate
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE: status epilepticus
SEM: standard error of the mean
sIPSCs: spontaneous inhibitory postsynaptic currents
siRNA: small interfering RNA
SOC: super optimal broth
SSRIs: selective serotonin reuptake inhibitors
TBI: traumatic brain injury
THDOC: allotetrahydrodeoxycorticosterone
THIP: 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol
TMDs: transmembrane domains

TUBB: Tubulin β Class I
VGCCs: voltage gated calcium channels

Chapter 1: Introduction

Epilepsy disorders are a growing concern for our society as 3.4 million people in the United States are reported to be currently living with epilepsy (Centers for Disease Control and Prevention). Epilepsy is characterized by unprovoked and recurring seizure activity in the brain (B. S. Chang & Lowenstein, 2003). Conventional drugs used as treatments for epilepsy are benzodiazepines—modulators of $GABA_A$Rs (Ochoa & Kilgo, 2016). However, treatment with benzodiazepines is not always effective. In status epilepticus (SE), a severe epileptic disorder, patients exhibit persistent seizures that fail to terminate and become resistant to benzodiazepines (Lowenstein & Alldredge, 1998). Thus, there is a need for better therapeutic interventions. Endogenous compounds produced in the brain, known as neurosteroids, also positively modulate $GABA_A$R activity (E.E. Baulieu & Robel, 1990; Neil L. Harrison & Simmonds, 1984). But, they preferentially act on a subset of $GABA_A$Rs distinct from those that bind benzodiazepines (Belelli & Lambert, 2005). For this reason, neurosteroids may be a better and more powerful treatment option than benzodiazepines for epilepsies.

Using synthetic neurosteroids as a potential therapy for epilepsy is what a company in Cambridge, Massachusetts known as SAGE Therapeutics, is currently exploring. For my PhD, I have collaborated with SAGE Therapeutics to investigate the therapeutic potential of using synthetic neurosteroids and the mechanism by which they affect the GABAergic system. Neurosteroids have long been characterized by their modulation of $GABA_A$R currents (on the timescale of milliseconds), but other studies have demonstrated that neurosteroids can also exhibit longer term effects (minutes to hours) by affecting $GABA_A$R

expression within cells (Gulinello, Gong, Li, & Smith, 2001; J. Maguire & Mody, 2007). Expanding on this finding, our lab previously demonstrated that the neurosteroid THDOC enhanced insertion of $GABA_A$Rs at the plasma membrane due to increased phosphorylation of $GABA_A$ subunits (Abramian et al., 2014). This was a pivotal finding because although other groups had demonstrated that phosphorylation of $GABA_A$R subunits enhanced neurosteroid modulation of $GABA_A$R currents, this was the first study that demonstrated neurosteroids themselves could mediate phosphorylation of $GABA_A$R subunits and insertion of $GABA_A$Rs into the plasma membrane (Abramian et al., 2014; Fáncsik, Linn, & Tasker, 2000; Tasker, 2000). The data from this study provided further evidence for the long-term effects of neurosteroids on $GABA_A$R dynamics and clues about a potential mechanism involving phosphorylation.

THDOC was the first neurosteroid we investigated in modulating $GABA_A$R subunit phosphorylation and expression. However, it was unknown whether other neurosteroids could also facilitate phosphorylation of $GABA_A$Rs. This set the stage for the beginning of my PhD—I wanted to investigate the conserved feature of neurosteroids to mediate phosphorylation of $GABA_A$Rs. At the same time, SAGE Therapeutics was working on developing synthetic analogues for another neurosteroid—allopregnanolone. Allopregnanolone is the most abundant neurosteroid in the brain and has been implicated to play a role in stress, pregnancy, post-partum depression and other diseases (Eser, Schüle, Baghai, Romeo, & Rupprecht, 2006; J. Maguire & Mody, 2009; Purdy, Morrow, Moore, & Paul, 1991). SAGE Therapeutics was interested in using synthetic allopregnanolone as a therapy for epilepsy as well as for post-partum depression. We set up a collaboration with SAGE Therapeutics to test their synthetic analogues along with our endogenous steroids to investigate their role in phosphorylation mediated mechanisms. For my thesis work, I set

out to study the role of allopregnanolone on $GABA_AR$ phosphorylation and the impacts it had on $GABA_AR$ expression in the hippocampus—an area that is known to be susceptible to seizure activity (Heinemann et al., 1992; Luciano, 1993). Although our initial focus was looking at neurodevelopmental disorders that display co-morbidity with epilepsy, our aims shifted more closely with epilepsy and status epilepticus as our collaboration with SAGE Therapeutics grew. During my study, I also investigated the potential upstream factors involved in mediating neurosteroid action. Moreover, this became the turning point of my PhD, where I identified a key receptor facilitating allopregnanolone's effects on phosphorylation and expression of $GABA_AR$ subunits. My findings elucidated the mechanistic details of neurosteroid action, giving us a better understanding of using synthetic neurosteroids as treatment for epilepsies. Furthermore, recent data from a phase II study using SAGE Therapeutics synthetic allopregnanolone compound brexanolone, demonstrated long-term positive effects in patients diagnosed with post-partum depression (Kanes et al., 2017). Taken together, my findings implicate a novel pathway of neurosteroid mediated regulation of $GABA_ARs$ that may be utilized as a therapeutic option in a variety of disease states. Ultimately, my findings suggest that this key receptor-mediated pathway may serve as the missing link hypothesized in previous studies investigating the long-term effects of neurosteroids.

1.1 $GABA_ARs$

1.1.1 Structure

Fast inhibitory neurotransmission in the brain is mediated primarily by γ-aminobutyric acid type A receptors ($GABA_ARs$), members of the Cys-loop ligand-gated ion channel family (CLGIC), which also includes glutamate, nicotinic acetylcholine, serotonin ($5\text{-}HT_3$), glycine,

and zinc-activated receptors (Alexander et al., 2017; Bertrand & Changeux, 1995; C. N. Connolly & Wafford, 2004; Davies, Wang, Hales, & Kirkness, 2003). $GABA_A$Rs are heteropentameric, permeable to chloride and bicarbonate ions and assembled from eight different subunit classes: α(1-6), β(1-3),γ(1-3), δ, ε, θ, π and ρ(1-3), with the stoichiometric ratio 2α: 2β: 1γ or δ (Bormann, Hamill, & Sakmann, 1987; Fritschy & Panzanelli, 2014; Nusser, Sieghart, & Somogyi, 1998a; Sieghart & Sperk, 2002). $GABA_A$Rs containing α, β, γ subunits are localized to synaptic sites and mediate phasic inhibition, while α, β, δ-containing $GABA_A$Rs are localized to extrasynaptic sites and mediate tonic inhibition (Fritschy & Panzanelli, 2014, p. 201; Nusser et al., 1998a). The most prevalent type of $GABA_A$R found in the brain is α1β2γ2 (Sieghart and Sperk 2002). Receptors containing only α and β subunits also exist; 10% of pyramidal neurons in the hippocampus express αβ $GABA_A$Rs (Mortensen Martin & Smart Trevor G., 2006a). 50% of α4 containing $GABA_A$Rs in the forebrain have no γ or δ subunits, although may contain ε, θ, π and ρ (Bencsits, Ebert, Tretter, & Sieghart, 1999; Sieghart & Sperk, 2002). Each subunit of the $GABA_A$R consists of a large extracellular N-terminal domain, four transmembrane domains (TMDs) and a short C-terminal domain (Schofield et al., 1987). The five subunits are arranged around a central pore, with transmembrane domain two of each subunit facing the channel pore (Y. Chang, Wang, Barot, & Weiss, 1996, p. 1; Unwin, 1993). The intracellular loop between TMDs three and four, varies most in amino acid sequence between subunits and contains many sites for post-translational modifications as well as protein interactions, even between subunits of the same receptor (Moss, Doherty, & Huganir, 1992; Nymann-Andersen, Wang, Sawyer, & Olsen, 2002; Olsen & Sieghart, 2008; O'Toole & Jenkins, 2011; Wang, Bedford, Brandon, Moss, & Olsen, 1999). The β2 and γ2 subunit can be alternatively spliced into either β2S or β2L, or γ2S or γ2L, where β2L contains 38 extra amino acids and γ2L contains eight extra amino

acids in the intracellular loop between TM3 and TM4 (McKinley, Lennon, & Carter, 1995; Whiting, McKernan, & Iversen, 1990). Although there are many possibilities of combinations of $GABA_A$Rs, only certain combinations of receptors are expressed (McKernan & Whiting, 1996). The specific combination of subunits is what gives $GABA_A$Rs their distinct physiological and pharmacological properties (Alger & Nicoll, 1982; Olsen, 2018; Olsen & Sieghart, 2009).

1.1.2 Synaptic $GABA_A$Rs

Two molecules of GABA bind between both α-β subunit interfaces, resulting in a conformational change as the cys-loops in the extracellular domain interact with the TM2-TM3 linkers, bending the α helices in the TM2 regions outward, causing the ion channel to open (Baumann, Baur, & Sigel, 2002; Bera, Chatav, & Akabas, 2002; Lester, 2004). Chloride and bicarbonate ions are both permeant through the channel pore of $GABA_A$Rs (Kai Kaila, Price, Payne, Puskarjov, & Voipio, 2014). However, under most physiological conditions, the driving force of chloride is larger than that of bicarbonate, as the equilibrium potential for chloride lies near the resting potential of the neuron (E_{Cl} = ~ -70mV) and the equilibrium potential for bicarbonate is more positive (E_{HCO3^-} = -12mV) (Eccles, 1964; K Kaila, Pasternack, Saarikoski, & Voipio, 1989). When presynaptic neurons release GABA in millimolar concentrations, synaptic $GABA_A$Rs lying opposite the presynaptic terminal and across the synaptic cleft on the post synaptic neuron, bind neurotransmitter and allow chloride ions to permeate the channel, producing phasic current (Busch & Sakmann, 1990; Overstreet, Westbrook, & Jones, 2003). Synaptic $GABA_A$Rs have a low affinity for GABA, but are highly efficacious in gating GABA (Stell & Mody, 2002). Thus, their synaptic

localization allows them to respond to the high concentrations of transmitter in the synaptic cleft (Stell & Mody, 2002). Chloride influx through the $GABA_AR$ usually results in hyperpolarizing current due to the maintenance of low intracellular chloride levels via the activity of the neuronal specific potassium and chloride co-transporter KCC2 (Blaesse, Airaksinen, Rivera, & Kaila, 2009; Eccles, 1964; Payne, 1997).

1.1.3 Extrasynaptic $GABA_ARs$

Extrasynaptic type receptors, also referred to as perisynaptic, are located just outside the synapse. These receptors respond to low, ambient, nanomolar concentrations of GABA (Stell & Mody, 2002). The low or ambient concentrations of GABA that activate extrasynaptic type receptors can arise from a variety of sources: spillover from synaptic release, non-synaptic GABA release, and/or reduction in reuptake mechanisms (Engel et al., 1998; Rossi, Hamann, & Attwell, 2003; Semyanov, Walker, & Kullmann, 2003). Tonic current mediated by extrasynaptic receptors, is measured using different $GABA_A$ antagonists to quantify the change in holding current (Bright & Smart, 2013). However, the distinct pharmacological profiles of $GABA_A$ antagonists reflect the variability in tonic current measurements (Lee & Maguire, 2014). $GABA_A$ antagonists such as bicuculline and gabazine, compete for the GABA binding site, whereas $GABA_A$ antagonist picrotoxin blocks the receptor channel. Therefore, tonic current revealed by gabazine does not occlude spontaneous channel openings that contribute to tonic current (Lee & Maguire, 2014; Wlodarczyk et al., 2013).

Regions in the brain that demonstrate modulation by tonic inhibition include: the hippocampus, neocortex, amygdala, striatum, thalamus, hypothalamus, cerebellum and spinal cord (Brickley, Cull-Candy, & Farrant, 1996; Lee & Maguire, 2014). Within the dentate gyrus

of the hippocampus, the majority of tonic current is believed to be mediated by δ-containing $GABA_A$Rs in dentate gyrus granule cells (DGGCs) (Glykys, Mann, & Mody, 2008). However, αβ-containing receptors without δ subunits may also contribute to tonic current in this region (Mortensen Martin & Smart Trevor G., 2006a). α4β and α4βδ receptor signaling can be differentiated pharmacologically (Stórustovu & Ebert, 2006). α4β3 receptors are inhibited by Zn^{2+} (IC_{50}=62 nM) but α4β3δ receptors are less sensitive to Zn^{2+} inhibition (IC_{50}=5.3μM) (Stórustovu & Ebert, 2006). In the CA1, principal neurons with α5β3γ-containing $GABA_A$Rs are highly expressed and are responsible for mediating the majority of tonic inhibition (Caraiscos et al., 2004; Sperk, Schwarzer, Tsunashima, Fuchs, & Sieghart, 1997). Although δ-containing subunits in pyramidal neurons are also expressed in the CA1, they respond to lower concentrations of GABA, while α5-containing receptors in pyramidal neurons respond to higher concentrations of ambient GABA (Scimemi, Semyanov, Sperk, Kullmann, & Walker, 2005).

1.1.4 Function of $GABA_A$Rs

GABAergic signaling is necessary for brain function. GABA mediated inhibition generates inhibitory postsynaptic potentials (IPSPs) that are summated along with excitatory post synaptic potentials (EPSPs) in both time and space to inhibit the probability of firing an action potential. Fast GABAergic signaling, mediated by a brief exposure to high concentrations of GABA, allows for "point to point communication" between neurons, controlling neuronal action potential firing and circuit oscillations (Cherubini, 2012). $GABA_A$R function plays a critical role in terminating seizure activity, which occurs as a result of aberrant excitatory discharges (Bromfield, Cavazos, & Sirven, 2006; Treiman, 2001). Thus,

GABAergic inhibition plays a role in maintaining the excitatory and inhibitory balance in the brain (Treiman, 2001).

Tonic inhibition mediated by extrasynaptic receptors primarily regulates the excitability of cells; the persistent activation of extrasynaptic $GABA_A$Rs reduces the probability of firing an action potential. At depolarizing voltages, tonic current decreases membrane resistance, weakening the contribution of excitatory input at synapses (Lee & Maguire, 2014). Tonic conductance in pyramidal cells in the CA1 is more prominent during cell firing but not at subthreshold membrane potentials (Pavlov, Savtchenko, Kullmann, Semyanov, & Walker, 2009). The impact of tonic current on the neuronal network is complex since both principal cells and interneurons express extrasynaptic $GABA_A$ (Lee & Maguire, 2014). For example, interneurons in the CA1 can fire action potentials when excitatory transmission is inhibited, suggesting that ambient GABA concentrations activate cell firing in these neurons (Song, Savtchenko, & Semyanov, 2011).

The subcellular localization of $GABA_A$Rs also plays a role in modulating the output of the neuron. In the cortex, inhibitory neurons (parvalbumin + basket cells) specifically synapse on the soma of pyramidal neurons, allowing a higher degree of control over cell excitability (Kubota, Karube, Nomura, & Kawaguchi, 2016). This is further extended by parvalbumin + chandelier cells synapsing on α2-containing $GABA_A$Rs at the axon initial segment, where the action potential is generated (Coombs J. S., Curtis D. R., & Eccles J. C., 1957; Kubota et al., 2016; Nyíri Gábor, Freund Tamás F., & Somogyi Péter, 2001).

1.2 Phosphorylation of $GABA_A$Rs

Phosphorylation plays a critical role in the regulation of $GABA_A$Rs (Comenencia-Ortiz, Moss, & Davies, 2014; Saliba, Kretschmannova, & Moss, 2012; Vithlani & Moss, 2009). Many phosphorylation sites on $GABA_A$R subunits have been identified along with their functional roles (see Nakamura, Darnieder, Deeb, & Moss, 2015 for full review). Phosphorylation is a type of post-translational modification, where the terminal phosphate of an adenosine triphosphate (ATP) molecule is transferred to the protein of interest. Phosphorylation serves as a signal in many intracellular mechanisms—where enzymes known as kinases catalyze the transfer of the phosphate group and conversely, phosphatases are enzymes that remove the phosphate groups. The phosphates are predominantly added to serine, threonine or tyrosine residues in proteins. The majority of phosphorylation sites identified on $GABA_A$Rs lie in the intracellular loop domain between TM 3-4 (N. J. Brandon, 2000; N. J. Brandon, Delmas, Hill, Smart, & Moss, 2001; Nicholas J. Brandon et al., 1999; B. J. McDonald & Moss, 1994; Mcdonald & Moss, 1997; Moss et al., 1992). The majority of the phosphorylated residues are found in β and γ subunits (N. J. Brandon, 2000; B. J. McDonald & Moss, 1994; Mcdonald & Moss, 1997; Bernard J. McDonald et al., 1998). However, α subunits can also be phosphorylated; T375 on the α1 and S443 on the α4 subunit (Abramian et al., 2014; Mukherjee et al., 2011; Nakamura et al., 2015).

1.2.1 Phosphorylation of β subunits

The initial studies on phosphorylation of β subunits were carried out using glutathione S-transferase fusion proteins expressing solely the intracellular loop of β subunits that carry post-translational modification sites (Mcdonald & Moss, 1997; Moss et al., 1992).

Serine 409 on the β1 subunit has been shown to be phosphorylated by a variety of kinases—PKA, PKC, CaMKII, and PKG (B. J. McDonald & Moss, 1994; Moss et al., 1992). The β2 subunit is phosphorylated at serine 410 by the same kinases (Mcdonald & Moss, 1997). The β3 subunit also contains serine 409, similar to the β1 subunit, which also can be phosphorylated by PKA, PKC, PKG and CaMKII. Interestingly, the β3 subunit contains a phosphorylation site not found in either β1 or β2 subunits, serine 408 (Mcdonald & Moss, 1997). Serine 408 has been shown to be phosphorylated by both PKC and PKA (Mcdonald & Moss, 1997; Bernard J. McDonald et al., 1998). It is important to note that serine 408 and serine 409 can be phosphorylated independently, demonstrated by using single point mutations that did not prevent either one site from being phosphorylated (Bernard J. McDonald et al., 1998). A third phosphorylation site on β3, serine 383 (serine 384 in β1) has also been identified and is phosphorylated by CaMKII (B. J. McDonald & Moss, 1994).

1.2.1.1 Functional role of $\beta3^{S408/409}$ phosphorylation

Phosphorylation of both 408 and 409 residues is important for the enhancement of GABA-mediated currents in HEK293 cells (Bernard J. McDonald et al., 1998). α1β3γ2S receptors were transfected into HEK293 cells and 10μM GABA evoked currents were recorded in the presence or absence of cyclic adenosine monophosphate (cAMP) in the patch pipette. Intracellular application of cAMP, which activates PKA kinases, enhanced GABA responses from these cells by approximately 30% and maintained this enhancement for 30 minutes of recording. In α1β3S408Aγ2S expressing HEK293 cells however, there was a reduction in GABA evoked current 10-15 minutes after cAMP application. Although the S408A mutant significantly reduced GABA-evoked responses in the presence of cAMP,

S409A expressing HEK293 cells prevented cAMP modulation of GABA-mediated currents. Mutation of both residues in α1β3S408AS409Aγ2S transfected cells, also exhibited no change in response to cAMP levels. Together, this suggests that these two phosphorylation sites exhibit different functions, but phosphorylation of both residues is required for enhancing $GABA_A$Rs currents (Bernard J. McDonald et al., 1998).

It is also known that phosphorylation of S408/409 residues reduces receptor endocytosis from the plasma membrane (Kittler et al., 2005). Kittler et al., (2005) demonstrated that the μ2 subunit of the AP2 complex, known to regulate endocytosis, binds the intracellular loop of β3 subunit between residues 395-410 in glutathione S-transferase (GST) fusion proteins. They found this binding was phospho-dependent, as *in vitro* phosphorylation with PKA or PKC of GST fusion protein containing the intracellular loop of β3, prevented binding of μ2. To implicate a role in AP2 binding in the modulation of $GABA_A$ currents, Kittler et al., (2005) internally applied a β3 peptide containing the μ2 binding domain and demonstrated enhanced amplitude and frequency of miniature inhibitory postsynaptic currents (mIPSCs) in cultured neurons. In contrast, internal application of a phosphorylated β3 peptide, did not enhance mIPSC frequency or amplitude (Kittler et al., 2005).

Phosphorylation of the β3 subunit has been implicated to play a role in a mouse model of status epilepticus (Terunuma et al., 2008). Terunuma et al., (2008) demonstrate that phosphorylation of $\beta3^{S408/409}$ is significantly reduced in SE, concurrent with a decrease in PKC binding to the β3 subunit. $GABA_A$R subunit expression itself is altered, including a reduction in β3 surface levels. Terunuma et al., further confirmed that the loss of S408/409 phosphorylation was a result of increased association with AP2. Thus, blocking endocytosis

or activating PKC dependent S408/409 phosphorylation, restored the surface stability of β3 containing receptors in SE (Terunuma et al., 2008).

Conversely, compromising the phosphorylation state of these residues may play a role in autism spectrum disorders (Vien et al., 2015). S408A/S409A knock-in mice were generated in which both serine residues were mutated to alanines. These mice exhibited autism spectrum like phenotypes including decreased social interaction and repetitive-like behaviors (Vien et al., 2015). The mutation of serine to alanine usually mimics a constitutively dephosphorylated phenotype at a phosphorylation site of a protein (Dissmeyer & Schnittger, 2011). The S408/409A knock-in mutation resulted in alterations of $GABA_A$ subunit levels in the hippocampus--a reduction in α4 but an increase in α2 subunit expression at the plasma membrane. This was consistent with an increase in phasic and a decrease in tonic inhibition within the hippocampus. Together, this suggests that phosphorylation of S408/409 may contribute to the assembly as well as the trafficking of extrasynaptic and synaptic $GABA_A$Rs (Vien et al., 2015).

1.2.1.2 Functional role of $\beta3^{S383}$ phosphorylation

The phosphorylation of S383 on the β3 subunit specifically enhances the insertion of $GABA_A$Rs into the plasma membrane without affecting receptor endocytosis (Saliba et al., 2012). Activation of L-type voltage gated calcium channels (VGCCs) results in an influx of Ca^{2+} that promotes CaMKII to phosphorylate $\beta3^{S383}$. L-type VGCC agonist (Bay K8644) significantly enhanced the number of β3-containing receptors at the plasma membrane within 2 minutes of treatment. The increase in phosphorylation and β3 surface levels were blocked by pretreatment with CaMKII inhibitor, (KN-93). Furthermore, Bay K8644

treatment had no effect on insertion of phospho-null mutant $\beta3^{S383A}$ into the neuronal membrane, whereas surface expression of WTβ3 was significantly enhanced. Lastly, blocking endocytosis with myristoylated dynamin inhibitory peptide, did not prevent the Ca^{2+} mediated increase in β3 surface levels (Saliba et al., 2012).

1.3. Dynamic regulation of $GABA_A$Rs at the plasma membrane

$GABA_A$Rs are dynamic entities at the plasma membrane—they are constantly inserted, endocytosed and recycled either to the surface or for degradation (Kittler et al., 2000). Vesicles containing $GABA_A$Rs fuse with the plasma membrane predominantly at extrasynaptic sites, where receptors then laterally diffuse to their respective locations (Bogdanov et al., 2006). The subunit composition of receptors dictates their synaptic and extrasynaptic location at the plasma membrane (Fritschy & Panzanelli, 2014; Nusser, Sieghart, & Somogyi, 1998b). $GABA_A$Rs are endocytosed from the membrane via clathrin-coated pits by their interactions with the adaptor protein 2 (AP2) complex. Both β and γ2 subunits can bind α and β subunits of the AP2 complex through their intracellular loop domains (Kittler et al., 2000). There is constant turnover of $GABA_A$Rs and a proportion of them are recycled back to the membrane (Kittler et al., 2004a). Thirty percent of $GABA_A$Rs that are endocytosed are recycled back to the membrane within 5 minutes, and 70% of them are reinserted within an hour. This interaction may be dependent on β subunits binding to huntingtin-associated protein 1(HAP-1) proteins that enhance their recycling back to the plasma membrane. Those receptors that are *not* recycled are destined for lysosomal degradation over longer periods of time (6 hours) (Kittler et al., 2004b).

1.4 $GABA_AR$ signaling in the hippocampus

The hippocampus has been shown to play a role in anxiety and epilepsy disorders--both of which involve dysfunction of the GABAergic system (Crestani et al., 1999; Engin & Treit, 2007; Luciano, 1993). Intrahippocampal infusion of $GABA_AR$ modulators, such as benzodiazepines and neurosteroids, diminishes anxiety phenotypes in rats (Engin & Treit, 2007). However, reducing $GABA_AR$ activity by knockdown of the γ2 subunit in mice, enhances anxiety-like behaviors (Chandra, Korpi, Miralles, De Blas, & Homanics, 2005; Crestani et al., 1999).

The hippocampus is well known for its susceptibility to seizure activity (Heinemann et al., 1992; Luciano, 1993). The dentate gyrus of the hippocampus is known to play a key role in filtering excitatory activity coming into the trisynaptic loop of the hippocampus (Heinemann et al., 1992; Strien, Cappaert, & Witter, 2009). The main cells of the dentate gyrus, DGGCs, exhibit hyperpolarized resting membrane potentials, low input resistance and higher threshold for action potential firing (Heinemann et al., 1992; Lothman, Stringer, & Bertram, 1992; Mody, Otis, Staley, & Köhr, 1992). Furthermore, DGGCs exhibit feedback and feedforward GABAergic inhibition that contributes to the low firing rate of these cells (Coulter & Carlson, 2007). Particularly, tonic inhibition mediated by extrasynaptic $GABA_ARs$ in this region, plays a critical role in the filtering function of the dentate gyrus (Coulter & Carlson, 2007; Stell & Mody, 2002). In disorders such as epilepsy, the function of the dentate "gate" may be compromised, resulting in a barrage of uncontrolled excitatory input or seizure activity (Heinemann et al., 1992; Lothman et al., 1992). In animal models of temporal lobe epilepsy, there is a reduction in δ subunit expression but an increase in α4 and

γ2 subunit expression in the dentate gyrus (Brooks-Kayal, Shumate, Jin, Rikhter, & Coulter, 1998; Peng, 2004). This suggests that under epileptic conditions, the composition of extrasynaptic $GABA_A$Rs may be altered (Coulter, 2001; Peng, 2004).

1.5 $GABA_A$R pharmacology

GABA binds the $GABA_A$R at the orthosteric binding site in the α-β interface of the extracellular domain (Baumann et al., 2002). Other ligands that bind the orthosteric site are muscimol, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol(THIP) and bicuculine (Curtis, Duggan, Felix, & Johnston, 1970; Johnston, Curtis, de Groat, & Duggan, 1968; P. Krogsgaard-Larsen, Johnston, Lodge, & Curtis, 1977). Both muscimol and THIP are GABA agonists (Johnston et al., 1968; P. Krogsgaard-Larsen et al., 1977). Muscimol is a conformationally restricted GABA analogue that is a potent agonist at both synaptic and extrasynaptic $GABA_A$Rs(Ebert et al., 1997; Johnston et al., 1968). However, muscimol demonstrates full agonism (compared to GABA) at α4β3δ receptors (Mortensen Martin, Ebert Bjarke, Wafford Keith, & Smart Trevor G., 2010). THIP, also known as gaboxadol, also demonstrates high potency at α4β3δ receptors (Povl Krogsgaard-Larsen, Frølund, Liljefors, & Ebert, 2004). Bicuculline is an antagonist of the $GABA_A$R, competing with GABA for the orthosteric binding site (Curtis et al., 1970). Alternatively, picrotoxin is a noncompetitive antagonist and inhibits $GABA_A$Rs by binding within the channel pore (Squires, Saederup, Crawley, Skolnick, & Paul, 1984; Xu, Covey, & Akabas, 1995).

1.5.1 Allosteric Modulators of $GABA_A$Rs

The subtype specificity of $GABA_A$Rs dictates the binding of various allosteric modulators. Positive allosteric modulators of the $GABA_A$R were identified through their physiological and pharmacological properties, mutagenesis of residues critical for binding and or potentiation, and by radio-affinity labeling using unlabeled agonists and antagonists (Neil L. Harrison & Simmonds, 1984; Olsen, 2015). Benzodiazepines, ethanol and pyrazoloquinolines all bind in the extracellular domain of the $GABA_A$R, whereas etomidate, propofol, octanol, barbiturates, volatiles and neurosteroids bind in transmembrane domains (see Olsen, 2015 for full review).

Benzodiazepines were one of the first discovered positive allosteric modulators of the $GABA_A$R, shown to have anxiolytic effects by enhancing GABAergic transmission through increasing the affinity for GABA (Mohler & Okada, 1977). Benzodiazepines bind between the α-γ interface and potentiate $GABA_A$ currents by increasing the frequency of $GABA_A$R channel openings and the mean open time of channels (Study & Barker, 1981). Barbiturates are compounds that also potentiate GABA responses through enhancing channel open time while however, reducing the frequency of openings (Study & Barker, 1981). In cerebellar granule cells that highly express α6β3δ extrasynaptic receptors, "behaviorally-relevant" ethanol concentrations (~15 mM) significantly enhanced tonic inhibition (Hanchar, Dodson, Olsen, Otis, & Wallner, 2005).

Neurosteroids are *endogenous* allosteric modulators of the $GABA_A$R that can both potentiate and inhibit GABA-mediated responses (E.E. Baulieu & Robel, 1990). Potentiating neurosteroids such as allopregnanolone and THDOC bind near the lipid interface of α-β transmembrane domains, where inhibitory neurosteroids such as dehydroepiandrosterone sulfate (DHEAS) and pregnenolone sulfate are thought to bind within a subunit TM (Hosie,

Wilkins, Silva, & Smart, 2006; Laverty et al., 2017; Majewska, Demirgo¨ren, Spivak, & London, 1990; Majewska, Harrison, Schwartz, Barker, & Paul, 1986; Majewska, Mienville, & Vicini, 1988). Hosie et al., (2006) first identified the Q241 residue in the α subunit as a critical site for the allosteric potentiation by neurosteroids (Hosie et al., 2006). Potentiating neurosteroids enhance GABAergic inhibition through augmenting channel mean open time which results in prolonging the decay of the IPSC and increasing tonic current (Haage & Johansson, 1999). Nanomolar concentrations of neurosteroids potentiate $GABA_A$ currents through allosteric modulation, whereas, micromolar concentrations of neurosteroids can directly activate $GABA_A$Rs (Majewska et al., 1986). Neurosteroids potentiate both phasic and tonic current, however $GABA_A$Rs containing the δ subunit are particularly sensitive to lower concentrations of neurosteroids (Belelli et al., 2009; Belelli & Lambert, 2005; N. L. Harrison, Vicini, & Barker, 1987).

1.6 Neurosteroids

1.6.1 Neurosteroid synthesis and function

Steroids are synthesized from cholesterol in the ovaries (progesterone), testes (testosterone) and kidneys (aldosterone and cortisol) (Doodipala Samba Reddy, 2010). These steroids from the periphery can then enter the brain due to their lipophilic nature and exert their effects on steroid receptors that regulate transcription of genes (Evans, 1988; Selye, 1941). However, Baulieu and Robel (1990) demonstrated that the brain itself contains steroidogenic enzymes to convert steroid precursors into active metabolites. Thus, Baulieu and Robel termed steroids that can be synthesized within the brain, "neurosteroids" (E.E. Baulieu & Robel, 1990). These steroids can be categorized into three groups based on their

structure; pregnane neurosteroids, androstane neurosteroids, and sulfated neurosteroids (Doodipala Samba Reddy, 2010). Although classical steroid signaling occurs through transcriptional changes that typically occur over a time scale of hours to days, steroids can also mediate effects through neurotransmitter receptors in the brain within milliseconds to minutes (Paul & Purdy, 1992). Paul and Purdy termed these steroids as "neuroactive." Providing an understanding of the original work of Hans Selye in the early 1940's, Harrison and Simmons (1984) demonstrated that the sedative, hypnotic and anxiolytic effects of specific steroids are mediated via the GABAergic system (Neil L. Harrison & Simmonds, 1984). Neurosteroids are synthesized from either progesterone, deoxycorticosterone or testosterone via enzymatic reduction of their A-rings by 5α-reductase and 3α-hydroxysteroid dehydrogenase (3α-HSD) to yield allopregnanolone (3α,5α-THP), allotetrahydrodeoxycorticosterone (THDOC) and 3α-androstanediol (3α-diol), respectively (Carver & Reddy, 2013). The parent molecules progesterone and deoxycorticosterone, are derived from enzymatic conversion of pregnenolone via 3β hydroxysteroid dehydrogenase (Carver & Reddy, 2013). Pregnenolone is synthesized from cholesterol by cytochrome P450 in mitochondrial membranes (Lambeth, Seybert, Lancaster, Salerno, & Kamin, 1982; Larroque, Rousseau, & van Lier, 1981). Cholesterol is mainly synthesized in the liver and its metabolites can cross the blood brain barrier, however the brain can also synthesize its own cholesterol de novo (Dietschy & Turley, 2001). Much of the cholesterol the brain synthesizes is used for myelin and construction of plasma membranes (Chaves, Rusiñol, Vance, Campenot, & Vance, 1997; Snipes & Suter, 1997). 5α-reductase and 3α-HSD are widely expressed throughout the brain including regions such as cortex, hippocampus, olfactory bulb, striatum, thalamus, amygdala and cerebellum (Agís-Balboa et al., 2006). Both neurons and glia express steroidogenic enzymes, such as cytochrome P450, 3β-HSD, 5α-reductase

and 3α-hydroxysteroid dehydrogenase (Agís-Balboa et al., 2006; Compagnone & Mellon, 2000; Tsutsui, Ukena, Usui, Sakamoto, & Takase, 2000). Although most neurosteroid production seems to originate from neuronal populations, neurosteroid synthesis has also been found in glial cells (Agís-Balboa et al., 2006; E. E. Baulieu & Robel, 1991). Nevertheless, glutamatergic principal neurons show robust expression of steroidogenic enzymes, but no expression was detected in hippocampal and cortical GABAergic interneurons (Agís-Balboa et al., 2006).

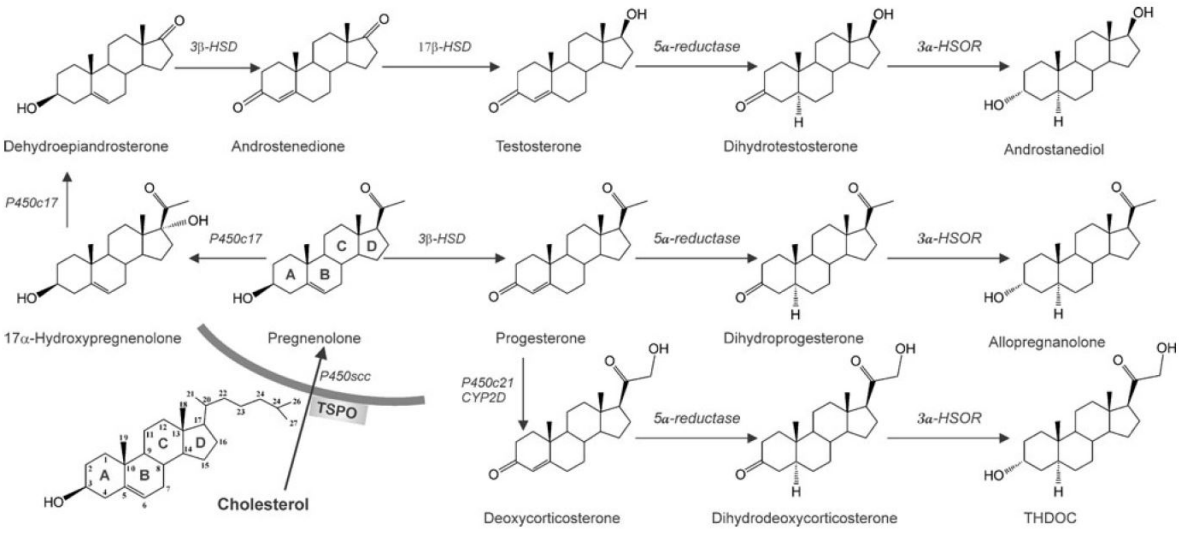

Figure 1.1
Neurosteroid synthetic pathways
Reprinted with permission from Carver, C. M., & Reddy, D. S. (2013). Neurosteroid interactions with synaptic and extrasynaptic $GABA_A$ receptors: regulation of subunit plasticity, phasic and tonic inhibition, and neuronal network excitability. *Psychopharmacology*, *230*(2), 151–188. https://doi.org/10.1007/s00213-013-3276-5

1.6.2 Physiological regulation of neurosteroids

Endogenous levels of neurosteroids are regulated in different physiological conditions including development, puberty, pregnancy, postpartum, stress and depression (Barnea, Hajibeigi, Trant, & Mason, 1990; Eser et al., 2006; J. Maguire & Mody, 2009; Purdy et al., 1991; Shen et al., 2010).

1.6.2.1 Stress

Purdy et al., (1991) were the first to quantify increases in brain steroid levels after acute stress. Particularly, they showed that levels of allopregnanolone and THDOC are enhanced in the cerebral cortex and plasma in male rats during swim stress (Purdy et al., 1991). The modulation of steroid levels during stress can also impact the GABAergic system; as seen with mice given a hypoxic challenge, in which δ- mediated tonic current in the dentate gyrus was enhanced (J. Maguire & Mody, 2007). Furthermore, stress can also elicit changes in $GABA_A$ mRNA and protein expression, such as in β1 and β3 subunits, (Cullinan & Wolfe, 2000; Orchinik, Weiland, & McEwen, 1995).

1.6.2.2 Pregnancy and post-partum

It is widely known that the steroid progesterone is essential for the maintenance of pregnancy. In addition, the metabolites of progesterone, allopregnanolone and THDOC, are also elevated during pregnancy (Concas et al., 1998). The elevation in neurosteroid levels are

correlated with a decrease in γ2 and δ containing $GABA_A$Rs in the dentate gyrus and a decrease in tonic and phasic current (J. Maguire, Ferando, Simonsen, & Mody, 2009; J. Maguire & Mody, 2008). Neurosteroid levels drop and GABAergic inhibition is restored after pregnancy and accompanied by an upregulation of δ subunits (J. Maguire & Mody, 2008). It is thought that the decline in neurosteroid levels and failure to upregulate δ subunit expression in the post-partum period may contribute to the depressive phenotypes of postpartum depression (Concas et al., 1998; J. Maguire & Mody, 2008). Furthermore, δ-knockout mice display abnormal maternal behavior demonstrated by improper construction of nests, dispersion of pups and increases in cannibalized offspring (J. Maguire & Mody, 2008). Enhancing neurosteroid levels in patients suffering from post-partum depression seems to be efficacious in relieving symptoms (Kanes et al., 2017). Surprisingly, a one-time infusion of brexanolone (synthetic allopregnanolone) resulted in long-lasting effects in patients scored on the Hamilton Depression Rating Scale (HAM-D) with scores improving 50% or greater compared to the placebo group at all time points up to 30 days (Kanes et al., 2017).

1.6.2.3 Depression

It has been shown that allopregnanolone levels are reduced in patients with major depression, but THDOC levels are enhanced (Romeo et al., 1998; Ströhle et al., 1999). Furthermore, antidepressant treatment with selective serotonin reuptake inhibitors (SSRIs) restored allopregnanolone levels, but levels of THDOC were unaffected by treatment (Ströhle et al., 1999). SSRIs are commonly prescribed anti-depressants, and although their mechanism of action is unclear, it is known that they can enhance neurosteroid synthesis

independent of their serotonin reuptake action (Pinna, Costa, & Guidotti, 2009). Thus, neurosteroids hold therapeutic potential in treating depression.

1.6.3 Metabotropic effects of neurosteroids on $GABA_A$Rs

In addition to allosterically potentiating $GABA_A$Rs, neurosteroids can independently activate metabotropic signaling to modulate $GABA_A$R expression, phosphorylation and receptor dynamics at the plasma membrane (Abramian et al., 2014; Adams, Thomas, & Smart, 2015; J. Maguire & Mody, 2007; Modgil et al., 2017a). For example, estradiol, a neurosteroid synthesized from androstenedione, can affect the clustering of synaptic $GABA_A$Rs at the plasma membrane in cultured cortical neurons (Mukherjee et al., 2017). Estradiol treatment reduces α2- containing $GABA_A$Rs clustered with post-synaptic scaffold protein gephyrin at synaptic sites, resulting in a reduction in the efficacy of synaptic inhibition (Mukherjee et al., 2017). These findings suggest that estradiol may affect the dynamics of gephyrin or $GABA_A$Rs at the synapse, possibly through phosphorylation mechanisms (Mukherjee et al., 2017). In addition to phospho-regulation of $GABA_A$R subunits, gephyrin also contains many phosphorylation sites within its C-domain (Herweg & Schwarz, 2012).

Neurosteroids can also mediate changes in the total protein level of $GABA_A$R subunits (Gulinello et al., 2001; J. Maguire & Mody, 2007). Maguire et al. (2005) demonstrated that steroid fluctuations during the ovarian cycle in late diestrus leads to an upregulation of δ -containing $GABA_A$ and an enhancement of tonic current within the dentate gyrus (J. L. Maguire, Stell, Rafizadeh, & Mody, 2005). In lieu with estrous cycle regulation of neurosteroid levels, chronic progesterone treatment in mice also led to enhancements in extrasynaptic $GABA_A$ α4 and δ subunit expression (Gulinello et al., 2001; J.

Maguire & Mody, 2007). Thirty minutes of THDOC treatment in hippocampal slices was also sufficient to increase δ subunit expression and exhibit an enhanced tonic current up to 3 hours following the 30 min treatment (J. Maguire & Mody, 2007).

The function of neurosteroids to mediate phosphorylation of $GABA_AR$ subunits was first demonstrated by Abramian et al. who showed that neurosteroid THDOC enhances phosphorylation of the α4 subunit and leads to the insertion of α4-containing receptors into the plasma membrane. This effect was not occluded by mutation of Q241, a residue critical for the potentiating effects of neurosteroids (Abramian et al., 2014). This suggests a role for neurosteroids independent from its allosteric modulation. Metabotropic effects were also demonstrated by Adams et al. (2015) who showed that THDOC can mediate phosphorylation of the β3 subunit at serine residues 408 and 409. In addition to THDOC, 20 minute treatment with allopregnanolone and SAGE-516 (synthetic analogue of allopregnanolone) also enhanced $\beta3^{S408/409}$ phosphorylation in the hippocampus (Modgil et al., 2017). Furthermore, allopregnanolone also enhanced β3-containing receptors at the plasma membrane. The increase in receptor number was consistent with enhancements in tonic current but not phasic current within the hippocampus. Although β3 subunits are present in both synaptic and extrasynaptic subtypes, an exclusive enhancement in tonic current suggested that allopregnanolone preferentially modulates extrasynaptic type receptors (Modgil et al., 2017).

1.6.4 Neurosteroid effects on membrane progesterone receptors

Membrane progesterone receptors (mPRs) were identified in 2003 as the long sought-after receptors of progesterone that could explain many of the non-genomic effects that have been well described throughout the literature (Moussatche & Lyons, 2012; Singh,

Su, & Ng, 2013; Zhu, Rice, Pang, Pace, & Thomas, 2003). mPRs are G-protein-like receptors that are expressed in a variety of tissue including brain, lung, liver, kidneys, ovaries and testes (Kasubuchi et al., 2017; Zhu, Bond, & Thomas, 2003). They are activated by their respective progestins that are endogenous to cell type (Thomas, 2008). For example, the endogenous progestins progesterone, 20β-S and 17,20βDHP demonstrate the highest affinity for human, seatrout and zebrafish mPRα, respectively (Thomas, 2008). In humans, allopregnanolone also shows affinity for mPRα, although at 7.6% binding affinity of progesterone (Kelder et al., 2010). However, the mPRα synthetic agonist 10-ethenyl-19-norprogesterone (ORG OD 02-0), has a greater binding affinity than both progesterone and allopregnanolone (Kelder et al., 2010).

1.6.4.1 Characterization of mPRs

mPRs were first cloned from fish oocytes, where Zhu et al., (2003) found that these ~40 kDa proteins showed a topology of seven transmembrane domains, indicative of G protein coupled receptors (Zhu, Rice, et al., 2003). These receptors were eventually categorized under the class II Progestin and AdipoQ Receptor family (PAQR) (Tang et al., 2005). Class I and Class III PAQRs do not respond to progesterone (Smith et al., 2008). Five subtypes of mPRs were identified, with mPRα, β, γ characterized first, followed by δ and ε (Pang, Dong, & Thomas, 2013; Smith et al., 2008; Zhu, Bond, et al., 2003; Zhu, Rice, et al., 2003). All mPR subtypes seem to be expressed in the human brain, quantified by mRNA levels (Pang et al., 2013). mPRα mRNA is found in a variety of tissues including lung, kidney, testis, ovary and brain in both invertebrate and vertebrate animals (Kasubuchi et al., 2017; Zhu, Bond, et al., 2003). In the mouse brain, mPRα protein has been identified in the cortex,

hypothalamus, hippocampus and striatum and mainly in neuronal populations (Meffre et al., 2013).

1.6.4.2 Function of mPRs

Many studies demonstrate that mPRs function via coupling to G proteins (Pang & Thomas, 2010; Sleiter et al., 2009; Thomas et al., 2007; Tubbs & Thomas, 2009). Other studies however, have identified G protein-independent effects as well (Pang et al., 2013). mPRα couples to G_i proteins in seatrout and breast cancer cells and G_{olf} proteins in fish sperm to inhibit and activate cAMP levels, respectively (Thomas, 2008; Thomas et al., 2007; Tubbs & Thomas, 2009). mPRβ has also been shown to interact with $G_{i/o}$ proteins, however mPRγ interaction with G proteins has not been well characterized (Karteris et al., 2006). mPR δ and ε couple to G_s proteins and enhance cAMP levels in human breast adenocarcinoma cells (MDA-MB-231) (Pang et al., 2013). In addition to G protein activation via mPRs, others have also identified mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) signaling downstream of mPR activation (Kasubuchi et al., 2017; Kelder et al., 2010; Pang, Dong, & Thomas, 2015; Salazar, Lerma-Ortiz, Hooks, Ashley, & Ashley, 2016; Tan & Thomas, 2014). MAPK and AKT pathways can be activated through the βγ dimer that dissociates from the inhibitory G protein via mPRα activation (Crespo, Xu, Simonds, & Gutkind, 1994; Leopoldt et al., 1998; Pace & Thomas, 2005). Although Kasubuchi et al., demonstrate mPR activation in pheochromocytoma of rat adrenal medulla cells (PC12) only increases extracellular signal-regulated kinase (ERK) phosphorylation with no G protein activation, another study shows both MAPK signaling and G protein activation via mPRs in MDA-MB-231 cells (Kasubuchi et al., 2017; Pang et al., 2013). Taken

together, mPRs can activate a variety of downstream signaling pathways however, in a cell type specific manner.

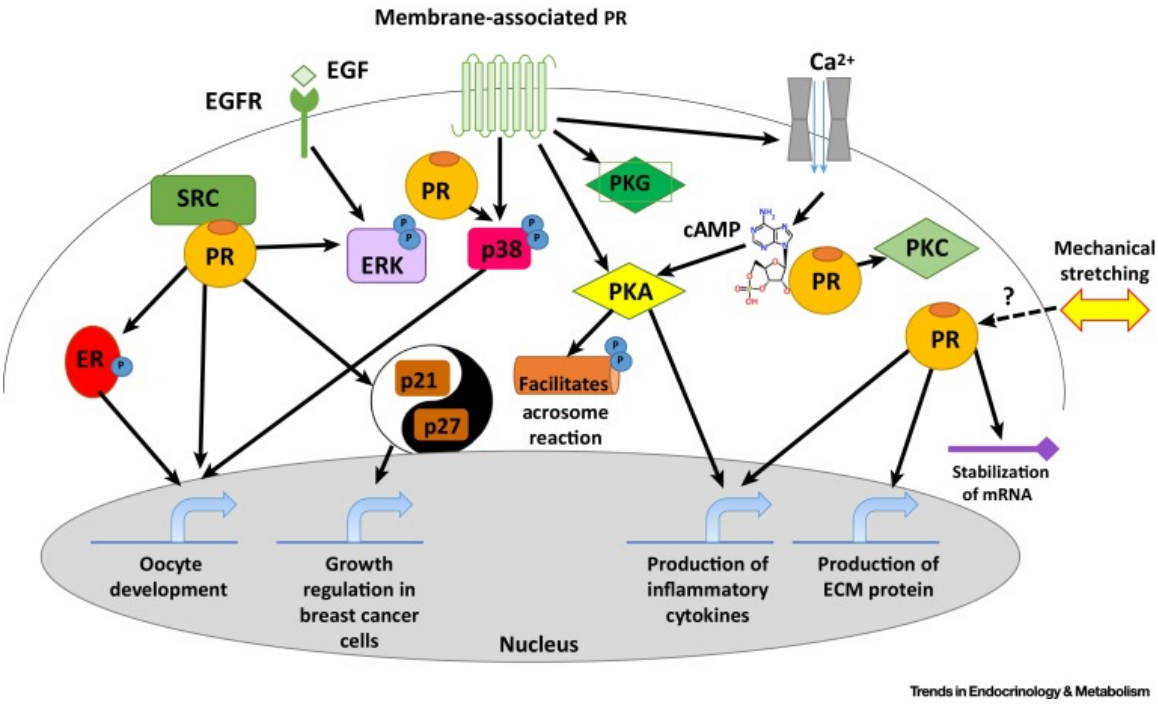

Figure 1.2
Membrane progesterone receptor signaling pathways
Reprinted with permission from Garg, D., Ng, S. S. M., Baig, K. M., Driggers, P., & Segars, J. (2017). Progesterone-Mediated Non-Classical Signaling. *Trends in Endocrinology & Metabolism*, *28*(9), 656–668. https://doi.org/10.1016/j.tem.2017.05.006

1.6.4.3 Subcellular localization of mPRs

The controversy over mPR coupling to G proteins is further debated due to the inconsistency in current literature over this protein's subcellular localization. Zhu et al., 2003 demonstrate that mPRα is localized to plasma membranes in oocytes, follicle cells, sperm and MDA-MB-231 cells (Zhu, Rice, et al., 2003). More definitive evidence comes from Foster et al. (2010) who used immunogold labeling in electron microscopy to show

membrane localization of mPRα in human myometrial cells (M11) (Foster et al., 2010). However, in other cell types such as human embryonic kidney (HEK293), African green monkey kidney (COS-1) and human endometrial adenocarcinoma (Ishikawa), mPRα was found in intracellularly or in the ER (Krietsch et al., 2006; Lemale et al., 2008). Taken together, the discrepancies in the data can be explained by potential retention motifs in the C-terminus of mPRα (Lemale et al., 2008). Localization may be dependent on lysine 343 in the C-terminus, where when mutated allows mPRα to exit the ER. Furthermore, it is thought that adaptor proteins such as progesterone receptor membrane component 1 (PGRMC1), may mask this motif and allow translocation of mPRα to the plasma membrane (Lemale et al., 2008). Thomas et al., (2014) demonstrated that stably transfected PGRMC1 enhances mPRα expression in MDA-MB-231 cells at the plasma membrane (Thomas, Pang, & Dong, 2014). Taken together, mPR expression on the plasma membrane is dependent on cell type and may require the help of adaptor proteins.

1.6.4.4 Physiological roles of mPRs

mPR signaling was first investigated in reproductive tissues, where activation of mPRs has been implicated in oocyte maturation and sperm motility (Roy, Wang, Rana, Nakashima, & Tokumoto, 2017; Tokumoto, Nagahama, Thomas, & Tokumoto, 2006; Tubbs & Thomas, 2009). Estrogen signaling in zebrafish oocytes increases cAMP levels which arrests cells at the G2/M phase and prevents their maturation (Pang & Thomas, 2010). However, it is thought that mPRα activation via progesterone signaling removes the block on maturation via inhibiting cAMP levels (Pang & Thomas, 2010). 20-β S activation of mPRs in Atlanta Croaker sperm increase cAMP levels and induce motility of sperm (Tubbs

& Thomas, 2009). mPRs also play a role in cell proliferation, where mPRα mRNA was found to be upregulated in breast cancer tissue and mPRα protein promoted survival of breast cancer cells through anti-apoptotic mechanisms (Dressing, Alyea, Pang, & Thomas, 2012; Dressing, Goldberg, Charles, Schwertfeger, & Lange, 2011). Knockdown of mPRα with siRNA prevented the decrease in cell death seen with progesterone treatment (Dressing et al., 2012). And more recently, mPRs have also been shown to play a role in neuroprotection in the brain after traumatic brain injury (Guennoun et al., 2015). The protective effects of progesterone are thought to be partly mediated by mPRα—there is an upregulation of mPRα in glia including oligodendrocytes, astrocytes and reactive glia and a downregulation of mPRα in neurons (Meffre et al., 2013). The adaptor protein PGRMC1, has been shown to play a more direct role in traumatic brain injury (TBI), by promoting neuroprogenitor cell proliferation, axon growth and survival of neurons (Liu et al., 2009; Sakamoto et al., 2004). mPR signaling in the brain is largely unknown, thus more studies need to be done to investigate its role in physiological functions and in disease.

Chapter 2: Materials and Methods

2.1 Animals

C57BL/6J mice (The Jackson Laboratory) were housed under constant temperature and humidity on a 12-h light/dark cycle with standard rodent food and water *ad libitum*. Initially, 3-5 week old male mice were used for electrophysiology studies investigating the therapeutic potential of neurosteroids in neurodevelopmental disorders. Later, 8-12 week old male mice were used in biochemistry studies to investigate the therapeutic potential of neurosteroids as anti-epileptic drugs in adult animals. All animal protocols were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Institutional Animal Care and Use Committee of Tufts University.

2.2 Cell Culture

2.2.1 HEK293T cells

HEK-293T cells from ATCC were passaged in DMEM 1X +4.5g/L D-Glucose +L-Glutamine (Thermo Fisher Scientific, catalog no. 11964-092) with 10% Fetal Bovine Serum (FBS) (Fisher, catalog no.SH3007003HI) from P0-P22.

2.2.2 GT1-7 cells

GT1-7 cells were generously provided by the Guennoun lab at Institut National de la Santé et de la Recherche Médicale (INSERM) Paris, France. GT1-7 cells were passaged in DMEM 1X +4.5g/L +L-Glutamine + sodium pyruvate (Corning, catalog no. 10-013-CV) with 10% FBS (Gemini Bio-Products, catalog no. 900-108) and 1% penicillin/streptomycin antibiotic (Thermo Fisher Scientific, catalog no.15140-122) from P7-P20.

2.3 DNA

2.3.1 Sequencing of Constructs

Table 2.1 Constructs & Primers

DNA	Vector	Source	Primers used to validate sequences
α4	pEYFP-N1	Moss lab	**Primer 1:** 5'-GTTTCTGTCCAGAAGG-3' **Primer 2:** 5'-GAGAAATGGCACTAT-3' **Primer 3:** 5'-GTTTGCTGCTGTCAA-3' **Reverse primer 1:** 5'-ATCAGAAACGGGTCC-3'
β3	pEYFP-N1	Moss lab	**Sp6:** 5'-ATTTAGGTGACACTATAGA-3' **Reverse primer 1:** 5'-CTTCAACCGAAAACTC-3' **Reverse primer 2:** 5'-ACAAAGGCGTACTCC 3'
mPRα-MYC-FLAG	pCMV6	Origene catalog no. MR229384	**T7:** 5'-TAATACGACTCACTATAGG-3' **XL39:** 5'-TAGGACAAGGCTGGTGG-3'
Empty plasmid	pRK5	Addgene	**N/A**

α4 and β3 cDNA was transfected into HEK-293T cells to study the effects of neurosteroids on extrasynaptic type $GABA_A$Rs. (Mortensen Martin & Smart Trevor G., 2006b).

2.3.2 Maxipreps

1-2μL of DNA was transformed into 20μL of DH5α (Thermo Fisher Scientific catalog no. 18258012) and incubated in 100-200μL SOC media for one hour before plating onto ampicillin or kanamycin agar plates. One single colony was chosen per plate and grown in 2

mLs of LB with antibiotic (1:1000 ampicillin or 1:2000 kanamycin) in a shaking incubator for 6-8 hours. The 2 mLs of bacterial culture was then transferred to a larger culture (in 300mLs LB with antibiotic) overnight. Bacteria was pelleted at 6,000G for 15 minutes and plasmid purification was carried out with Qiagen Plasmid Maxi Kit (Qiagen catalog no. 12163). DNA concentration was measured using a Nanodrop 1000 (Thermo Fisher Scientific).

2.4 Transfection

2.4.1 Biochemistry and immunocytochemistry experiments

600,000 HEK-293T cells were plated in a 6-well plate 24 hours before transfection for biochemistry experiments. Three micrograms total DNA was transfected per well containing the following constructs: 1.0μg α4, 1.0μg β3 and 1.0μg mPRα (or 1.0μg empty pRK5) using FUGENE HD Transfection Reagent (Promega, catalog no.E2311) (1:3 DNA to FUGENE in 100μL of media).

50,000 HEK-293T cells were plated on poly-L-lysine treated coverslips the day before transfection for immunocytochemistry experiments. 0.75μg total DNA was transfected per coverslip containing the following constructs: 0.25μg a4, 0.25μg β3, 0.25μg mPRα using FUGENE HD Transfection Reagent (1:3 DNA to FUGENE in 100μL of media).

2.4.2 Electrophysiology experiments

1x10^6 – 1.5x10^6 HEK-293T cells were electroporated with 4 μg total DNA containing the following constructs: 1.0μg α4, 1.0μg β3, 1.0μg mPRα and 1.0μg GFP. Cells were electroporated with a single 25.0ms pulse at 110V in 2mm cuvettes using Gene pulser xcell

(Bio-rad, catalog no. 1652660). 15,000 HEK-293T cells were plated into 35mm dishes for experiments.

2.5 Drugs & Reagents

Table 2.2 Drugs & Reagents

Drug	**Source**	**Concentration**
Allopregnanolone	Tocris catalog no. 3653	100nM
SAGE-516	SAGE Therapeutics	100nM
Progesterone	Tocris catalog no. 2835	100nM
ORG OD 02-0	Axon Medchem 2085	300nM
GF 109203X or GFX	Tocris catalog no. 0741	10uM
KT 5720	Tocris catalog no. 1288	1uM
DMSO	Sigma catalog no. 67-68-5	N/A

2.6 Antibodies

Table 2.3 Primary Antibodies

Primary Antibody (IgG)	**Source**	**Dilution**
Polyclonal anti-phospho Ser408/409 $GABA_A$ β3	Cocalico Biologicals antisera no. UP 2030	1:5000-1:10,000
Polyclonal anti-$GABA_A$ β3	PhosphoSolutions catalog no. 863-GB3C	1:1000
Polyclonal anti-α4	Cocalico Biologicals antisera no. T114	1:10,000
Monoclonal anti-$GABA_A$ β2,3 clone BD17	Millipore catalog no. MAB341	1:100
Monoclonal anti-transferrin	Thermo Fisher Scientific catalog no. 13-6800	1:1000
Monoclonal anti-DDK (FLAG)	Origene Catalog no. TA50011-100	1:4000
Monoclonal anti-β-actin	Sigma catalog no. A1978	1:25,000
Polyclonal anti-mprα	Guennoun lab INSERM, France	1:30,000

Table 2.4 Secondary Antibodies

Secondary antibody (IgG)	**Source**	**Dilution**
Polyclonal Peroxidase AffiniPure Goat Anti-Rabbit	Jackson Immunoresearch 111-035-144	1:7000
Polyclonal Peroxidase AffiniPure Goat Anti-Mouse	Jackson Immunoresearch 115-035-003	1:7000-12:000
Polyclonal Goat Anti-Mouse IgG (H+L), Alexa Fluor 488	Thermo Fisher Scientific Catalog no. R37120	1:500
Polyclonal Goat Anti-Mouse IgG (H+L), Alexa Fluor 568	Thermo Fisher Scientific Catalog no. A-11004	1:500

2.7 *In vitro* brain slice preparation for biochemistry

2.7.1 Acute hippocampal slices

Acute hippocampal slices from 8-10 week old C57BL/6J male mice were prepared by first immersing brains in ice-cold sucrose-based cutting solution containing (in mM): 87 NaCl, 3 KCl, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 7 $MgCl_2$, 50 sucrose, 25 D-glucose, and 25 $NaHCO_3$ equilibrated with 95% O_2 and 5% CO_2. Then 350 μm coronal hippocampal slices were sectioned using a Leica vibratome and the hippocampus was dissected out of the slice. For experiments using dentate gyrus slices, the dentate gyrus (including the molecular layer) was dissected out of the hippocampus. Dissected slices were then allowed to recover for 1 hour in normal artificial cerebrospinal fluid (nACSF) containing in (mM) 126 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 D-glucose, 1.5 pyruvate, 1 L-glutamine, (300-310 mOsm) at 31°C. After recovery, slices were treated with either control (DMSO) or neurosteroids in oxygenated nACSF for 20 minutes in bacterial centrifuge tubes (Carolina Biological Supply Company, catalog no. 215088). For kinase inhibitor experiments, slices were pretreated for 10 minutes with GFX and KT 5720 followed by treatment with neurosteroids for 10 minutes as described above.

2.8 Immunocytochemistry

2.8.1 Surface staining

Coverslips with transfected HEK-293T cells were treated with ORG OD 02-0 for 5 minutes and fixed in 4%PFA/PBS for 20 minutes. PFA was quenched with 0.1M glycine for 5 minutes and washed 3x with 1X PBS. Surface and intracellular staining was then followed as in Connolly et al., 1999 using anti-$GABA_A$ β2,3 clone BD17 antibody (Millipore) (Christopher N. Connolly et al., 1999).

2.8.2 Image analysis of surface stained HEK-293T cells

The outline of cells was drawn using the drawing tool in Image J and the average density of fluorescence for each channel (emission 488 nm and 568 nm) was determined. Surface levels were quantified as follows: (surface fluorescence)/ (surface fluorescence+ intracellular fluorescence). Surface levels were normalized to those in control treated cells. (Christopher N. Connolly et al., 1999)

2.9 Protein Biochemistry

2.9.1 Lysate Preparation

2.9.1.1 Lysate preparation for hippocampal slices

Slices treated with neurosteroids were snap frozen and stored at -80°C in Eppendorf tubes. The following day, slices were briefly thawed on ice and homogenized using a hand-held motorized tissue grinder (Fisher, catalog no. 12-1413-61) in lysis buffer containing the following in mM: 20 Tris-Cl pH 8.0, 150 NaCl, 5 EDTA pH 8.0, 10 NaF, 2 sodium

orthovanadate, 10 sodium pyrophosphate, 0.1%SDS. 2% Triton-X (v/v) was added to the samples before solubilizing on a rotating wheel at 4°C for at least 2 hours.

2.9.1.2 Lysate preparation for HEK-293T and GT1-7 cells

Cells were solubilized in lysis buffer containing the following in mM: 20 Tris-Cl pH 8.0, 150 NaCl, 5 EDTA pH 8.0, 10 NaF, 2 sodium orthovanadate, 10 sodium pyrophosphate and 1% Triton-X on a rotating wheel at 4°C for one hour. Samples were centrifuged at 15,000 RPM for 15 minutes and protein concentration was determined using the Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, catalog no. 5000006) and reading absorbance at 595nm on a Smart spec plus spectrophotometer (Bio-rad, catalog no. 1702525). Samples were boiled in 4X sample buffer containing the following: 24 mLs 1M Tris pH 6.8, 28mL diH_20, 8g SDS, 40mL glycerol, 0.009g BPB, 10% 2-mercaptoethanol for 1-3 minutes at 95°C.

2.9.2 Western Blotting

2.9.2.1 SDS-PAGE

Twenty-five micrograms of protein was loaded on 8-10% SDS-polyacrylamide gels and run at 100V in 1X running buffer (25mM Tris, 192mM glycine,1%SDS) for 1.5 hours in a vertical electrophoresis cell (Mini Protean tetra vertical electrophoresis cell, Bio-rad, catalog no. 1658004).

2.9.2.2 Wet Transfer

Protein was transferred onto nitrocellulose membranes (Bio-rad, catalog no. 1620115) using transfer tanks (Mini Trans-Blot Electrophoretic Transfer Cell, Bio-rad, catalog no. 1703930) in 1X transfer buffer (48mM Tris, 39mM glycine,0.37%SDS) at 45 mA overnight at 4°C. Membranes were stained with Ponceau for 5 seconds and then washed twice with 1X PBS-T(0.1% Tween).

2.9.2.3 Blocking

Membranes were incubated with 6% milk in PBS-T for all antibodies except anti-phospho Ser408/409 $GABA_A$ β3, for which 6% BSA in PBS-T was used.

2.9.2.4 Incubation with Antibodies

Blots were incubated with primary antibodies (containing 1:1000 of 20% sodium azide) in blocking solution on a shaker overnight at 4°C. Blots were washed 3x for 10 minutes each in PBS-T before being incubated in secondary antibodies in blocking solution for 1.5 hours.

2.9.2.5 Developing Blots

Blots were washed three times for 5 minutes in PBS-T before being incubated in SuperSignal West Dura Extended Duration Substrate ECL (Thermofisher, catalog no. 34075) for 30seconds to 1 minute and developed using chemiluminescence on the CCD-based ChemiDoc XRS system (catalog no. 1708265).

2.9.3 Biotinylation

Cells or slices were incubated on ice for 20-30 minutes with 1mg/mL EZ-link Sulfo-NHS-SS-Biotin(Thermo Fisher Scientific, catalog no. 21331) in 1X PBS or nACSF, respectively. Excess biotin was quenched in 2 mLs of 50mM glycine and 0.1%BSA in PBS/nACSF three times, followed by three washes in ice-cold PBS or nACSF. Cell and tissue lysates were prepared as described above. 350-500ug of protein lysate were incubated with Pierce NeutrAvidin Agarose (Thermo Fisher Scientific, catalog no. 29200) for 18-24 hours at 4°C. Agarose was washed twice with 150mM NaCl 1% Triton lysis buffer, twice with 500mM NaCl 1%Triton lysis buffer, followed by a final wash with 150mM NaCl 1%Triton lysis buffer. Bound material was eluted with sample buffer and subjected to SDS-PAGE and then immunoblotted with antibodies as described above.

2.9.4 In vitro p32 kinase assay

Acute hippocampal slices were prepared and treated with neurosteroids as described above, or neurosteroids were directly added to immunoprecipitated protein on beads. Slices were lysed in lysis buffer described above used for HEK-293T and GT1-7 cells and solubilized on a rotating wheel at 4°C for one hour. The β3 subunit was immunoprecipitated with anti-$GABA_A$ β3 (PhosphoSolutions) overnight followed by incubation with Protein A-Sepharose beads(VWR, catalog no. 95016-902) on a rotating wheel for 2 hours at 4°C. Beads were washed 3x with lysis buffer containing in mM : 150 NaCl, 10 triethanolamine, 5 EGTA, 5 EDTA, 50 NaF, 10 sodium pyrophosphate, 1 sodium orthovanadate and 3x with kinase buffer containing in mM: 20 Tris pH 7.4, 20 $MgCl_2$, 1 EDTA, 1EGTA, 1 sodium orthovanadate, 0.1 DTT, 2 $MnCl_2$. 0.1mCi/uL ATP [γ-^{32}P] was added to beads together

with 0.5mM cold ATP in kinase buffer with either 1mM GFX or DMSO for 30 minutes at 30°C. Samples were centrifuged at 2200 RPM and beads were boiled in in 2X sample buffer for 3 minutes at 100°C. Supernatant was run on an 8% SDS-polyacrylamide gel and the gel was fixed for one hour in ratio of (50 ethanol: 3 o-phosphoric acid:47 diH_2O) followed by three 5 minute washes in diH_2O. The gel was then washed for one hour in Neuhoff reagent (10% o-phosphoric acid, 10%ammonium sulphate) followed by colloidal coomassie stain: (10% o-phosphoric acid, 10% ammonium sulphate, 0.12% coomassie brilliant blue G250, 25% methanol added fresh) overnight. Gels were destained in 1% acetic acid/diH20 for 2 hours and then dried for 2 hours using a gel dryer (Bio-rad catalog no. 1651745) Gels were exposed to film in radiography cassettes at -80°C for 1-5 days. Film was developed using a mini-medical developer (AFP imaging catalog no. 9992305300-U) and imaged on ChemiDoc XRS.

2.9.5 Quantification of immunoblots

Blots were quantified using densitometric analysis on Image J. Phospho-protein and total protein levels were divided by respective loading (actin) controls and normalized to control (DMSO) or empty plasmid conditions. Phospho-protein and total protein values were divided by the average of drug treatments to account for variability in the control treated conditions. These values were then used for statistical analysis (Student's t-test or One-way ANOVA) on GraphPad Prism 7.

2.10 RNA

2.10.1 Quantitative PCR

RNA was extracted from HEK293T cells using the RNeasy Plus Mini Kit (Qiagen). cDNA was synthesized using SuperScript® IV First-Strand Synthesis kit (Life Technologies) with random hexamers following manufacturer's instructions. For qPCR, cDNA was amplified using 600nM primers and 2x SYBR Green master mix in a volume of 25 µl in the Mx3000P system (Agilent) in triplicates using the primers listed below in Table 5. The gene dose was calculated based on the standard curve method relative to *TUBB*.

Table 2.5. Primers for qPCR

Gene	Primer sequence
PAQR5 (mPRγ)	**Forward Primer** 5'ATGTGCACCACTTTCCATGA **Reverse Primer** 5'TGTGCTTCTGGTGGTACGAG
PAQR7 (mPRα)	**Forward Primer** 5'GTGAATGTCTGGACCCACCT **Reverse Primer** 5'CACTGCCAAACTGGTACACG
PAQR8 (mPRβ)	**Forward Primer** 5'GTCAACGTCTGGACCCATTT **Reverse Primer** 5'GTGGGAGAGCTCTGACTTGG
PAQR9 (mPRε)	**Forward Primer** 5' CAAGAGCCGTACCGACTGG **Reverse Primer** 5' CCGATAATGTCGAAAAGACCCG
TUBB (β-tubulin)	**Forward Primer** 5' GAGGAGTTCCCAGACCGCATC **Reverse Primer** 5'CAGGGGTGCGAAGCCG

2.11 Electrophysiology

All electrophysiological studies for this project were done by Amit Modgil, Yihui Zhang and Jen Yoo.

2.11.1 In vitro brain slice preparation for electrophysiology

Brain slices were prepared from 3-5 week-old male C57BL/6J mice. Mice were anesthetized with isoflurane, decapitated, and brains were rapidly removed and submerged in ice-cold cutting solution containing (mM): 126 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 glucose, 1.5 sodium pyruvate, and 3 kynurenic acid. Coronal 310 μm thick slices were cut with the vibratome VT1000S (Leica Microsystems, St Louis, MO, USA). The slices were then transferred into incubation chamber filled with prewarmed (31-32°C) oxygenated nACSF of the following composition (in mM): 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 10 glucose, 1.5 sodium pyruvate, 1 glutamine, 3 kynurenic acid and 0.005 M GABA bubbled with 95% O_2 -5% CO_2. Exogenous GABA was added to standardize ambient GABA in the slice and provide an agonist source for newly inserted extrasynaptic $GABA_A$Rs. Slices were allowed to recover at 32°C for at least 30 min before exposure to neurosteroids.

2.11.1.1 Neurosteroid incubation

Hippocampal slices were incubated for 15 min in a chamber containing either control or neurosteroids dissolved in nACSF that did not contain kynurenic acid. Following this incubation, slices were transferred to a submerged, dual perfusion recording chamber

(Warner Instruments, Hamden, CT, USA) on the stage of an upright microscope (Nikon FN-1) with a 40x water immersion objective equipped with DIC/IR optics. Slices were maintained at 32ºC and gravity-superfused with nACSF solution (with kynurenic acid) throughout experimentation and perfused at rate of 2 ml/min with oxygenated (O_2/CO_2 95/5%) nACSF. Slices were perfused for 30 to 60 mins before recordings were started.

2.11.2 Electrophysiology recordings in dentate gyrus granule cells

Whole-cell currents were recorded from the dentate gyrus granule cells (DGGCs) in 310-µm-thick coronal hippocampal slices. Patch pipettes (5–7 MΩ) were pulled from borosilicate glass (World Precision Instruments) and filled with intracellular solution of the composition (in mM) as follows: 140 CsCl, 1 MgCl2, 0.1 EGTA, 10 HEPES, 2 Mg-ATP, 4 NaCl and 0.3 Na-GTP (pH = 7.2 with CsOH). A 5 min period for stabilization after obtaining the whole-cell recording conformation (holding potential of -60 mV) was allowed before currents were recorded using an Axopatch 200B amplifier (Molecular Devices), low-pass filtered at 2 kHz, digitized at 20 kHz (Digidata 1440A; Molecular Devices), and stored for off-line analysis.

2.11.2.1 Electrophysiology Analysis

For tonic current measurements, an all-points histogram was plotted for a 10 s period before and during 100µM picrotoxin application, once the response reached a plateau level. Recordings with unstable baselines were discarded. Fitting the histogram with a Gaussian distribution gave the mean baseline current amplitude and the difference between the amplitudes before and during picrotoxin was considered to be the tonic current. The negative section of the all-points histogram which corresponds to the inward IPSCs was not

fitted with a Gaussian distribution (Kretschmannova et al., 2013; Nusser & Mody, 2002). Series resistance and whole-cell capacitance were continually monitored and compensated throughout the course of the experiment. Recordings were eliminated from data analysis if series resistance increased by >20%. Spontaneous inhibitory post-synaptic currents (sIPSCs) were analyzed using the mini-analysis software (version 5.6.4; Synaptosoft, Decatur, GA). Minimum threshold detection was set to 3 times the value of baseline noise signal. To assess sIPSC kinetics, the recording trace was visually inspected and only events with a stable baseline, sharp rising phase, and single peak were used to negate artifacts due to event summation. Only recordings with a minimum of 200 events fitting these criteria were analyzed. sIPSCs amplitude, and frequency from each experimental condition was pooled and expressed as mean ± SEM. To measure sIPSC decay we averaged 100 consecutive events and fitted the decay to a double exponential and took the weighted decay constant (σ). Statistical analysis was performed by using Student t-test (paired and unpaired where appropriate), where $p<0.05$ is considered significant.

2.11.3 Current Run-down assays in HEK-293T and GT1-7

HEK293 cells expressing $GABA_A$Rs composed of $\alpha4\beta3$ subunits along with mPRα, and GT1-7 cells, were superfused, at a rate of 2 mL/min at 32–33°C, with an extracellular solution containing (in mM) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, and 11 glucose, and adjusted to pH 7.4 with NaOH. Borosilicate glass patch pipettes (resistance 2–5 MΩ) contained (in mM) 140 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 Hepes, 1 GTP, 2.5 creatine phosphate, and 2 ATP (Mg^{2+} salt) and adjusted to pH 7.4 with KOH. Experiments were started 3–5 min after achieving the whole-cell configuration. GABA (1 μM, ~EC_{50}) was

applied once every 60 seconds via a fast-step perfusion system (Warner Instruments). All experiments were carried out at 32°C using recording chamber and in-line perfusion heaters (Warner Instruments). Cells were voltage-clamped at –60 mV. Neurosteroids were applied to the cell internally via passive dialysis from the pipette solution. Internal application of neurosteroids has been established to have no effect on basal $GABA_AR$ function nor impacts on the ability of external applications of neurosteroids to allosterically modulate receptor function (Lambert, Peters, Sturgess, & Hales, 1990).

2.11.3.1 Analysis of run-down current

Current rundown is seen in all whole-cell recordings and for $GABA_ARs$ is thought to reflect a loss in the activity/number of $GABA_ARs$ (Jacob, Moss, & Jurd, 2008). GABA-evoked current amplitudes were measured at each time point and expressed as a percentage of the first current measured (defined as t=0 min and 100%). Current amplitudes from cells exposed to vehicle control or neurosteroids were compared at t=20 min using Student *t*-tests.

Chapter 3: Results

3.1 A novel role for neurosteroids in mediating phosphorylation of extrasynaptic $GABA_A$Rs and enhancing tonic current in the hippocampus

Neurosteroids have long been characterized by their actions on $GABA_A$Rs, specifically in enhancing $GABA_A$R activity (Neil L. Harrison & Simmonds, 1984; Majewska, 1992). Neurosteroids are particularly efficacious in potentiating tonic currents at extrasynaptic type $GABA_A$Rs, which are known to be highly enriched in the hippocampus (Fritschy & Brünig, 2003; Nusser & Mody, 2002). Although it has been demonstrated that neurosteroids can also affect $GABA_A$R expression, the mechanism by which this occurs has been largely unexplored (Gulinello et al., 2001; J. Maguire & Mody, 2007). Our lab was the first to demonstrate neurosteroids' ability to mediate phosphorylation of extrasynaptic $GABA_A$ subunits, leading to an enhancement of extrasynaptic $GABA_A$Rs at the plasma membrane (Abramian et al., 2014). Specifically, Abramain et al., (2004) have shown that neurosteroid THDOC mediates PKC-dependent phosphorylation of $GABA_A$-α4 subunits at serine 443. This phosphorylation in turn, enhances α4-containing $GABA_A$R insertion into the plasma membrane (Abramian et al., 2014). Interestingly, this phosphorylation effect is specific for α4-subunit containing extrasynaptic type $GABA_A$Rs, as α1 and α5 subunits, were not phosphorylated by THDOC. In line with enhancement of extrasynaptic type $GABA_A$Rs, Abramian et al., demonstrated a PKC-dependent enhancement of tonic current in *in vitro* hippocampal neurons in response to neurosteroid treatment (Abramian et al., 2014). Surprisingly, these phosphorylation effects seem to be *independent* from the allosteric effects of neurosteroids on $GABA_A$Rs. When a residue important for allosteric potentiation was mutated on the α4 subunit (Q241), THDOC treatment still resulted in enhancements in

$GABA_AR$ expression (Abramian et al., 2014; Hosie et al., 2006). Thus, neurosteroids can have sustained effects on GABAergic inhibition, in addition to the fast potentiating effects.

3.1.1 Do neurosteroids other than THDOC, also show a similar profile in phosphorylating $GABA_ARs$ in the hippocampus?

Although THDOC is predominantly known to be a stress steroid, I wanted to investigate whether other neurosteroids such as allopregnanolone, the most abundant neurosteroid in the brain, could also mediate sustained effects on $GABA_ARs$ (Corpéchot et al., 1993; Doodipala S. Reddy, 2003; Sandhu, Anjum, Mukhtar, Hussain, & Khan, 2013). I decided to test the ability of allopregnanolone and its synthetic analog, SGE-516 (SAGE Therapeutics) to mediate $GABA_AR$ phosphorylation in the hippocampus. α4,β3, β2 and δ subunit containing receptors are the most common type of $GABA_ARs$ found in the dentate gyrus of the hippocampus (Nusser & Mody, 2002; Stell, Brickley, Tang, Farrant, & Mody, 2003). Previous studies in our lab demonstrated that both α4 and β3 subunits contain PKC-dependent phosphorylation sites, so we hypothesized that if α4 could be phosphorylated through a neurosteroid mechanism, then the β3 subunit may also be phosphorylated. The β3 subunit is phosphorylated at serines 383, 408 and 409 on the intracellular loop between transmembrane domains three and four (Mcdonald & Moss, 1997). Brandon et al., 1999 identified serines 408 and 409 as PKC substrate sites, which led to the development of a phospho specific antibody in our lab against these residues (Nicholas J. Brandon et al., 1999). Shortly after, Adams et al., 2015 demonstrated that THDOC does in fact phosphorylate serines 408/409 on the β3 subunit in a1β3γ2 transfected HEK-293T cells (Adams et al., 2015).

I decided to investigate whether this property was specific for THDOC, or also conserved in allopregnanolone. In Modgil et al., 2017, I treated acute hippocampal slices from 8-12 week old male C57BL/6J mice with either control DMSO, 100nM allopregnanolone or 100 nM SGE-516 (synthetic analogue of allopregnanolone) for 20 minutes. The tissue was then lysed and immunoblotted for phospho 408/409 and total-β3 levels. Treatment with 100nM allopregnanolone enhanced S408/409 phosphorylation to 141 ± 10% of control ($n=10$, $p < 0.01$) and 100nM SGE-516 enhanced S408/409 phosphorylation to 143± 14% of control ($n=4$, $p < 0.05$) (Figure 3.1 A-B).

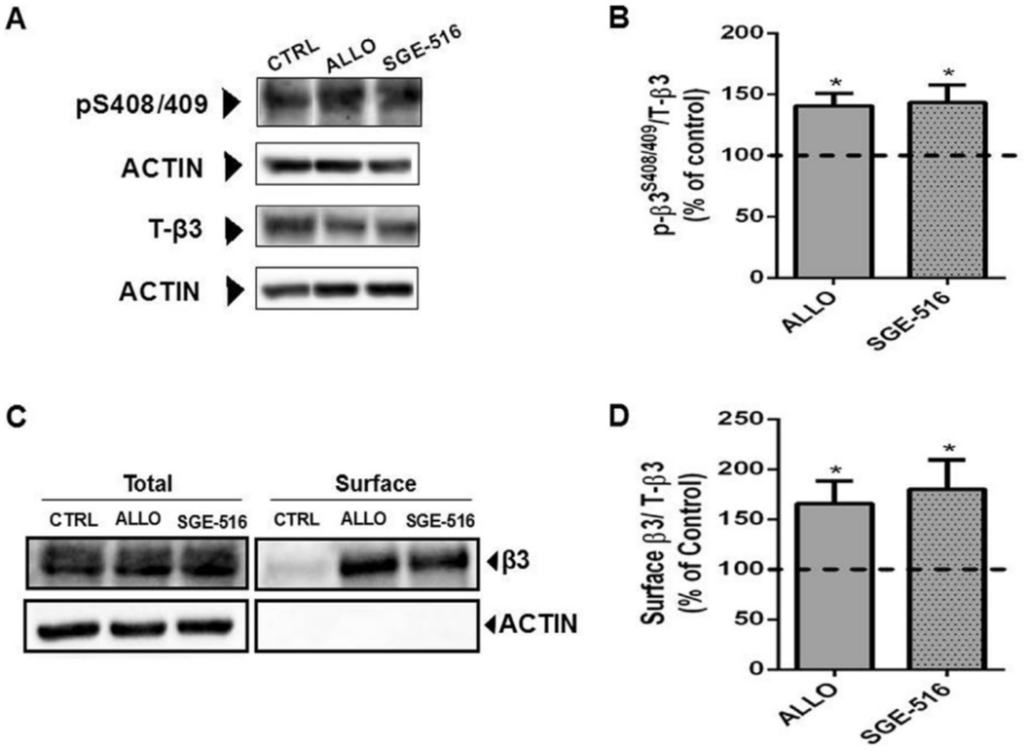

Figure 3.1
Allopregnanolone and SGE-516 exposure increases phosphorylation and surface expression of β3 subunits. (A) Exposure to 100 nM of the neurosteroids, ALLO or SGE-516, for 20 min increases β3 S408/409 phosphorylation in acute hippocampal slices. (B) The ratio of p-β3/T-β3 was measured and values were normalized to those in control (100%). Asterisks represent a significant difference from control (ALLO: $p < 0.01$, Student's t-test, n=10 slices, from 10 mice; SGE-516: $p < 0.05$, Student's t-test, n=4 slices, from 4 mice). (C) Exposure to 100 nM ALLO or SGE-516 for 20 min increases $GABA_A$-β3 containing receptors at the plasma membrane in acute hippocampal slices. (D) The ratio of surface β3/T-β3 was measured and values were normalized to cell surface levels in control treated slices (100%). Asterisks represent a significant difference from control (ALLO: $p < 0.05$, Student's t-test, n=8 slices; SGE-516:$p < 0.05$, Student's t-test, n=4 slices).

Reprinted with permission from Modgil, A., Parakala, M. L., Ackley, M. A., Doherty, J. J., Moss, S. J., & Davies, P. A. (2017). Endogenous and synthetic neuroactive steroids evoke sustained increases in the efficacy of GABAergic inhibition via a protein kinase C-dependent mechanism. *Neuropharmacology*, *113*, 314–322. https://doi.org/10.1016/j.neuropharm.2016.10.010

3.1.2 Does allopregnanolone-mediated phosphorylation also enhance $GABA_AR$ number at the plasma membrane?

Previous studies in our lab have demonstrated S408/409 phosphorylation on the β3 subunit results in decreased endocytosis of β3-containing $GABA_ARs$ from the plasma membrane (Kittler et al., 2005). Conversely, dephosphorylation of these residues results in increased interaction with clathrin adaptor protein AP2 and signals its endocytosis from the membrane (Kittler et al., 2005). To confirm that allopregnanolone mediated S408/409 phosphorylation enhances β3-containing $GABA_ARs$ at the plasma membrane, I used cell surface biotinylation to measure β3 subunit levels after allopregnanolone, SGE-516 or control treatment for 20 minutes. β3 subunit levels were increased to 166± 22% of control ($n=8$, $p < 0.05$) and 180 ± 29% of control ($n=4$, $p < 0.05$) for allopregnanolone and SGE-516, respectively (Figure 3.1 C-D).

Since THDOC was previously shown to have a significant effect on tonic current in hippocampal neurons, I decided to investigate whether the enhancement in surface β3 levels by allopregnanolone and its synthetic analogue SGE-516, led to an enhancement in tonic current in the hippocampus (Abramian et al., 2014). To distinguish changes in tonic current presumed to arise from increased surface expression, from the allosteric potentiating effects of neurosteroids, we first treated hippocampal slices for 10-15 minutes with allopregnanolone, SGE-516 or control DMSO. We then washed off the neurosteroid for 30-50 minutes and recorded tonic current from the dentate gyrus granule cells of the hippocampus (Figure 3.2 A). Control slices exhibited a tonic current of 43.9 ± 5.7 pA ($n = 12$), whereas 100nM allopregnanolone and 100nM SGE-516-treated slices had tonic currents of 95.8 ± 10.8 pA ($n=4$, $p=0.0005$) and 123.0 ± 22.2 pA, ($n=6$, $p=0.0003$), respectively (Figure 3.2 B-C). The enhancement of tonic current by both allopregnanolone and SGE-516

was prevented with PKC inhibitor (GFX); tonic current was not significantly different from control treated slices (Figure 3.2 B-C).

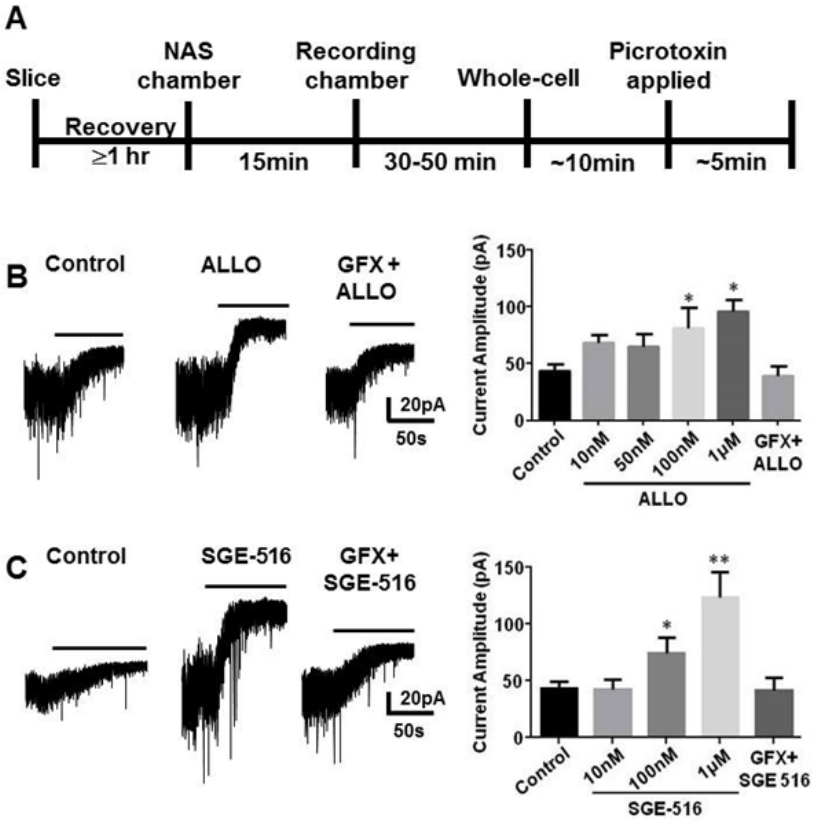

Figure 3.2
Neurosteroid mediated metabotropic enhancement of tonic inhibitory current in DGGC neurons. (A) Scheme demonstrating experimental protocol. Hippocampal slices were allowed to recover for at least 1 h following slicing. Slices were then incubated for 15 min in a chamber containing neurosteroids dissolved in nACSF. Slices were then transferred to the recording chamber of the microscope followed by a wash period between 30 and 60 min of continuous perfusion of neurosteroid-free nACSF before recordings were started. Recordings were made from DGGCs in hippocampal slices from postnatal 3-5 week C57BL/6J mice in the presence of 5 μM GABA followed by 100 μM picrotoxin and the difference in holding current was then determined. Example tonic currents from slices following exposures to vehicle (control) or 100 nM ALLO (B) or 100 nM SGE-516 (C) for 15 min. No change in tonic current was observed in slices pre-incubated for 15 min with GFX followed by ALLO, or SGE-516. Bar above current represents application of picrotoxin (100 μM). Bar graph shows average tonic current was significantly enhanced following exposure to different concentrations of ALLO(B) and SGE-516(C). In all panels * =significantly different to control ($p < 0.05$; un-paired t-test, n=4-12 cells). **Data by Amit Modgil**

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https://doi.org/10.1016/j.neuropharm.2016.10.010

However, in hippocampal slices, treatment with 10 μM GFX alone trended towards enhancing S408/409 phosphorylation (156.7 ± 33.85% of control, n=7, p=0.0595) (Figure 3.3). Although not significant, this trend suggests another kinase may compensate for phosphorylating these residues. McDonald and Moss 1997 identified serines408/409 as predominantly PKC substrate sites, but PKA has also been shown to phosphorylate the β3 subunit (Nicholas J. Brandon et al., 1999; Mcdonald & Moss, 1997). Thus, by inhibiting both PKA and PKC kinases with GFX and KT5720 respectively, I was able to prevent allopregnanolone mediated S408/409 phosphorylation (3α,5α-THP 120.5 ± 3.209% of control, n=3, p<0.01) (GFX+KT+3α,5α-THP 99.4 ± 2.753% of control, n=3, p=0.7458) (Figure 3.4).

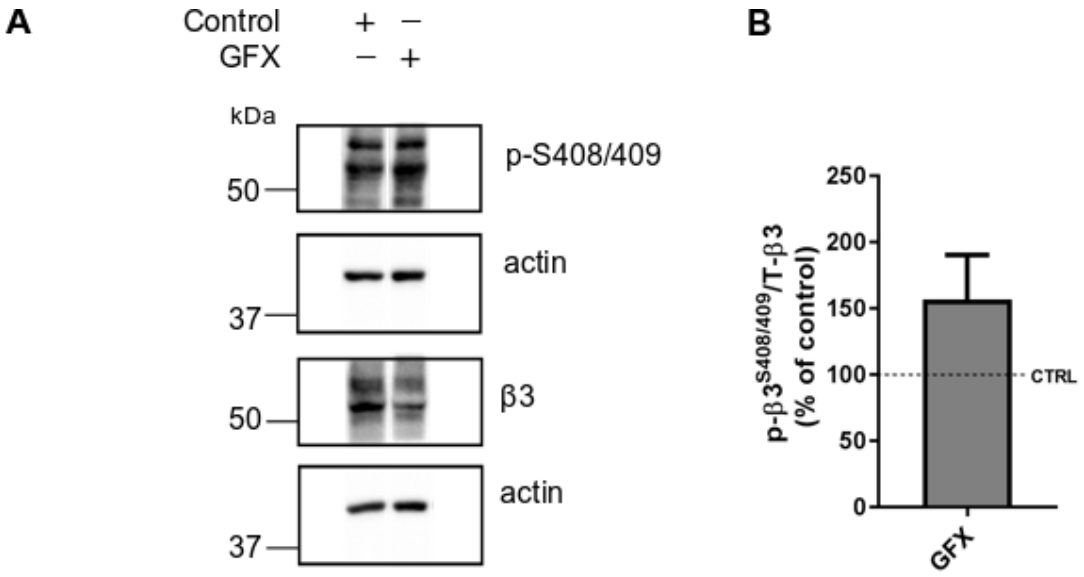

Figure 3.3
PKC inhibition may affect β3 S408/409 phosphorylation in the hippocampus. (A) Exposure to 10μM GFX or control (DMSO) for 10 minutes increases β3 S408/409 phosphorylation in acute hippocampal slices. (B) The ratio of p-β3/T-β3 was measured and values were normalized to those in control (100%). (GFX: 156.7% of control, n=7 mice (3 slices each), p=0.0595, Student's t-test.

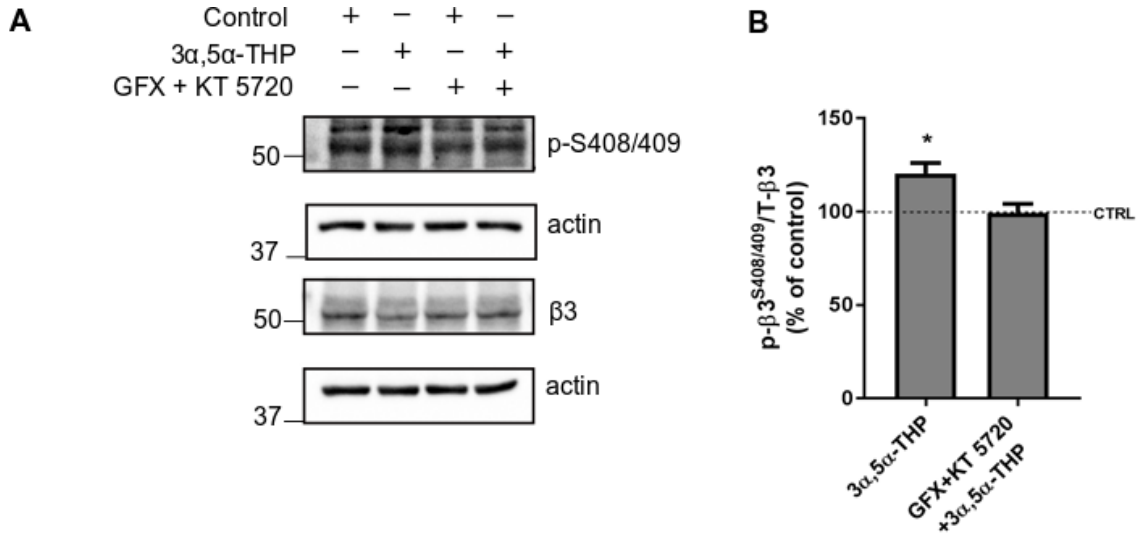

Figure 3.4
PKC and PKA inhibition prevents allopregnanolone-mediated increase in β3 S408/409 phosphorylation. (A) Exposure to 100 nM 3α,5α-THP or control (DMSO) for 10 minutes or pretreatment with 10μM GFX+1μM KT 5720 or control (DMSO) followed by 3α,5α-THP or control in hippocampal slices. (B) The ratio of p-β3/T-β3 was measured and values were normalized to control or GFX+KT 5720 (100%). (3α,5α-THP 120.5 ± 3.209% of control, n=3 mice (3 slices each), $p<0.01$) (GFX+KT+3α,5α-THP 99.4 ± 2.753% of control, n=3 mice (3 slices each), $p=0.7458$) Student's t-test.

3.1.3 Is allopregnanolone's effect on phosphorylation restricted to the dentate gyrus of the hippocampus?

Tonic inhibition in the dentate gyrus has been hypothesized to function as a "gate" to filter excitatory activity from the performant pathway entering the hippocampus proper (Coulter & Carlson, 2007; Heinemann et al., 1992). I hypothesized that since α4βδ type receptors are heavily expressed in the dentate gyrus, neurosteroid-mediated phosphorylation of $GABA_AR$s would be greatly enhanced in the dentate gyrus compared to other regions of the hippocampus (CA1-CA3) (Nusser & Mody, 2002). I dissected the dentate gyrus out of acute hippocampal slices and treated with either control or 100nM allopregnanolone for 20 minutes. The remaining tissue containing CA1-CA3 regions was also treated as above.

Allopregnanolone significantly enhanced S408/409 phosphorylation in the dentate gyrus (126.2% of control, n=4, p<0.01) but not in CA1-CA3 regions (90.6% of control, n=4, p=0.0784) (Figure 3.5).

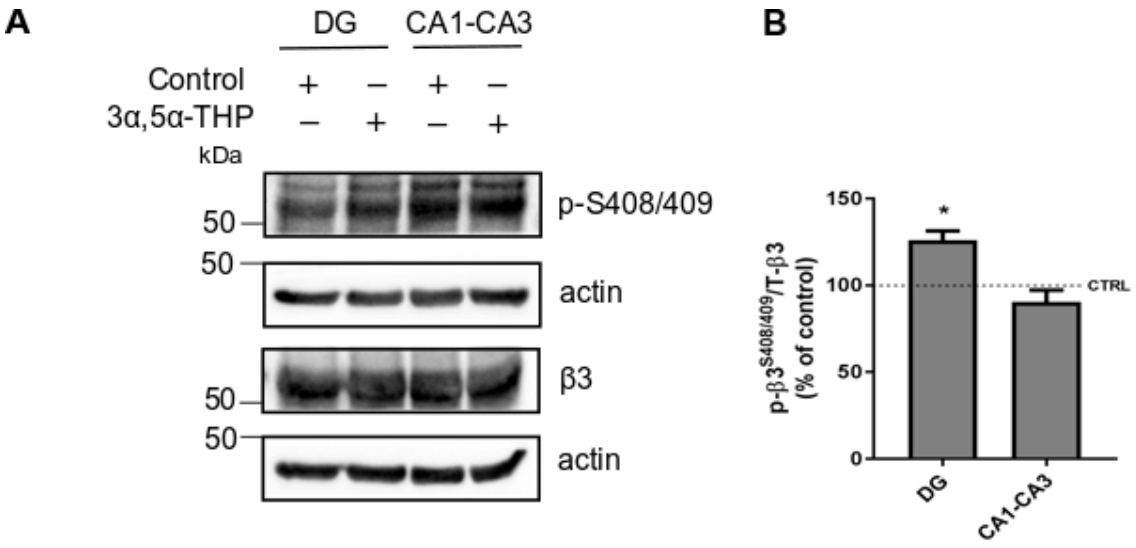

Figure 3.5
Allopregnanolone enhances β3S408/409 phosphorylation in the dentate gyrus but not in the CA1-CA3 regions of hippocampus. The dentate gyrus was dissected out of the hippocampus and treated with (A) 100nM 3α,5α-THP or control (DMSO) for 20 minutes. The remaining CA1-CA3 regions were treated similarly. (B) The ratio of p-β3/T-β3 was measured and values were normalized to control in the dentate gyrus or CA1-CA3 respectively (100%). 3α,5α-THP significantly enhanced S408/409 phosphorylation in the dentate gyrus (126.2% of control, n=4 mice (5 slices each), p<0.01) but not in CA1-CA3 regions (90.6% of control, n=4 (5 slices each), p=0.0784) Student's t-test.

3.1.4 Does allopregnanolone recruit other kinases beside PKC and PKA to mediate phosphorylation of $GABA_AR$s?

Since allopregnanolone has been shown to activate both PKC and PKA signaling mechanisms, I wanted to investigate whether other kinases such as CaMKII, are also activated by neurosteroids. Saliba et al., 2012 have demonstrated that unlike serines 408 and 409, serine 383 on the β3 subunit is phosphorylated by CaMKII (Saliba et al., 2012). To investigate whether allopregnanolone activates CaMKII signaling, I treated dissected dentate gyrus slices as above, with either vehicle or 100nM allopregnanolone. Allopregnanolone treatment significantly enhanced serine 383 phosphorylation in the dentate gyrus (131.9% of control, n=3, p<0.01) but not in the CA1-CA3 (114.7% of control, n=3, p=0.5106) (Figure 3.6).

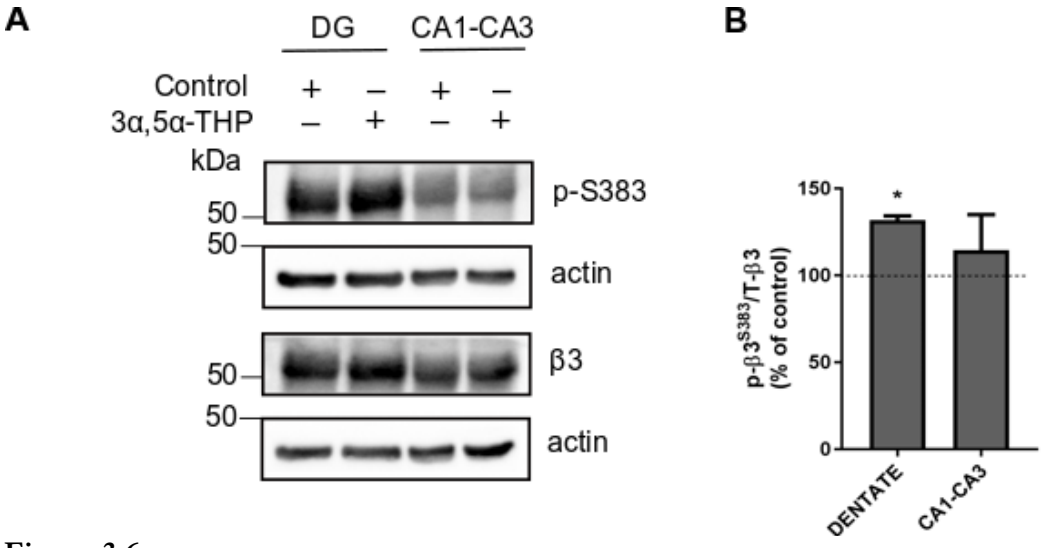

Figure 3.6
Allopregnanolone enhances β3S383 phosphorylation in the dentate gyrus but not in the CA1-CA3 regions of hippocampus. The dentate gyrus was dissected out of the hippocampus and treated with (A) 100nM 3α,5α-THP or control (DMSO) for 20 minutes. The remaining CA1-CA3 regions were treated similarly. (B) The ratio of p-β3/T-β3 was measured and values were normalized to control in the dentate gyrus or CA1-CA3 respectively (100%). 3α,5α-THP significantly enhanced S383 phosphorylation in the dentate gyrus (131.9% of control, n=3 mice (5 slices each), p<0.01) but not in the CA1-CA3 (114.7% of control, n=3 mice (5 slices each), p=0.5106) Student's t-test.

3.1.5 Do neurosteroids directly activate kinases?

Conventional PKC kinases are activated by Ca^{2+} and diacylglycerol (DAG) and translocate to the plasma membrane to mediate phosphorylation of substrates (L. Coussens et al., 1986; Lisa Coussens, Rhee, Parker, & Ullrich, 1987). I hypothesized that neurosteroids cause the intracellular activation of PKC, either by direct binding or through second messengers. In basal conditions, PKC binds the β3 subunit --PKC isoform βII has been shown to bind the intracellular loops of β1 and β3 subunits within residues 405-415 (Nicholas J. Brandon, Jovanovic, Smart, & Moss, 2002; Nicholas J. Brandon et al., 1999). To confirm whether neurosteroids directly activate kinases bound to the β3 subunit, I immunoprecipitated β3 subunits from acute hippocampal slices, added 100nM allopregnanolone and radiolabeled ATP, and measured phosphorylation of the β3 subunit. In vitro addition of allopregnanolone did not significantly enhance β3 phosphorylation (80.66% of control, n=3, p=0.0662) compared to control application (Figure 3.7).

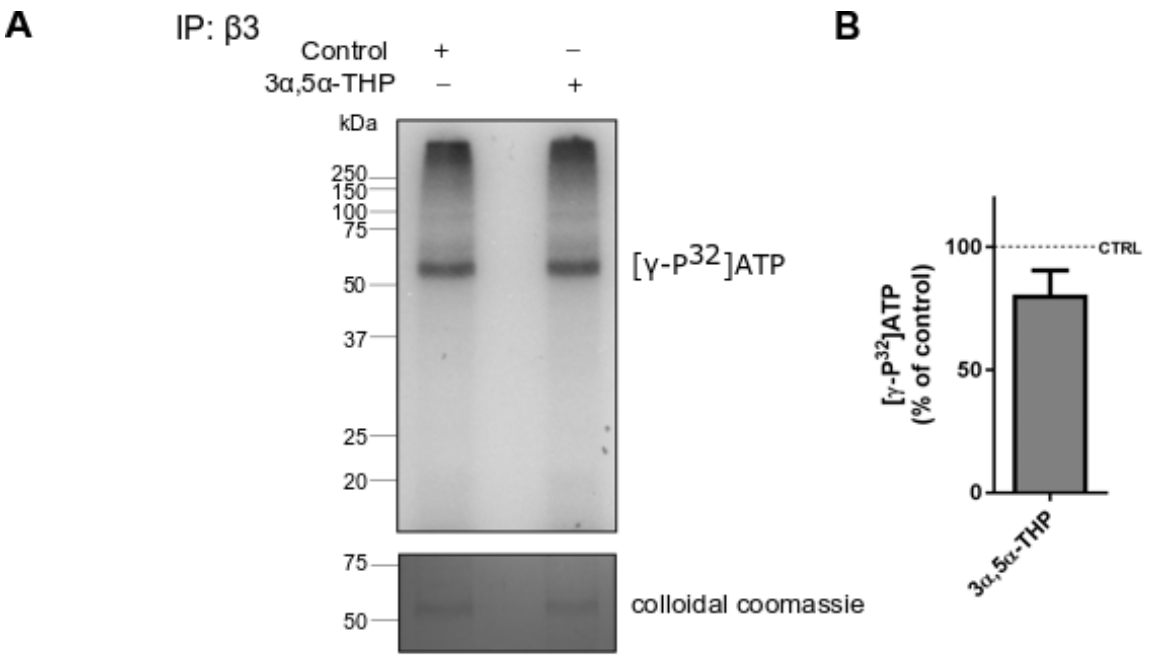

Figure 3.7
Allopregnanolone does not directly activate kinases bound to $GABA_A$-β3. (A) β3 proteins were immunoprecipitated from hippocampal slices to which either control (DMSO) or 3α,5α-THP was added to the IP mixture in the presence of radiolabeled ATP. (B) The ratio of [γ-P^{32}]ATP/ IP-β3 per lane visualized by colloidal coomassie was measured and values were normalized to control (DMSO)(100%). In vitro addition of 3α,5α-THP did not significantly enhance β3 phosphorylation (80.66% of control, n=3 mice, p=0.0662) compared to vehicle application. Student's t-test.

3.2 The long-term effects of neurosteroids are mediated by membrane progesterone receptors (mPRs)

Since my results suggest that neurosteroids do not directly activate kinases, I hypothesized that they may mediate downstream signaling through (1) binding $GABA_ARs$, leading to a conformational change exposing the substrate site for PKC phosphorylation or (2) binding a receptor in either the plasma membrane or inside the cell that activates downstream kinase signaling. Allopregnanolone has been shown to bind mPRs in human breast adenocarcinoma cells (MDA-MB-231) with a relative binding affinity of 7.6% to that of progesterone (Kelder et al., 2010). Progesterone's non-genomic actions through membrane progesterone receptors activate downstream G proteins, decrease cAMP levels and activate MAPK signaling in MDA-MB-231 (Zhu, Rice, et al., 2003).

3.2.1 Does allopregnanolone bind to membrane progesterone receptors to mediate intracellular kinase activation?

If mPRs are activated by both allopregnanolone and progesterone, I hypothesized that progesterone would also mediate S408/409 phosphorylation. I treated acute hippocampal slices as described previously, with 100nM progesterone for 20 minutes. Progesterone treatment significantly enhanced S408/409 phosphorylation in the hippocampus (146.6% of control, n=3, p=0.0374, 150.3% of control, n=3, p=0.0248 for allopregnanolone) (Figure 3.8).

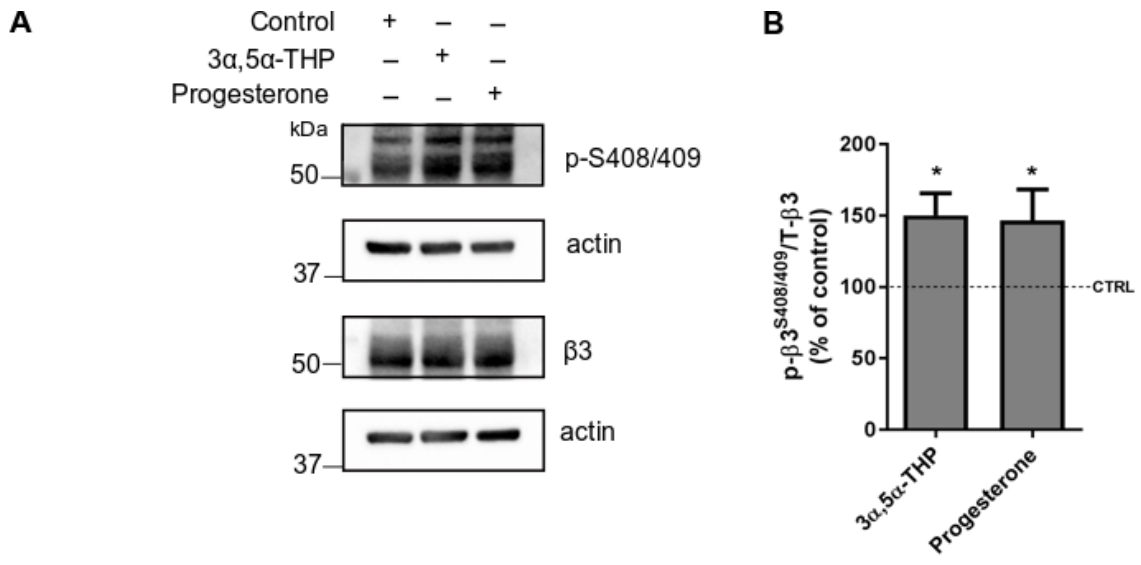

Figure 3.8
Progesterone enhances β3S408/409 phosphorylation in the hippocampus. (A) Hippocampal slices were treated with 100 nM 3α,5α-THP, 100nM progesterone or control (DMSO) for 20 minutes. (B) The ratio of p-β3/T-β3 was measured and values were normalized to control (100%). Progesterone treatment significantly enhanced S408/409 phosphorylation in the hippocampus (146.6% of control, n=3 mice (4 slices per treatment), adjusted p=0.0374, 3α,5α-THP 150.3% of control, n=3 mice (4 slices per treatment), adjusted p=0.0248) One-way ANOVA p=0.0260, Dunnet's post-hoc.

3.2.2 Is progesterone's effect on β3 phosphorylation through mPRs or nPRs?

Progesterone's classical action of signaling is via nuclear progesterone receptors (nPRs) that induce gene transcription (Evans, 1988). Only in the past two decades have the non-genomic actions of progesterone been identified (Garg, Ng, Baig, Driggers, & Segars, 2017; Moussatche & Lyons, 2012; Singh et al., 2013). Progesterone exerts its non-genomic signaling through two membrane receptors, membrane progesterone receptors (mPRs) and PGRMC1, progesterone receptor membrane component 1 (Falkenstein, Meyer, Eisen, Scriba, & Wehling, 1996; Zhu, Rice, et al., 2003). Although allopregnanolone does not have any affinity for nPRs, its metabolite 5α-DHP can bind nPRs and induce transcriptional

changes (Rupprecht et al., 1993). To investigate whether both allopregnanolone and progesterone mediate their actions through mPRs and not nPRs, I used an immortalized mouse cell-line (GT1-7) with very low expression of nPRs (Sleiter et al., 2009). GT1-7 cells are immortalized mouse hypothalamic neurons that endogenously express mPRα,β,δ,ε and $GABA_A$ subunits α1, β1 and β3 (Hales, Kim, Longoni, Olsen, & Tobin, 1992; Thomas & Pang, 2012). I treated GT1-7 cells with 100nM allopregnanolone, 100nM progesterone or vehicle DMSO for 15 minutes. Both allopregnanolone and progesterone significantly enhanced β3 phosphorylation in these cells, (133.1% of control, n=5, $p<0.01$) and (126.6% of control, n=5, $p<0.05$), respectively (Figure 3.9).

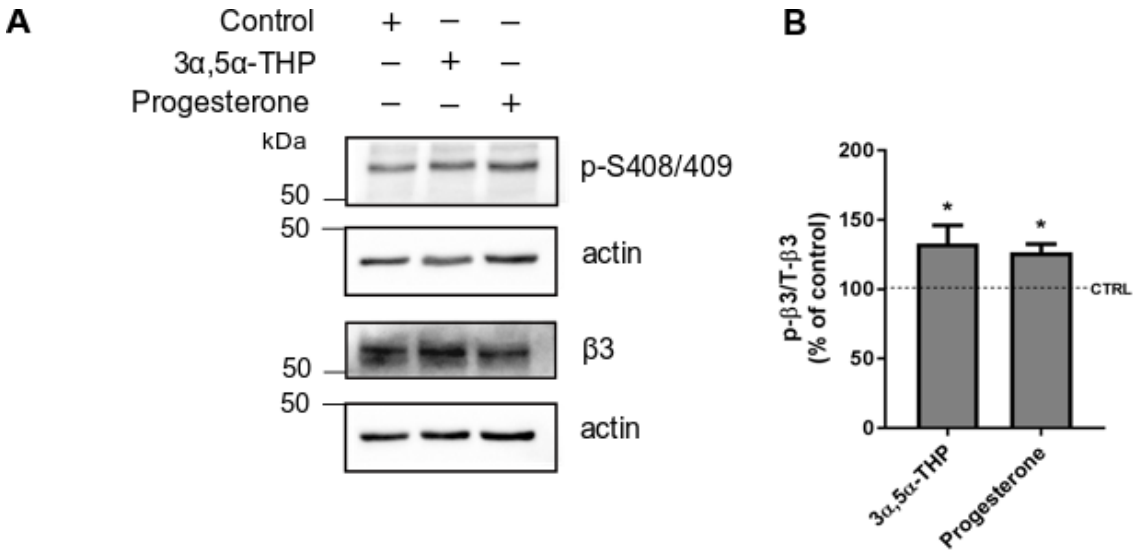

Figure 3.9
Progesterone and allopregnanolone enhance β3 S408/409 phosphorylation in GT1-7 cells that have negligible nuclear progesterone receptor expression. (A) GT1-7 cells were treated with 100nM 3α,5α-THP, 100nM progesterone or control (DMSO) for 15 minutes. (B) The ratio of p-β3/T-β3 was measured and values were normalized to control (100%). Both 3α,5α-THP and progesterone significantly enhanced β3 phosphorylation in these cells, (133.1% of control, n=5, adjusted $p<0.01$) and (126.6% of control, n=5, adjusted $p<0.05$), respectively. One-way ANOVA p=0.01, Dunnet's post-hoc.

3.2.3 Does allopregnanolone enhance $GABA_AR$ trafficking in GT1-7 cells?

Previous studies in our lab measured trafficking of $GABA_ARs$ in HEK-293T cells using GABA-evoked current run-down assays (Abramian et al., 2010). Run-down of current is a well characterized phenomenon in cells, where dialysis of cell contents with the intracellular pipette solution results in loss of ATP, GTP and other energy dependent molecules important for maintaining cell surface $GABA_ARs$ by balancing rates of exo- or endocytosis of $GABA_ARs$. Loss of cell surface $GABA_ARs$ overtime leads to "run-down" of currents (Horn & Korn, 1992). Abramian et al., (2010) demonstrate that treatment with PDBu (PKC activator), results in prevention of current run-down compared to control (DMSO) treatment (Abramian et al., 2010). This suggests an increase in receptor number, allowing for maintenance of GABA-evoked current. We used a similar assay to investigate the effects of allopregnanolone on GT1-7 cells (Figure 3.10). Using intracellular application of allopregnanolone, we observed that GABA-evoked current was significantly enhanced compared to control over the course of 20 minutes. In GT1-7 cells, at 20 min after the start of the experiment, control current amplitude was 74 ± 7% (n=7) of the initial GABA-mediated response compared to 108 ± 8% (n=6; p=0.02) in the presence of internal allopregnanolone (Figure 3.10).

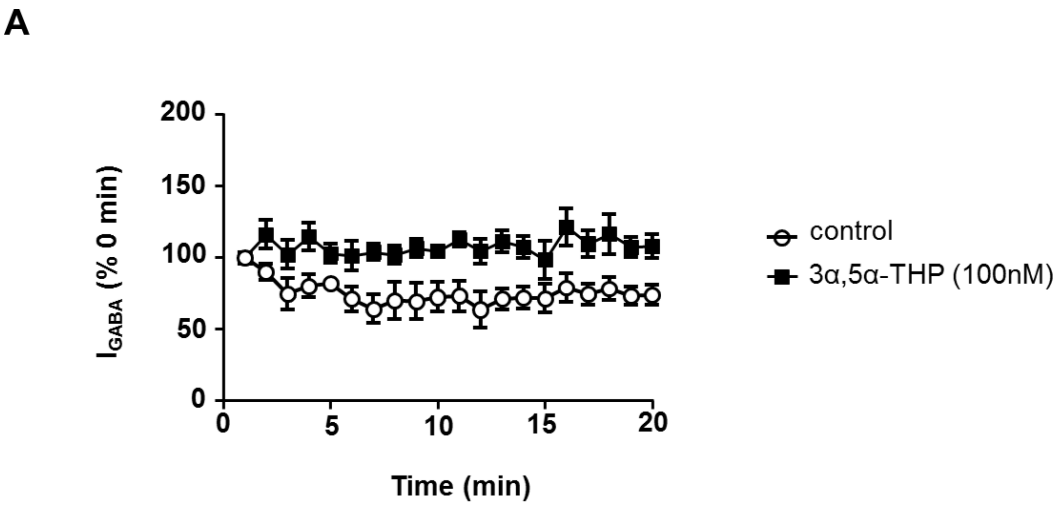

Figure 3.10
Allopregnanolone prevents run-down of current in GT1-7 cells. (A) Run-down of current in GT1-7 cells was measured in the presence of 3α,5α-THP or control (DMSO) applied intracellularly. At 20 minutes, the GABA-mediated response in the presence of 3α,5α-THP was 108 ± 8% (n=6; p=0.02) of the response at 0 min. Current amplitude of control treated cells was 74 ± 7% (n=7) of the response measured at 0 min. Student's t-test.
Data by Jen Yoo

3.2.4 Does mPR agonist ORG OD 02-0 also allosterically potentiate $GABA_A$ currents?

Previously published data in Abramian et al., 2014 demonstrate that the sustained effects of neurosteroids (30 minutes-1hour) reveal an independent mechanism from the fast allosteric potentiating effects (milliseconds) (Abramian et al., 2014). My hypothesis was: if mPRs are responsible for $GABA_AR$ phosphorylation events and subsequent enhancement of tonic current, then mPR agonist, ORG OD 02-0, should enhance tonic inhibitory current while having no allosteric potentiating effect on $GABA_AR$, since it is not a known modulator of $GABA_ARs$. In HEK293 cells expressing α4β3 $GABA_ARs$, EC_{10} (1μM) GABA-evoked currents were allosterically potentiated by co-application of allopregnanolone (100nM, 511.22 ± 246% of control, n=4). Co-application of 100nM ORG OD 02-0 failed to

allosterically potentiate (116.47 ± 28.9% of control, n=3 (Figure 3.11). In agreement, we have also shown in hippocampal slices that co-application of GABA and allopregnanolone results in potentiation of tonic currents, where no potentiation was observed with co-application of ORG OD 02-0 (not shown here). However, tonic current was enhanced after a "chronic application" of ORG OD 02-0 (as in Modgil et al., 2017) in dentate gyrus granule cells (52.60 ± 12.93 pA, n=8, for control) (90.65 ± 12.03 pA, n=10, $p<0.05$) for 300nM ORG OD 02-0) (Figure 3.12).

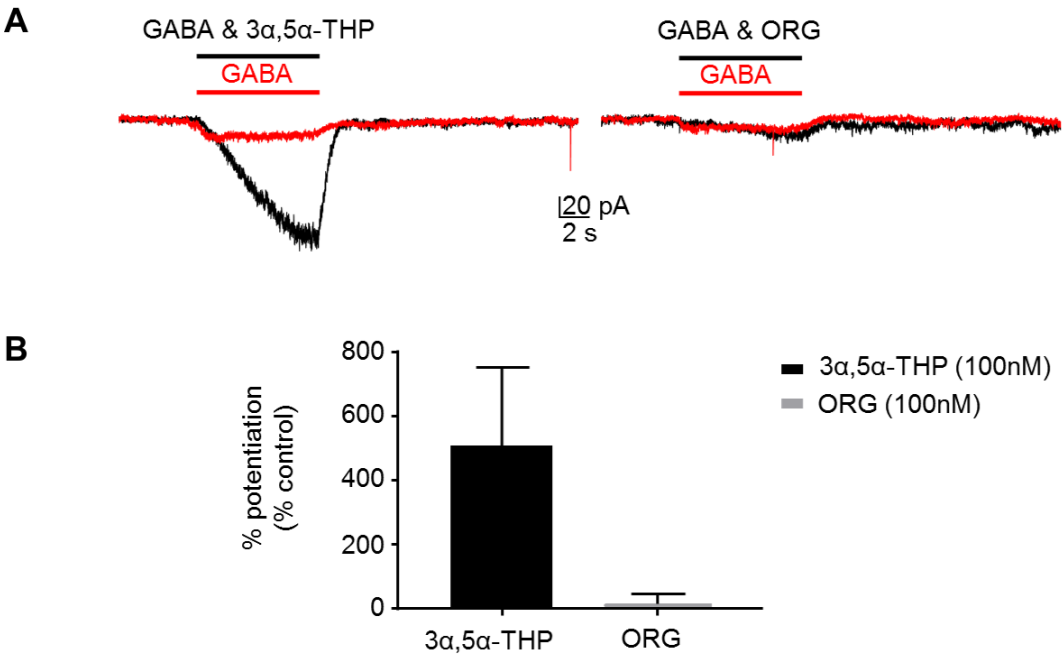

Figure 3.11
ORG OD 02-0 is not an allosteric modulator of the $GABA_AR$. On the left (A) is a whole-cell recording from α4β3 transfected HEK-cells where 3α,5α-THP was co-applied with 1μM GABA. (B) 100 nM 3α,5α-THP potentiated GABA-mediated current (511.22 ± 246% of GABA alone control, n=4), where ORG OD 02-0 (right trace) showed no potentiation of GABA (116.47 ± 28.9% of control, n=3). (n.s.) **Data by Jen Yoo**

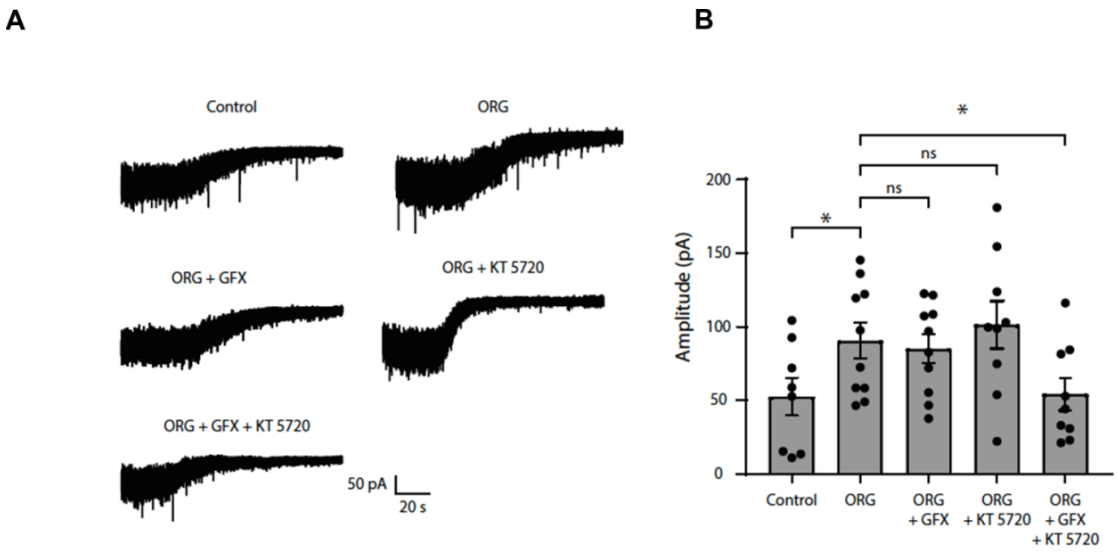

Figure 3.12

ORG OD O2-0 enhances tonic $GABA_AR$-mediated current via PKC/PKA signaling in DGGCs.

(A) Chronic treatment of hippocampal slices as in Modgil et al., 2017 with mPR agonist ORG OD 02-0 alone, or with PKC (GFX) and PKA (KT 5720) inhibitors. (B) ORG OD 02-0 significantly enhanced tonic current in dentate gyrus granule cells compared to control (DMSO) treatment (52.60 ± 12.93 pA, n=8, for control) (90.65 ± 12.03 pA, n=10, $p<0.05$) for 300nM ORG OD 02-0). ORG OD 02-0 mediated increase in tonic current was only abolished by treatment with both PKC (GFX) and PKA (KT 5720) inhibitors (54.19 ± 10.97 pA, n=9, p=0.04 for ORG 02-0+GFX+KT 5720 compared to 90.65 ± 12.03 pA for ORG OD 02-0) Student's t-test. **Data by Yihui Zhang**

3.2.5 Does ORG OD 02-0 activate PKC/PKA signaling to mediate phosphorylation of $GABA_A$-β3 phosphorylation?

Inhibition of PKC and PKA kinases prevented allopregnanolone mediated increase of $\beta3^{S408/409}$ phosphorylation (Figure 3.4). Thus, I decided to confirm if mPR activation via ORG OD 02-0 also enhanced PKC and PKA signaling. 300nM ORG OD 02-0 treatment of acute hippocampal slices enhanced β3 phosphorylation compared to control (138.9% of control, n=6, $p<0.05$) (Figure 3.13). This effect was blocked with GFX and KT 5720 (94.7% of control, n=6, p=.2811) (Figure 3.13). The increase in tonic current with chronic ORG OD 02-0 treatment was also blocked by these kinase inhibitors (52.60 ± 12.93 pA, n=8 for control) (54.19 ± 10.97 pA, n=9, p=0.04 for ORG 02-0+GFX+KT 5720) (Figure 3.12).

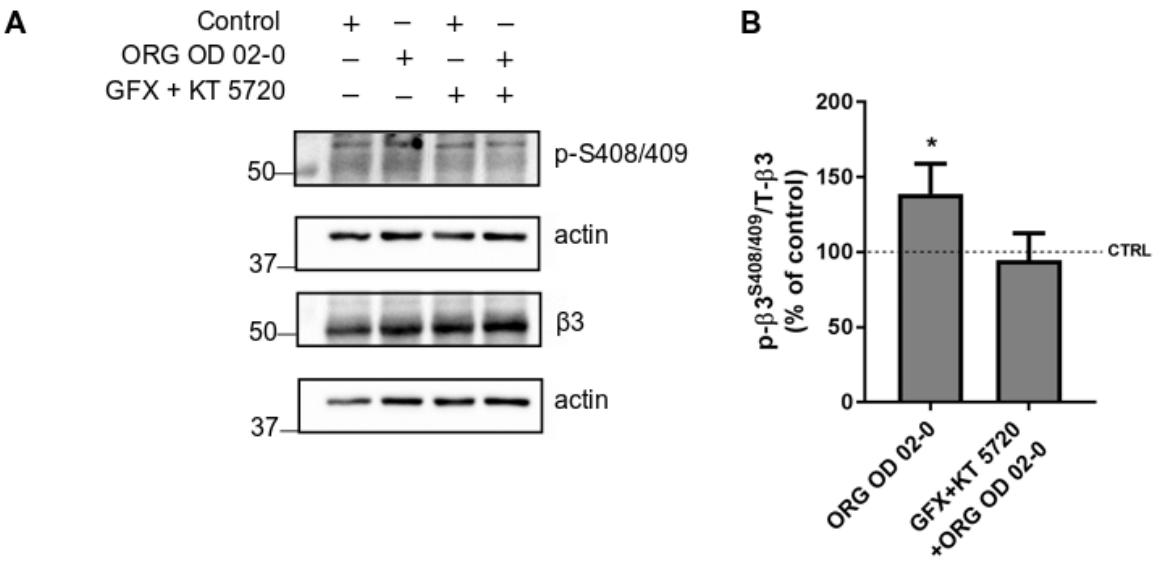

Figure 3.13
ORG OD 02-0 enhances β3S408/409 phosphorylation via PKC/PKA in the hippocampus. (A) Hippocampal slices treated with control (DMSO) or 300nM ORG OD 02-0 in the presence or absence of PKC (GFX) and PKA (KT 5720) inhibitors. (B) The ratio of p-β3/T-β3 was measured and values were normalized to control or GFX+KT 5720 alone (100%). β3 phosphorylation is increased by ORG OD 02-0 treatment compared to control (138.9% of control, n=6 mice (3 slices per treatment), $p<0.05$). Pretreatment with GFX and KT 5720 blocks ORG OD 02-0 mediated increase in β3 phosphorylation (94.7% of control, n=6, p=.2811) Student's t-test.

3.2.6 Are β3-containing $GABA_A$Rs enhanced at the plasma membrane as a result of ORG OD 02-0 treatment?

ORG OD 02-0 treatment enhanced S408/409 phosphorylation and increased β3 surface levels (141.4% of control, n=6, $p<0.01$) (Figure 3.14). In Abramian et al., 2014, THDOC treatment significantly enhanced α4-$GABA_A$R insertion into the plasma membrane in hippocampal neurons (Abramian et al., 2014). I decided to investigate whether ORG OD 02-0 also demonstrated effects on α4 surface levels in hippocampal slices. Twenty minutes of ORG OD 02-0 treatment enhanced α4-containing $GABA_A$Rs at the plasma membrane (114.1% of control, n=5, $p<0.05$) (Figure 3.15).

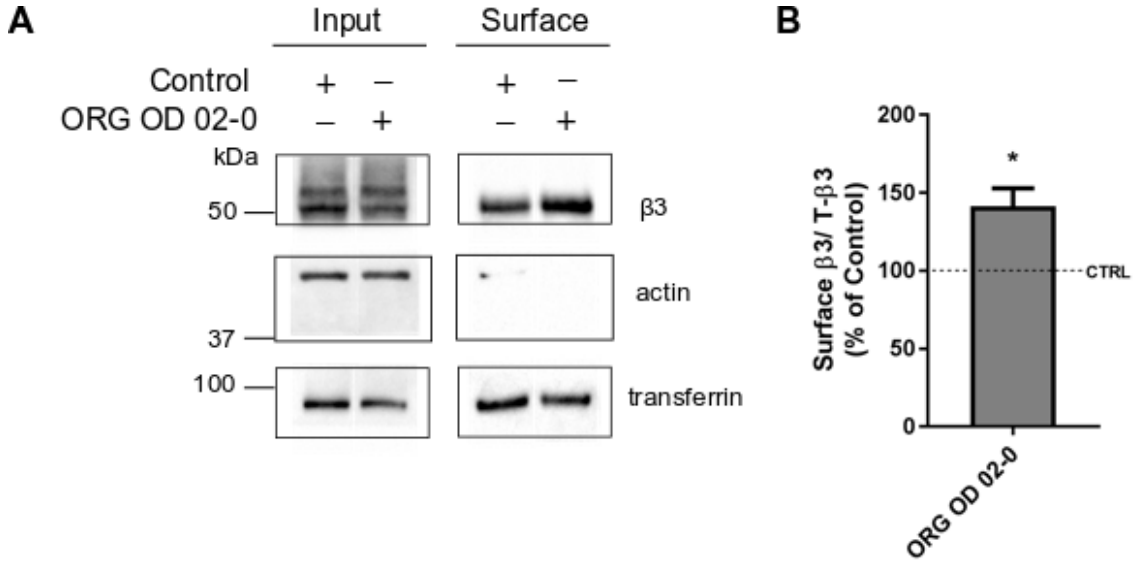

Figure 3.14
ORG OD 02-0 enhances β3 surface levels in the hippocampus. (A) Hippocampal slices treated with 300 nM ORG OD 02-0 or control (DMSO) for 20 minutes and labeled with NHS-biotin to isolate membrane receptors with neutravidin. Input represents ~10% of total lysate. Positive and negative membrane protein controls are shown (transferrin and actin, respectively). (B) The ratio of surface β3/T-β3 was measured and values were normalized to cell surface levels in control treated slices (100%). 20 minute ORG OD 02-0 exposure increases $GABA_A$-β3 containing receptors at the plasma membrane in acute hippocampal slices

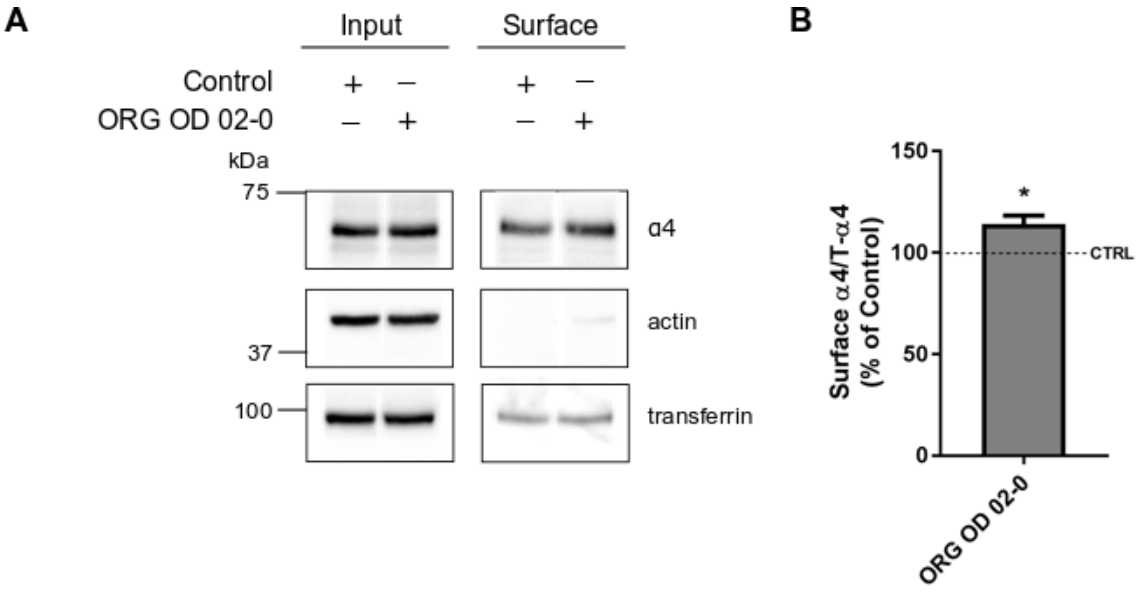

Figure 3.15
ORG OD 02-0 enhances α4 surface levels in the hippocampus. (A) Hippocampal slices treated with 300 nM ORG OD 02-0 or control (DMSO) for 20 minutes and labeled with NHS-biotin to isolate membrane receptors with neutravidin. Input represents ~10% of total lysate. Positive and negative membrane protein controls are shown (transferrin and actin, respectively). (B) The ratio of surface α4/T-α4 was measured and values were normalized to cell surface levels in control treated slices (100%). Twenty minute ORG OD 02-0 exposure increases $GABA_A$-α4 containing receptors at the plasma membrane in acute hippocampal slices (114.1% of control, n=5 mice (4 slices per treatment), $p<0.05$) Student's t-test.

3.3 Activation of membrane progesterone receptor alpha (mPRα) mediates β3$^{S408/409}$ phosphorylation, enhancement of β3-containing receptors and prevents run-down of GABA-evoked current

mPRs activate intracellular signaling involving PKC, PKA, and elevation in cytoplasmic Ca^{2+}(Rekawiecki, Kowalik, Slonina, & Kotwica, 2008). mPRs have been categorized as putative GPCR receptors, activating pertussis toxin sensitive $G_{i/o}$ proteins in MDA-MB-231 cells overexpressing human mPRα (Thomas et al., 2007). Since allopregnanolone has been shown to bind mPRα in MDA-MB-231 cells and activate G protein signaling, I hypothesized that mPRα may play a role in mediating β3 phosphorylation through downstream kinase activation. I decided to use HEK-293T cells for my overexpression studies since these cells are easy to culture and transfect (compared to GT1-7 cells). I first wanted to validate whether HEK-293T cells endogenously express mPRs.

3.3.1 Do HEK-293T cells express membrane progesterone receptors?

Due to lack of good antibodies against mPR subytpes except for mPRα, we decided to validate mRNA expression of mPRs using qPCR. We used primers against mPRα, β, γ, and ε listed in (Table 5). mPRδ is known to be expressed exclusively in the brain so we omitted this gene in our qPCR screen of HEK-293T cells (Tang et al., 2005). We found that mPRα and mPRβ mRNAs were robustly expressed in HEK-293T, with mPRβ mRNA being the most abundantly expressed (Figure 3.16).

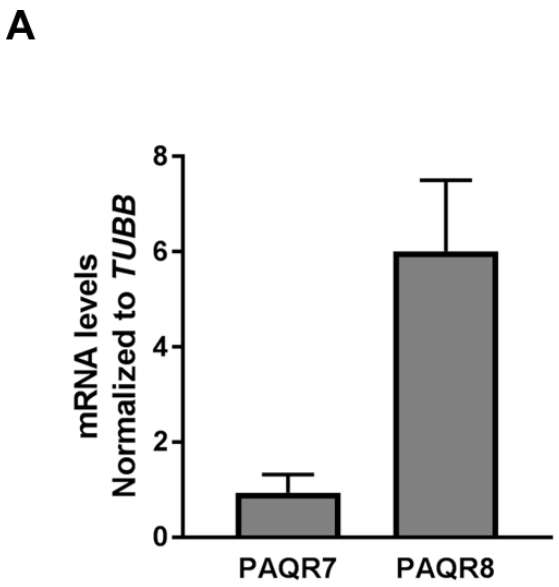

Figure 3.16
qPCR of mPR genes PAQR5(mPRγ), PAQR7 (mPRα), PAQR8 (mPRβ), PAQR9 (mPRε) in HEK-293T cells (A) Only mPRα and mPRβ were robustly expressed in HEK-293T. Relative abundance was normalized to β-tubulin (house-keeping gene) for all replicates. **Data by Jayashree Chadchankar**

3.3.2 Do HEK-293T cells overexpressing membrane progesterone receptor alpha, enhance β3 phosphorylation and GABA-mediated current?

When HEK-293T cells overexpressing α4β3 $GABA_A$Rs were treated with 100nM allopregnanolone, there was no enhancement of β3 phosphorylation (105.4% of control, n=5, p=0.4876) (Figure 3.17). Although our qPCR data confirmed mRNA expression of mPRα in HEK-293T (Figure 3.16), I decided to investigate the subcellular localization of mPRα proteins. Using cell surface biotinylation, I found mPRα levels to be very low at the plasma membrane (32.29% of over-expressed mPRα, n=5, $p<0.01$), even though total expression was robust (Figure 3.18). However, overexpression of mPRα-FLAG led to robust expression of mPRα at the plasma membrane (Figure 3.18).

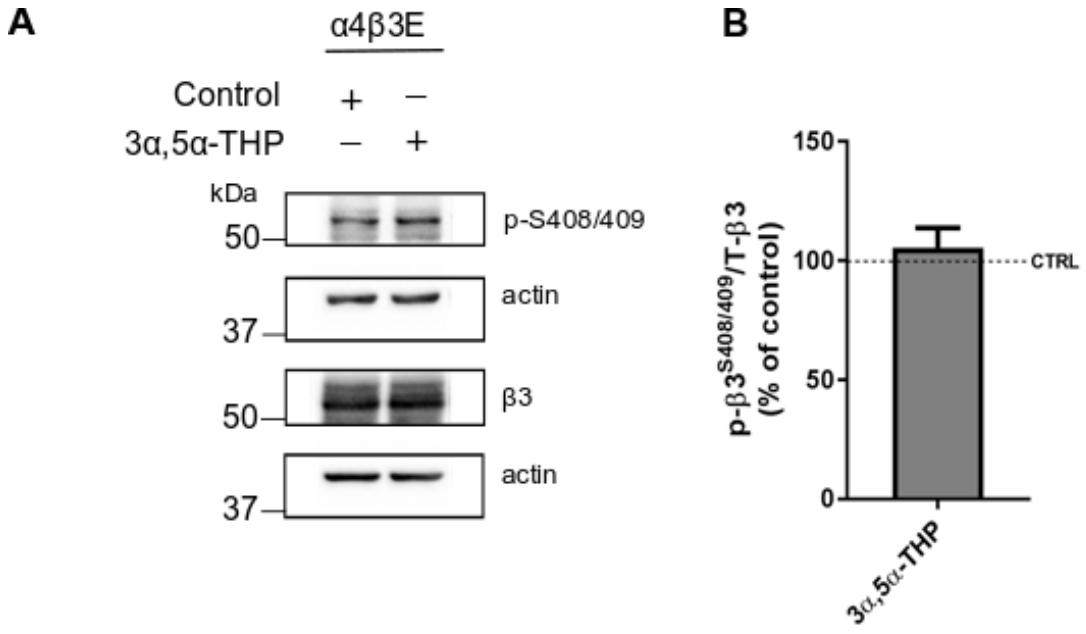

Figure 3.17
Allopregnanolone does not enhance β3 phosphorylation with transfection of α4β3 in HEK-293T. (A) HEK-293T cells co-transfected with α4 and β3 $GABA_A$ subunit cDNA (and empty plasmid) were treated with either control (DMSO) or 3α,5α-THP for 5 minutes. (B) The ratio of p-β3/T-β3 was measured and values were normalized to control (100%). Exposure to 3α,5α-THP did not show any enhancement in β3 phosphorylation (105.4% of control, n=5, p=0.4876). Student's t-test.

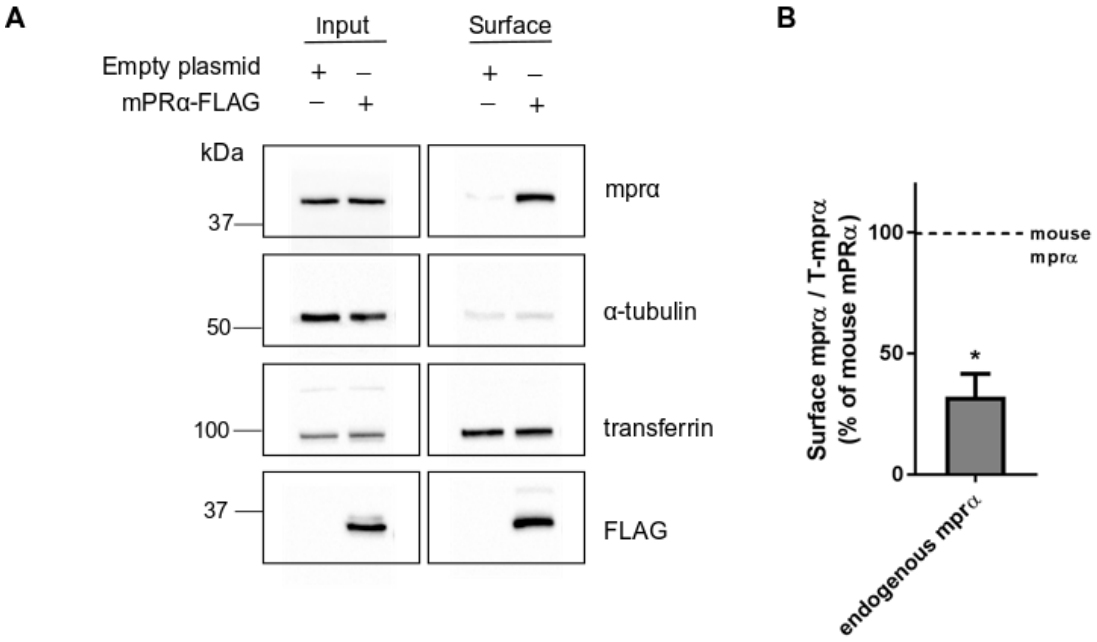

Figure 3.18
Low levels of mPRα are expressed at the plasma membrane in HEK-293T cells.
(A) Empty plasmid or mouse mPRα-FLAG cDNA was transfected into HEK-293T cells and cell surface receptors were labeled with NHS-biotin to isolate membrane receptors with neutravidin. Input represents ~10% of total lysate. Positive and negative membrane protein controls are shown (transferrin and α-tubulin, respectively). (B) The ratio of endogenous surface mPRα/T-mPRα was measured and values were normalized to mPRα-FLAG levels (100%). Endogenous mPRα in HEK-293T is not robustly expressed at the plasma membrane (32.29% of over-expressed mPRα, n=5, $p<0.01$). Student's t-test.

Thus, I decided to repeat neurosteroid treatment in these conditions—with overexpression of α4β3 together with mPRα. Under conditions of co-expressing mPRα, both allopregnanolone and ORG OD 02-0 significantly enhanced β3 phosphorylation (146.1% of control, n=5, $p<0.01$, 139.2% of control, n=3, $p<0.01$), respectively (Figure 3.19 & 3.20); this increase was prevented by PKC and PKA kinase inhibition (72.65%, n=3, $p<0.05$) (85.1%, n=3, $p=0.2115$), respectively (Figure 3.21 & 3.22).

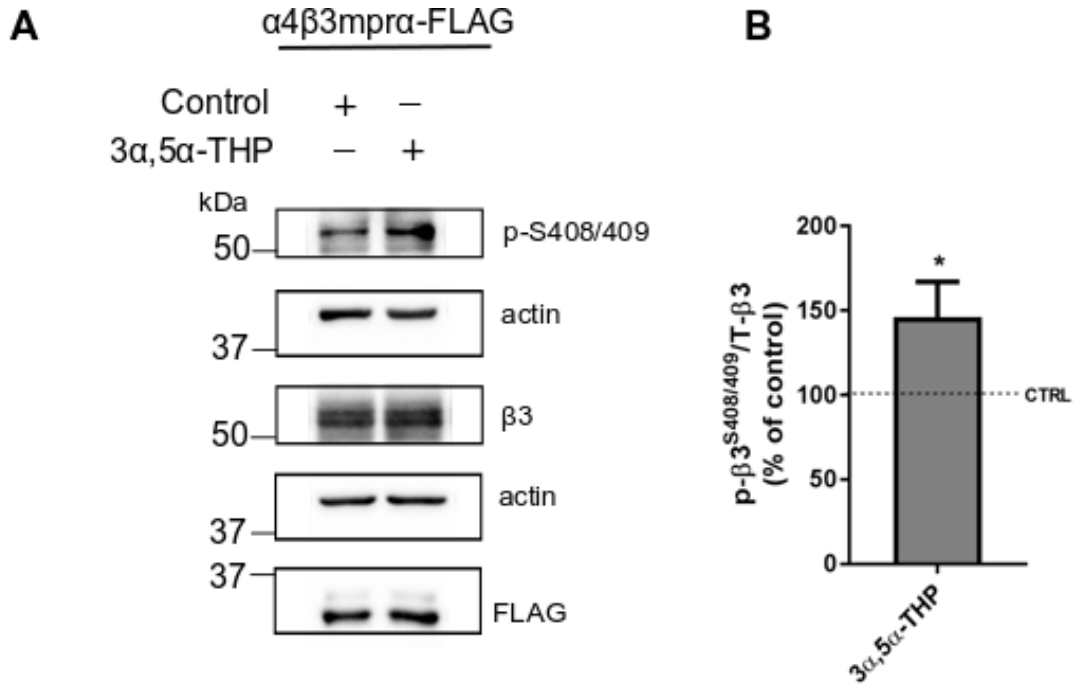

Figure 3.19
Allopregnanolone enhances β3 phosphorylation with overexpression of α4β3mPRα in HEK-293T cells. (A) Treatment of α4β3mPRα transfected HEK-293T cells with control (DMSO) or 3α,5α-THP for 5 minutes. (B) The ratio of p-β3/T-β3 was measured and values were normalized to control (100%). 3α,5α-THP treatment significantly enhanced β3 phosphorylation in HEK-293T cells (146.1% of control, n=5, $p<0.01$). Student's t-test.

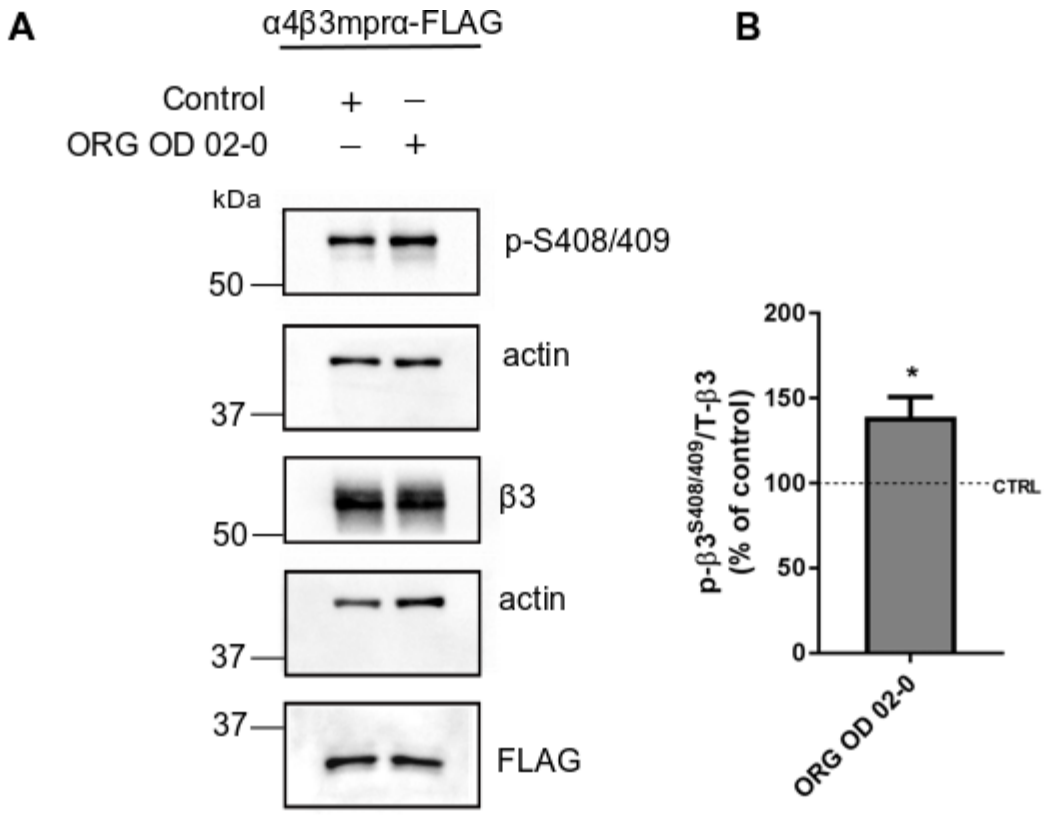

Figure 3.20
ORG OD 02-0 enhances β3 phosphorylation with overexpression of α4β3mPRα in HEK-293T cells. (A) Treatment of α4β3mPRα transfected HEK-293T cells with control (DMSO) or ORG OD 02-0 for 5 minutes. (B) The ratio of p-β3/T-β3 was measured and values were normalized to control (100%). ORG OD 02-0 treatment significantly enhanced β3 phosphorylation in HEK-293T cells (139.2% of control, n=3, $p<0.01$). Student's t-test.

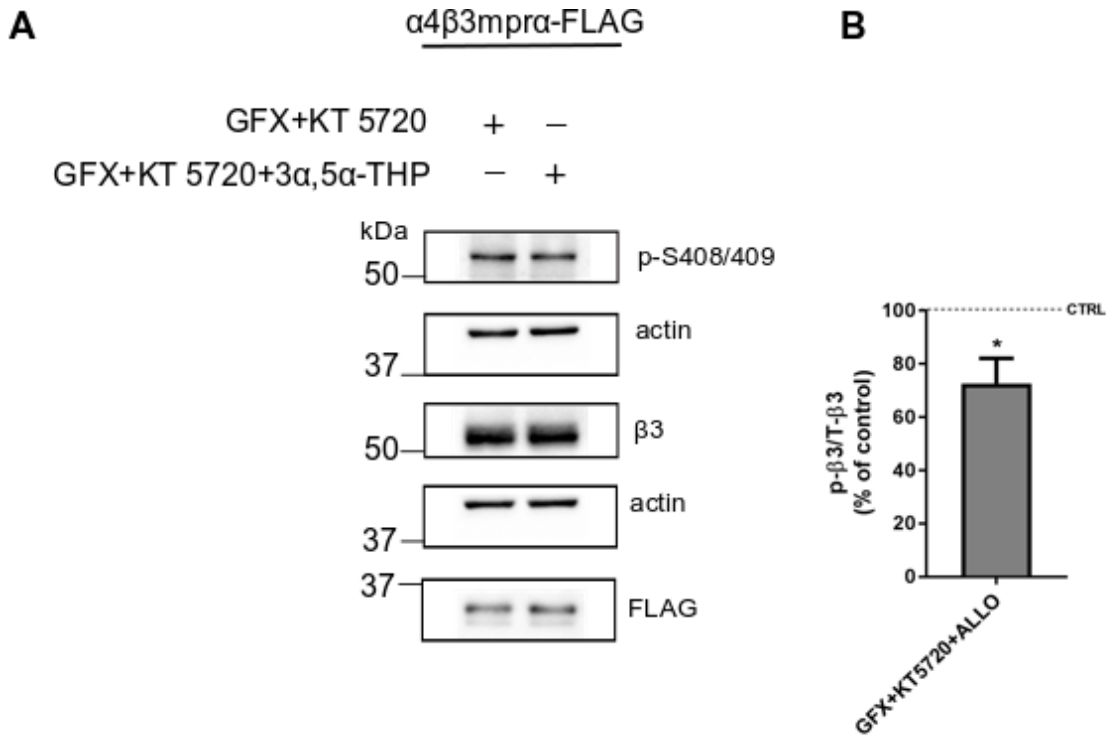

Figure 3.21
PKC/PKA inhibition blocks allopregnanolone mediated increase in β3 phosphorylation in α4β3mPRα expressing HEK-293T cells. (A) Pretreatment with PKC (GFX) and PKA (KT 5720) kinase inhibitors for 10 minutes followed by 5 minute treatment with control (DMSO) or 3α,5α-THP. (B) The ratio of p-β3/T-β3 was measured and values were normalized to GFX + KT 5720 alone (100%). PKC and PKA inhibition prevent 3α,5α-THP mediated increase in β3 phosphorylation in HEK-293T (72.65%, n=3, $p<0.05$). Student's t-test.

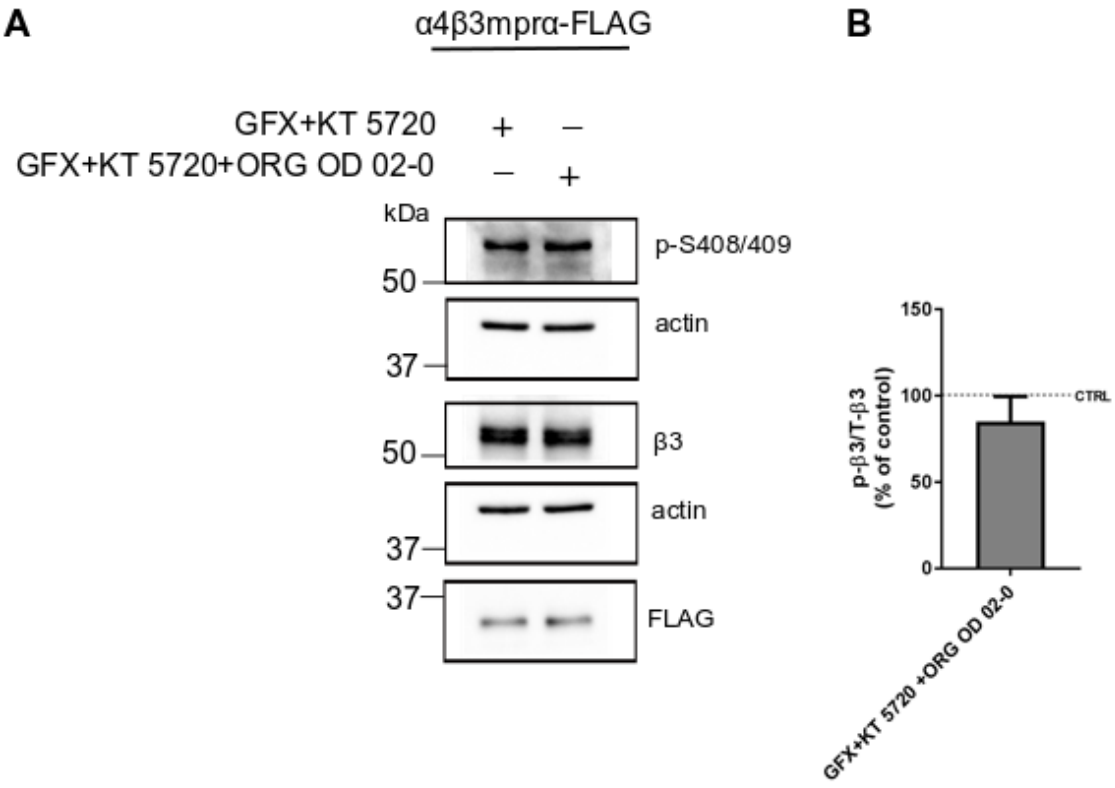

Figure 3.22
PKC/PKA inhibition blocks ORG OD 02-0 mediated increase in β3 phosphorylation in α4β3mPRα expressing HEK-293T cells. (A) Pretreatment with PKC (GFX) and PKA (KT 5720) kinase inhibitors for 10 minutes followed by 5 minute treatment with control (DMSO) or ORG OD 02-0. (B) The ratio of p-β3/T-β3 was measured and values were normalized to GFX + KT 5720 alone (100%). PKC and PKA inhibition prevent ORG OD 02-0 mediated increase in β3 phosphorylation in HEK-293T (85.1%, n=3, p=0.2115). Student's t-test.

To determine if this increase in β3 phosphorylation translated into an increase of functional channels, run-down of GABA-mediated currents in HEK293T cells expressing α4β3 $GABA_AR$ and mPRα treated with allopregnanolone and ORG OD 02-0 was also examined (Figure 3.23 & 3.24). In experiments performed in HEK293T cells expressing α4β3 $GABA_ARs$ along with mPRα, control current at 25 min was 64.5 ± 11% (n=6) of the initial GABA-mediated response. Internal application through the patch pipette of the mPRα specific agonist, ORG OD 02-0 (112.4 ± 14% n=7, p=0.02, Fig. 3.24), and allopregnanolone (113.2 ± 18% n=6, p=0.04, Fig. 3.23) prevented current run-down. The inhibition of run down was correlated with an ORG OD 02-0 dependent increase in β3 surface levels as determined by ICC (117.2% of control, n=3, $p<0.05$) (Figure 3.25).

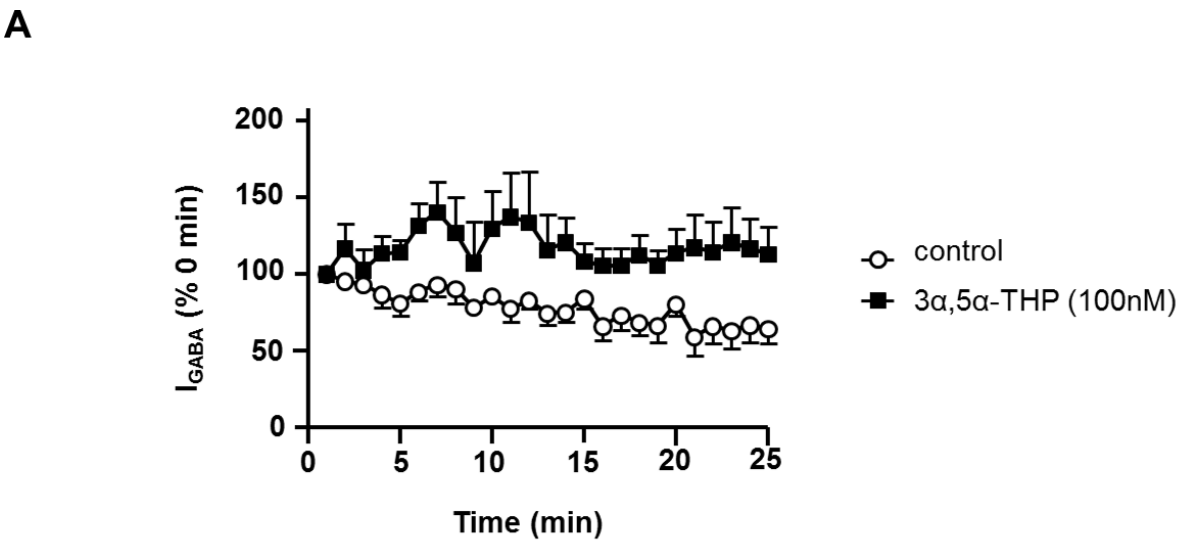

Figure 3.23
Allopregnanolone prevents run-down of current with overexpression of α4β3mPRα in HEK-293T cells. (A) Run-down of current in HEK-293T was measured in the presence of 3α,5α-THP or control (DMSO) applied intracellularly. Control currents at 25 min was 64.5 ± 11% (n=6) of the initial GABA-mediated response. Internal application of 3α,5α-THP through the patch pipette prevented current run-down (113.2 ± 18% n=6, p=0.04, Fig. 3.3.8). Student's t-test. **Data by Jen Yoo**

A

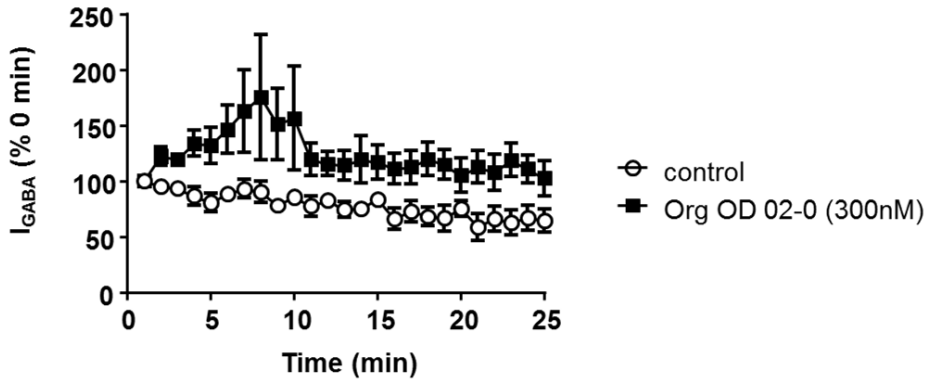

Figure 3.24
ORG OD 02-0 prevents run-down of current with overexpression of α4β3mPRα in HEK-293T cells. (A) Run-down of current in HEK-293T was measured in the presence of ORG OD 02-0 or control (DMSO) applied intracellularly. Application of ORG OD 02-0 through the patch pipette prevented current run-down (112.4 ± 14% n=7, p=0.02). Student's t-test. **Data by Jen Yoo**

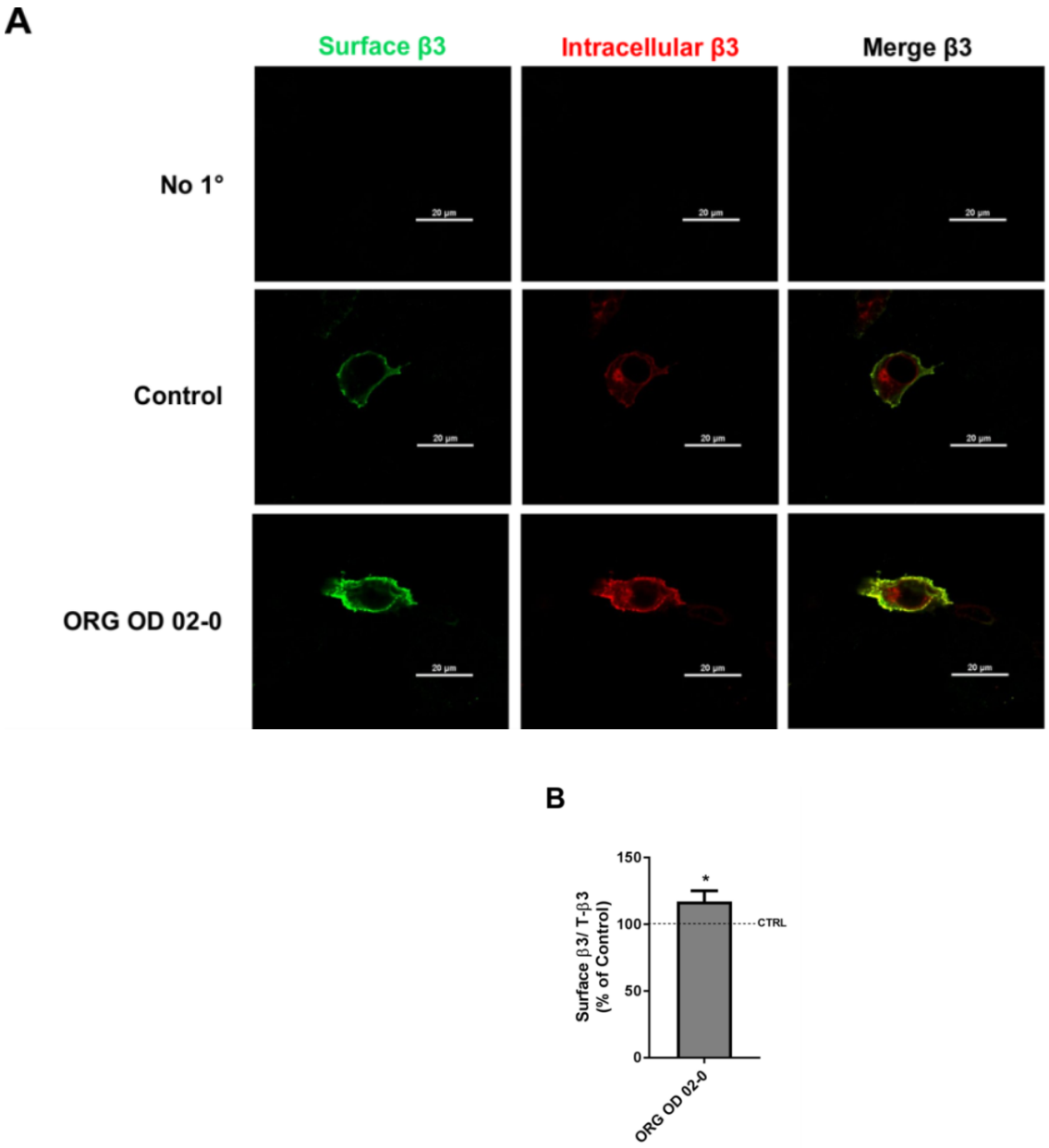

Figure 3.25
ORG enhances β3 surface levels with overexpression of α4β3mPRα in HEK-293T. (A) HEK-293T cells were transfected with α4β3mPRα and treated with either control (DMSO) or ORG OD 02-0 for 10 minutes. Cells were fixed and surface β3 subunits were labeled using a GFP secondary antibody. Cells were re-fixed, permeabilized and intracellular β3 subunits were labeled with a RFP secondary antibody. (B) 10 minute ORG OD 02-0 treatment significantly increased β3 surface levels (117.2% of control, n=3, $p<0.05$). Student's t-test.

Chapter 4: Discussion

GABAergic signaling allows for precise patterned firing of action potentials within brain circuits that is critical for proper cognition and behavior. This precision depends upon tightly controlled trafficking of different types of $GABA_A$Rs into the neuronal membrane. The information regarding extrasynaptic $GABA_A$R trafficking is limited compared with what is known for synaptic $GABA_A$Rs. There is a need for better understanding of the control and transport of this type of $GABA_A$R, considering that extrasynaptic $GABA_A$Rs appear to be a particularly key target for neurosteroids (Belelli et al., 2009; Belelli & Lambert, 2005). Endogenous neurosteroids play a critical role in controlling neuronal excitability (Stell et al., 2003). Synthetic neurosteroids have been shown to have long-term clinical efficacy at controlling depressive episodes and seizure activity (Doodipala Samba Reddy, 2010). We have previously shown that neurosteroids have prolonged effects on inhibition through a metabotropic mechanism; yet details of this mechanism remain elusive (Modgil et al., 2017). My thesis work presented here sheds light on a novel signaling mechanism through which endogenous and synthetic neurosteroids manipulate the levels of inhibition.

The regulation of β3-containing $GABA_A$Rs is important for proper inhibition in the brain (Christopher N. Connolly, Krishek, McDonald, Smart, & Moss, 1996; Terunuma et al., 2008). Alterations in levels of the β3 subunit have been implicated in various neurodevelopmental and neurological diseases such as Fragile X Syndrome, Angelman's syndrome and epilepsy (Clayton-Smith, 1992; Deidda, Bozarth, & Cancedda, 2014; Terunuma et al., 2008). The accumulation of $GABA_A$Rs is regulated by both insertion and endocytotic pathways; where phosphorylation of the different $GABA_A$R subunits plays a key

role (N. J. Brandon, 2000; Mcdonald & Moss, 1997). Phosphorylation of residues in both the β3 and γ2 subunit prevents $GABA_A$Rs from being endocytosed from the plasma membrane (Kittler et al., 2005). Previous studies in our lab have shown that phosphorylation of serine residues 408 and 409 on the β3 subunit prevents clathrin dependent AP2 protein from binding to the β3 subunit and signaling endocytosis (Kittler et al., 2005). Furthermore, in a model of status epilepticus, there is an increase in AP2 binding to β3 subunits in CA1 neurons, resulting in reductions in both β3 surface levels and mIPSC amplitude (Terunuma et al., 2008). Intracellular application of a β3 peptide containing the AP2 binding motif competed with membrane β3 subunits to limit their AP2-mediated endocytosis and enhanced the amplitude of mIPSCs in these neurons (Terunuma et al., 2008). Phosphorylation of serine residue 383 on the β3 subunit also contributes to an increase in $GABA_A$R *insertion* into the plasma membrane from the Golgi (Saliba et al., 2012). Activation of voltage-gated calcium channels results in CaMKII phosphorylation of $\beta3^{S383}$ and increased insertion of β3 and α5-containing receptors (Saliba et al., 2012). Thus, phosphorylating β3 subunits is one way of enhancing GABAergic inhibition in the brain.

Neurosteroids can also enhance GABAergic inhibition through allosteric potentiation and more recently, via metabotropic mechanisms that modulate the trafficking of $GABA_A$Rs (Abramian et al., 2014; Adams et al., 2015; E.E. Baulieu & Robel, 1990; Modgil et al., 2017a). Our lab has shown that these two pathways are independent; where the potentiating effects are revealed by acute neurosteroid treatment and the metabotropic effects through chronic treatment (Modgil et al., 2017). I have elaborated on the metabotropic signaling downstream of neurosteroids in my current thesis work. I first demonstrate that in addition to THDOC, allopregnanolone and its synthetic analogue SGE-

516, both enhance $\beta3^{S408/409}$ phosphorylation and β3 surface levels in the hippocampus. Allopregnanolone-mediated phosphorylation of S408/409 is activated by both PKC and PKA kinases, however allopregnanolone enhancement of tonic current is blocked with PKC alone. Abramian et al., (2014) previously demonstrated that neurosteroid THDOC can mediate α4 phosphorylation of serine 443, which is blocked with PKC inhibitor GFX (Abramian et al., 2014). Taken together, this suggests that although multiple kinases are recruited by neurosteroids to phosphorylate the β3 subunit, PKC-dependent α4 phosphorylation is also necessary for neurosteroid mediated increases in tonic current. This is further corroborated in experiments that show that THDOC treatment of $\alpha4^{S443A}\beta3$-containing receptors in HEK-293T cells fails to prevent the rundown of current seen with $\alpha4^{S443A}\beta3$-containing receptors (Abramian et al., 2014). To evaluate the contribution of both sites to neurosteroid enhancement of $GABA_AR$ expression at the plasma membrane, experiments with $\alpha4^{S443A}\beta3^{S408A/409A}$ need to be conducted.

The dentate gyrus of the hippocampus is sensitive to the effects of neurosteroids due to the large population of extrasynaptic $GABA_ARs$ expressed in this region (Stell et al., 2003). α4, β3, β2 and δ subunit-containing $GABA_ARs$ mediate the majority of tonic inhibition in the dentate gyrus (Mtchedlishvili & Kapur, 2006; Nusser & Mody, 2002). Tonic inhibition in the CA1 is largely mediated by α5-containing receptors, although δ-containing receptors have been shown to mediate about 30% of tonic current in this region (Glykys et al., 2008; Pirker, Schwarzer, Wieselthaler, Sieghart, & Sperk, 2000). α and β subunits expressed without δ or γ subunits also make up a proportion of $GABA_ARs$ in the hippocampus (Mortensen Martin & Smart Trevor G., 2006a). My results demonstrate that allopregnanolone significantly enhances β3 phosphorylation in the dentate gyrus but not in

the CA1-CA3 regions, suggesting that α4β2-β3 or α4β2- β3δ receptors are sensitive to metabotropic modulation by neurosteroids. Furthermore, CA1-CA3 regions show enhanced S408/409 phosphorylation at basal levels, suggesting reduced phosphatase activity in this region. PP2A is the predominant phosphatase that dephosphorylates S408/409 in cortical neurons although, PP1α can also dephosphorylate these residues, as shown with GST-β3 fusion proteins (Jovanovic, 2004; Terunuma et al., 2008).

Neurosteroids bind the $GABA_AR$ between α and β subunit interfaces, where residue Q241 on α subunits is critical for the allosteric potentiation by neurosteroids (Hosie, Wilkins, & Smart, 2007). Abramian et al., (2014) have shown that mutating this residue does not occlude the metabotropic effects of neurosteroids, suggesting that neurosteroid potentiation of $GABA_ARs$ is independent from the trafficking effects (Abramian et al., 2014). To rule out the hypothesis that neurosteroids can directly bind and activate kinases, I confirmed through *in vitro* kinase assays that allopregnanolone could not activate kinases bound to β3 subunits. Thus, this suggests that allopregnanolone may activate intracellular signaling to recruit kinases to β3 subunits. However, the components of this intracellular mechanism have largely been unexplored until now.

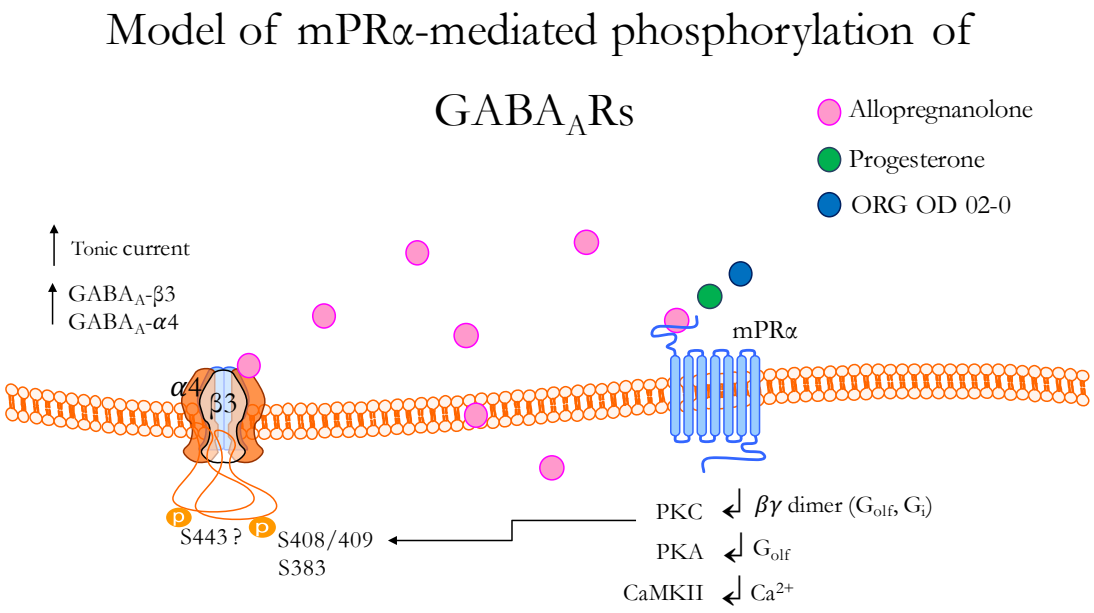

Figure 4.1
Proposed model of mPR-mediated phosphorylation of GABA$_A$Rs

I hypothesize that allopregnanolone mediated PKC, PKA and CaMKII signaling in the brain is a result of allopregnanolone binding to and activating membrane progesterone receptors (mPRs). My attention focused on mPRs after demonstrating that the parent steroid progesterone, also enhances β3 phosphorylation. Progesterone is not an allosteric modulator of GABA$_A$Rs, although its metabolites, allopregnanolone and THDOC show high potency in enhancing channel open time (Belelli & Lambert, 2005). Progesterone can bind to membrane progesterone receptors in the plasma membrane, as well as classical progesterone receptors in the cytoplasm that translocate to the nucleus to induce transcription (Brinton et al., 2008). To confirm that progesterone effects on GABA$_A$ phosphorylation are mediated from membrane receptors, I used a hypothalamic neuronal cell line (GT1-7 cells) that has negligible expression of nuclear progesterone receptors to test the effects of neurosteroid-mediated phosphorylation

(Sleiter et al., 2009). One caveat to using progesterone however, is that this molecule can be metabolized into allopregnanolone by 5α-reductase. Thus, to determine if progesterone alone can modulate β3 phosphorylation, future experiments should be done with co-application of 5α-reductase inhibitor finasteride, or with cell-impermeable progesterone. However, I circumvented the issue of progesterone metabolism by using the mPR synthetic agonist ORG OD 02-0 to specifically activate mPRs. ORG OD 02-0 treatment recapitulated allopregnanolone data in hippocampal slices by demonstrating enhancement of S408/409 phosphorylation through PKC and PKA kinases, enhancement in β3 surface levels, and enhancement in tonic inhibition. ORG OD 02-0 also increased surface levels of α4 $GABA_A$ subunits. We know that the α4 subunit is phosphorylated at serine 443 and phosphorylation of this residue results in increased insertion of α4-containing $GABA_A$Rs at the plasma membrane (Abramian et al., 2014). Although $\alpha4^{S443}$ phosphorylation is mediated by PKC, other kinases may also phosphorylate this site (Abramian et al., 2014). Further experimentation is necessary to investigate whether mPR-mediated enhancement of α4 surface levels is due to enhancement of S443 phosphorylation. It is also interesting to note that ORG OD-02-0, progesterone and allopregnanolone all enhance $\beta3^{S408/409}$ phosphorylation and surface expression of β3-containing receptors to similar levels. We know that ORG OD 02-0 demonstrates the greatest binding affinity to mPRs (IC_{50}=34 nM), followed by progesterone (IC_{50}=87 nM) and lastly with allopregnanolone (IC_{50}=1 uM) (Kelder et al., 2010). Although binding affinity may vary, the efficacy at which these steroids function may be similar.

All five subtypes of mPRs are expressed in the mammalian brain, with robust expression of mPRα and mPRε in the dentate gyrus of the hippocampus (Tang et al., 2005, Allen Brain Atlas). Since all mPR subtypes could theoretically be expressed within the same

cell, it would be difficult to study the contribution of a single mPR. To identify the mPR subtype important for mediating effects on $GABA_AR$ phosphorylation, I decided to use the well-established HEK-293T cells for transfection. These cells were useful to study effects of mPR subtype α (highly expressed in dentate gyrus) due to reduced expression of endogenous mPRα at the plasma membrane. Overexpression of C-terminally tagged mPRα demonstrated high levels of surface expression, suggesting that masking of a C- terminal lysine motif described by Lemale et al., 2008 dictates mPRα localization within the cell. Endogenously expressed adaptor proteins such as PGRMC1, may also mask this motif, allowing translocation of mPRα to the plasma membrane (Thomas et al., 2014). Overexpression of mPRα with α4β3 transfected receptors, enhanced allopregnanolone and ORG OD 02-0 mediated phosphorylation and cell surface expression of β3 subunits. The increase in β3 phosphorylation was prevented with PKC and PKA inhibition as shown in hippocampal slices.

Membrane progesterone receptors demonstrate both G protein-dependent and G protein-independent effects (Kasubuchi et al., 2017; Pang et al., 2013; Salazar et al., 2016; Sleiter et al., 2009; Thomas et al., 2007; Tubbs & Thomas, 2009). mPRα can activate G_i signaling that inhibits cAMP levels, but also can mediate effects through βγ dimers that activate phospholipase C and downstream PKC (Camps Montserrat et al., 2005). Our data suggests however, that PKA kinases are also involved in neurosteroid-mediated intracellular signaling. Although mPRα is known to activate G_i proteins and negatively modulate PKA activity, Tubbs and Thomas (2009) demonstrate that mPRα also couples to G_{olf} proteins in Atlanta croaker fish sperm to activate adenylyl cyclase and increase cAMP levels (Thomas et al., 2007; Tubbs & Thomas, 2009). Thus, mPRα may couple to G_{olf} proteins instead of G_i

proteins in the hippocampus. Further studies need to be done to tease apart the specific G protein involvement downstream of mPRα within the hippocampus. mPRε is also expressed in the dentate gyrus and couples to G_s proteins that activates PKA signaling (Pang et al., 2013, Allen Brain Atlas). Thus, mPRε signaling should also be investigated to explore its role in modulating $GABA_AR$ phosphorylation. mPR activation by allopregnanolone also resulted in an enhancement in S383 phosphorylation, a CaMKII substrate on the β3 subunit. Progestins binding to mPRs have been well known to increase intracellular calcium concentrations within seconds (Blackmore, Neulen, Lattanzio, & Beebe, 1991; Thomas, 2003). Thus, it is unsurprising that increases in intracellular calcium via mPR activation may activate CaMKII signaling to mediate $\beta3^{S383}$ phosphorylation (Shifman, Choi, Mihalas, Mayo, & Kennedy, 2006).

Binding affinity for allopregnanolone has only been tested with mPRα; therefore, other mPRs may also bind allopregnanolone (Kelder et al., 2010). In line with this, ORG OD 02-0 is a general agonist at mPRs thus, we cannot exclude effects from other mPR subtypes. In contrast to G protein effects, Kasubuchi et al., (2017) demonstrate that mPRs do not modulate cAMP levels in PC12 neuronal cells but activate AKT and MAPK signaling pathways downstream of mPRβ activation (Kasubuchi et al., 2017). Taken together, neurosteroid effects on intracellular signaling may be mediated through mPR pathways *and* other kinase signaling mechanisms. Thus, more experimentation is necessary to tease apart the distinct recruitment of PKC, PKA and CaMKII signaling downstream of neurosteroid action in the brain. Nevertheless, modulation of $GABA_AR$ phosphorylation by mPR activation suggests that these receptors play a role in enhancing $GABA_AR$ expression. Thus,

modulating mPR activity in aberrant physiological conditions such as postpartum depression and epilepsy could be a therapeutic avenue to restore GABAergic inhibition that is otherwise compromised.

Neurosteroids have been implicated in a variety of mood disorders including premenstrual dysphoric syndrome (PMS), bipolar disorder and post-partum depression (Bäckström Torbjörn et al., 2006; Carta, Bhat, & Preti, 2012; Hantsoo & Epperson, 2015). Neurosteroid levels are elevated in pregnancy; accompanied by a compensatory downregulation of $GABA_A$ δ subunits (J. Maguire & Mody, 2008). In the post-partum period, neurosteroid levels drop sharply and are correlated with increases in $GABA_A$ δ subunit expression. It is hypothesized that during post-partum depression, the down regulation of neurosteroids together with lack of upregulation of δ subunits, partly contributes to the depressive phenotypes (J. Maguire & Mody, 2008). In recent clinical studies, Sage Therapeutics has demonstrated that its lead compound brexanolone (allopregnanolone) has shown pronounced efficacy in Phase 3 clinical trials for post-partum depression (Kanes et al., 2017). We know that sustained effects of neurosteroids enhance $GABA_AR$ expression and GABAergic inhibition, however the precise mechanism through which this is achieved has been largely unknown-- until now.

For the first time, I identify a mechanism by which neurosteroids increase inhibition by activating membrane progesterone receptors, a mechanism that is *independent* of allosteric modulation of $GABA_ARs$. I hypothesize that mPR activation could partly contribute to the efficacy of the aforementioned brexanolone treatment in post-partum depression. However,

we do not know the details of the signaling mechanisms downstream of mPRs. Further characterization of mPR signaling is needed to better understand the physiological roles of neurosteroids and how they can be manipulated for treatments of neurological disorders.

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