Role of serotonergic signaling in $GABA_A$ receptor phosphorylation and functional expression

MANSI VITHLANI

A thesis submitted to UCL for the degree of Doctor of Philosophy

July 2009

Department of Neuroscience, Physiology and Pharmacology

UCL

I,  , confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. This copy has been supplied on the understanding that it is copyrighted material and that no quotation from the thesis may be published without proper acknowledgment.

To my mummy

Abstract

γ-aminobutyric acid type-A ($GABA_A$) receptors are heteropentameric ligand-gated chloride channels that mediate the majority of fast synaptic inhibition in the brain. Emerging evidence indicates that their functional expression is subject to dynamic modulation by phosphorylation. However, the cell signaling molecules responsible for regulating $GABA_A$ receptor phosphorylation and thus the efficacy of neuronal inhibition remain to be identified. The β subunits are of particular interest in this context as their intracellular domains contain conserved serine residues (S409 in β1, S410 in β2 and S408/9 in β3), known substrates for a number of protein kinases, including PKA and PKC. *In vitro* binding experiments showed that phosphorylation and/or mutation of these residues confers a reduction in binding of $GABA_A$ receptor β subunits to the μ2 adaptin of the clathrin adaptor protein (AP)-2 complex - a critical regulator of $GABA_A$ receptor endocytosis and surface number. Consistent with this, coimmunoprecipitation of AP2-μ2 adaptin with endogenous $GABA_A$ receptor β3 subunits was significantly reduced in cultured neurons treated with a potent inhibitor of S408/9 dephosphorylation that was accompanied by an increase in the stability of $GABA_A$ receptor β3 subunits at the cell surface.

Interestingly, recent studies have implicated PKA and PKC in the mediation of serotonergic modulation of $GABA_A$ receptor activity in the prefrontal cortex, suggesting that phosphorylation of $GABA_A$ receptor β subunits may underlie this regulation. To address this, a phospho-specific antibody directed against β3 at S408/9 was developed. Immunoblotting with anti-pS408/9-β3 demonstrated a

PKC-dependent increase in the phosphorylation state of $GABA_A$ receptor β3 subunits following enhanced 5-hydroxytryptamine type-2 ($5\text{-}HT_2$) receptor activation *ex vivo*. Moreover, *in vivo* biochemical and immunohistochemical studies revealed region-specific increases in $GABA_A$ receptor β3 subunit phosphorylation in mice dosed with the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Prozac™), a commonly prescribed antidepressant. Together, the results presented herein suggest that the phospho-dependent increase in $GABA_A$ receptor functional expression may underlie the therapeutic action of SSRIs.

Acknowledgments

First and foremost I would like to thank Professor S(inje) J(asper) Moss for being such a great mentor and friend. Steve's continual support, guidance and enthusiasm for my work have been invaluable during the course of my research, and I am very grateful to him for providing me with a home-away-from-home in the US and for always treating me like an 'Indian Princess'.

I would like to thank Wyeth Research (Princeton, NJ) for funding my studies and to express gratitude to Nick Brandon, who kindly let me run amok in his lab to carry out the focused microwave irradiation experiments. Special thanks to Miho Terunuma and Raquel Revilla-Sanchez for lending their scientific expertise, as well as Yolande Haydon for proofreading my thesis and for always having candy! I would also like to acknowledge everyone in the Moss lab for their scientific input and for putting up with my idiosyncrasies, in particular 'Japanese Princess', 'Boy', 'Jimmy' and 'Cookie'.

I am truly grateful for all my friends who have been there for the highs and lows of my Ph.D., particularly my friends back home in the UK - Rakhi, Sarbi, Nikhil and Fraser. Despite the thousands of miles that now separate us, you will always remain close to my heart. I am also thankful to Niv, Gauree, Amit, Taral and Kibwei for their unconditional love and generosity; I can no longer say that I have no family in the US! A big shout-out to everyone in Onda Latina, but especially F07 - Ooo! Ooo! To Nipa, Sonia, Akshita, Deepa, Tina, Ajay, Jay,

Sid, Raj and Ateet - I know that we've only known each other for a short while, but you all got me through one of the toughest times in my life. I feel extremely lucky to have met you and I know that I will always treasure our friendships.

Above all I am indebted to my loving family - Mum, brother, darling little niece (Priyanka) and nephew (Pranav), as well as my beloved grandparents, aunts, uncles and cousins, all of whom mean the world to me.

Contents

Title **1**

Dedication **3**

Abstract **4**

Acknowledgments **6**

Contents **8**

List of figures and tables **13**

Abbreviations **17**

Introduction **22**

GABA is the major inhibitory neurotransmitter in the brain **23**

GABA acts on three major receptor subtypes **25**

$GABA_A$ receptors 25

$GABA_B$ receptors 27

$GABA_C$ receptors 28

Pharmacology of $GABA_A$ receptors **29**

Structure and assembly of $GABA_A$ receptors **32**

$GABA_A$ receptor subunit expression within the brain **37**

Regional and cellular distribution 38

Subcellular localization 40

Functional significance of molecular heterogeneity **42**

Membrane trafficking of $GABA_A$ receptors **46**

Exocytosis of $GABA_A$ receptors to the plasma membrane.....46

Endocytosis of $GABA_A$ receptors from the plasma membrane.....52

Post-endocytic $GABA_A$ receptor sorting.....55

Postsynaptic targeting and clustering of $GABA_A$ receptors 56

Modulation of $GABA_A$ receptor function by post-translational modifications..... 59

Palmitoylation 59

Ubiquitination 60

Phosphorylation.....61

Phosphorylation-dependent $GABA_A$ receptor cross-talk..... 67

Methods & Materials 70

General materials..... 70

Chemicals and reagents.....70

Drugs 70

Equipment 70

Molecular biology..... 71

Constructs.....71

Growth media.....71

Agar plates 73

Bacterial strains.....73

Transformation of bacteria with plasmid DNA 73

Maxi preparation of plasmid DNA 74

Agarose gel electrophoresis 76

Cell Biology..... 77

Cell line culture 77
Primary neuronal culture 78
Histology 79
Animals 79
Acute PFC slice preparation 79
Focused microwave irradiation of the brain 80
Biochemistry 80
GST fusion protein purification 80
In vitro kinase assay 82
Antibodies 83
Antibody purification - affinity column assay 85
Preparation of cell and tissue extracts 87
SDS-PAGE 87
Coomassie stain of SDS-PAGE gels 88
Electro-transfer of SDS-PAGE gels 88
Western blotting 88
Dot blot assay 90
Overlay assay 91
Immunoprecipitation assay 91
Biotinylation cell surface assay 92
Immunohistochemistry 93
HRP staining of paraffin-embedded brain sections 93
Light microscopy 95

Results I: Development and characterization of phosphorylation sites S408/9-specific $GABA_A$ receptor β3 subunit antibody 97

Introduction 98

Results 102

Design and production of anti-pS408/9-β3 antibody 102

Characterization of anti-pS408/9-β3 antibody in western blotting 103

Focused microwave irradiation to measure *in vivo* $GABA_A$ receptor β3 subunit phosphorylation in the brain 116

Characterization of anti-pS408/9-β3 antibody in immunohistochemistry 116

Discussion 121

Results II: Phospho-dependent modulation of $GABA_A$ receptor β3 subunit functional expression 125

Introduction 126

Results 129

AP2-μ2 adaptin binding to $GABA_A$ receptor β subunits is negatively regulated by phosphorylation *in vitro* 129

S409 (but not S408) of the $GABA_A$ receptor β3 subunit is critical for AP2-μ2 adaptin binding *in vitro* 132

AP2-μ2 adaptin binding to the $GABA_A$ receptor β3 subunit is negatively regulated by phosphorylation in cultured neurons 135

Phosphorylation enhances the cell surface stability of $GABA_A$ receptor β3 subunits in cultured neurons 137

Discussion 141

Results III: Serotonergic modulation of $GABA_A$ receptor β3 subunit phosphorylation and cell surface stability 145

Introduction 146

Results 148

Serotonin enhances $GABA_A$ receptor β3 subunit phosphorylation via a PKC-mediated pathway in cultured neurons 148

Serotonin enhances $GABA_A$ receptor β3 subunit cell surface stability in cultured neurons 151

5-HT_2 receptors modulate PKC-mediated $GABA_A$ receptor β3 subunit in brain slices 154

Fluoxetine enhances $GABA_A$ receptor β3 subunit phosphorylation in a region-specific manner *in vivo* 157

Discussion 164

Discussion 178

Summary 167

S408/9 phosphorylation enhances the functional expression of β3 subunit-containing $GABA_A$ receptors 167

Modulation of $GABA_A$ receptor phosphorylation by serotonergic signaling may underlie the therapeutic efficacy of SSRIs 168

Future directions 170

References 185

List of figures and tables

Figure 1.1: GABA synthesis, uptake and metabolism.....24

Figure 1.2: $GABA_A$ receptor structure 34

Figure 1.3: Clathrin-mediated endocytosis.....53

Figure 3.1: Dot blot assay. UP-2028, UP-2029 and UP-2038 specifically recognize phospho- but not nonphospho-S408/9-β3 peptide.....104

Figure 3.2: UP-2028, UP-2029 and UP-2030 antisera specifically recognize GST-β3 phosphorylated in vitro by PKC.....106

Figure 3.3: Affinity purified anti-pS408/9-β3 antibody specifically recognizes GST-β3 phosphorylated in vitro by PKC.....107

Figure 3.4: Anti-pS408/9-β3 antibody specifically recognizes $GABA_A$ receptor β3 subunit in its phosphorylated state in COS-7 cells 109

Figure 3.5: Inhibiting PP1/PP2A activity increases anti-pS408/9-β3 immunoreactivity in cultured neurons 110

Figure 3.6: Phospho- but not nonphospho-S408/9-β3 peptide blocks immunodetection of phosphorylated $GABA_A$ receptor β3 subunit by anti-pS408/9-β3 antibody in cultured neurons.....112

Figure 3.7: λ-PPa treatment blocks immunodetection of phosphorylated $GABA_A$ receptor β3 subunit by anti-pS408/9-β3 and anti-total-β3 antibodies in cultured neurons.....113

Figure 3.8: Phospho- but not nonphospho-S408/9-β3 peptide blocks immunodetection of phosphorylated $GABA_A$ receptor β3 subunit by anti-pS408/9-β3 antibody in brain slices.....114

Figure 3.9: λ-PPa treatment blocks immunodetection of phosphorylated $GABA_A$ receptor β3 subunit by anti-pS408/9-β3 antibody in brain slices 115

Figure 3.10: Focused microwave irradiation of the brain preserves *in vivo* $GABA_A$ receptor β3 subunit phosphorylation.....117

Figure 3.11: Focused microwave irradiation minimizes *in vivo* $GABA_A$ receptor β3 subunit phosphorylation animal-animal variability in the brain 118

Figure 3.12: HRP staining of paraffin-embedded brain sections from mice fixed by focused microwave irradiation.....120

Figure 4.1: PKA-induced phosphorylation of GST-β1, β2 and β3 fusion proteins shifts ^{35}S-μ2 binding curves to the right *in vitro*.....131

Figure 4.2: PKA-induced phosphorylation decreases ^{35}S-μ2 binding to GST-β1, β2 and β3 fusion proteins *in vitro* 132

Figure 4.3: Selective mutation of S409 but not S408 decreases ^{35}S-μ2 binding to GST-β3 fusion proteins *in vitro*.....134

Figure 4.4: Selective PKC-induced phosphorylation of S409 but not S408 decreases ^{35}S-μ2 binding to GST-β3 fusion proteins *in vitro* 136

Figure 4.5: Inhibiting PP1/PP2A activity decreases coimmunoprecipitation of AP2-μ2 adaptin with $GABA_A$ receptor β3 subunits in cultured neurons 138

Figure 4.6: Inhibiting PP1/PP2A activity increases biotin-labeled $GABA_A$ receptor β3 subunits on the surface in cultured neurons 140

Figure 5.1: Serotonin increases $GABA_A$ receptor β3 subunit phosphorylation in cultured neurons 149

Figure 5.2: Serotonin-induced increase in $GABA_A$ receptor β3 subunit phosphorylation is potentiated by fluoxetine in cultured neurons 151

Figure 5.3: Serotonin increases PKC phosphorylation in cultured neurons 152

Figure 5.4: Serotonin-induced increase in $GABA_A$ receptor β3 subunit phosphorylation is blocked by calphostin C in cultured neurons 153

Figure 5.5: Serotonin increases biotin-labeled $GABA_A$ receptor β3 subunits on the surface in cultured neurons 155

Figure 5.6: DOI increases $GABA_A$ receptor β3 subunit phosphorylation in brain slices..... 156

Figure 5.7: DOI-induced increase in $GABA_A$ receptor β3 subunit phosphorylation is blocked by GFX in brain slices 158

Figure 5.8: Fluoxetine treatment increases $GABA_A$ receptor β3 subunit phosphorylation in the brain *in vivo* 159

Figure 5.9: Regional and cellular distribution of fluoxetine-induced increase in $GABA_A$ receptor β3 subunit phosphorylation in the PFC 160

Figure 5.10: Regional and cellular distribution of fluoxetine-induced increase in $GABA_A$ receptor β3 subunit phosphorylation in the hippocampal formation ...162

Table 1.1: $GABA_A$ receptor associated proteins 47

Table 1.2: $GABA_A$ receptor phsophorylation sites 63

Table 2.1: DNA constructs used in this study 72

Table 2.2: Antibodies used in this study 84

Table 2.3: Preparation of SDS-PAGE gels 89

Abbreviations

A	Absorbance
A293	Human embryonic kidney cell line
ACS	American Chemical Society
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
AIS	Axon initial segment
AKAP	A-kinase anchoring protein
AP	Adaptor protein
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BIG	Brefeldin A-inhibited guanine nucleotide exchange factor
BSA	Bovine serum albumin
CA	*Cornu Ammonis*
CaMKII	Calcium/calmodulin-dependent kinase II
CACA	*Cis*-4-aminocrotonic acid
β-CCE	β-carboline-3-carboxylate
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
COS-7	African green monkey kidney cell line
cpm	Counts per minute
D	Dopamine
DGC	Dystrophin glycoprotein complex

dH_2O	Deionized water
DIV	Days *in vitro*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOI	R(-)-dimethoxy-4-iodoamphetamine
DTT	Dithiothreitol
E18	Embryonic day 18
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERM	Ezrin/radixin/moesin
FBS	Fetal bovine serum
GABA	γ-aminobutyric acid
GABARAP	$GABA_A$ receptor associated protein
GABAT	GABA transaminase
GAD	Glutamic acid decarboxylase
GAT	GABA transporter
GEC	Guinea-pig endometrial cells
GFP	Green fluorescent protein
GFX	GF-109203X
GODZ	Golgi-specific DHHC zinc finger domain protein
G protein	Guanine nucleotide-binding protein
GRIP	Glutamate receptor-interacting protein
GST	Glutathione S-transferase

h	Hour(s)
HAP	Huntingtin-associated protein
HBSS	Hank’s Balanced Salt Solution
HEK-293	Human embryonic kidney cell line
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
5-HT	5-hydroxytryptamine
ICD	Intracellular domain
IgG	Immunoglobulin G
i.p.	Intraperitonealy
IP	Immunoprecipitation
IPSC	Inhibitory postsynaptic current
IPTG	Isopropylthio-β-D-galactoside
KCC	Potassium chloride cotransporter
kb	Kilobase
K_d	Dissociation constant
kDa	Kilodalton
Kir	Inwardly rectifying potassium channels
LB	Luria Bertani
LC	Light chain
MAP	Microtubule associated protein
min	Minute(s)
nACH	Nicotinic acetylcholine (receptor)
NG108-15	Neuroblastoma-glioma hybrid cell line

PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PDBu	Phorbol 12, 13-dibutyrate
PFC	Prefrontal cortex
PI	Protease inhibitor
PKA	cAMP-dependent protein kinase
PKB	Protein kinase B (also known as Akt)
PKC	Calcium/phospholipid-dependent protein kinase
PKG	cGMP-dependent protein kinase
PKM	A constitutively active catalytic domain of PKC
PLC	Phospholipase C
Plic	Protein that links integrin-associated protein with the cytoskeleton
PLL	Poly-L-lysine
PLP	Pyridoxal phosphate
PP (or PPa)	Protein phosphatase
PRIP	Phospholipase C-related catalytically inactive protein
PSD	Postsynaptic density
PSD-95	Postsynaptic density protein of 95 kDa
RACK	Receptor for activated C kinase
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rotations per minute
s	Second(s)

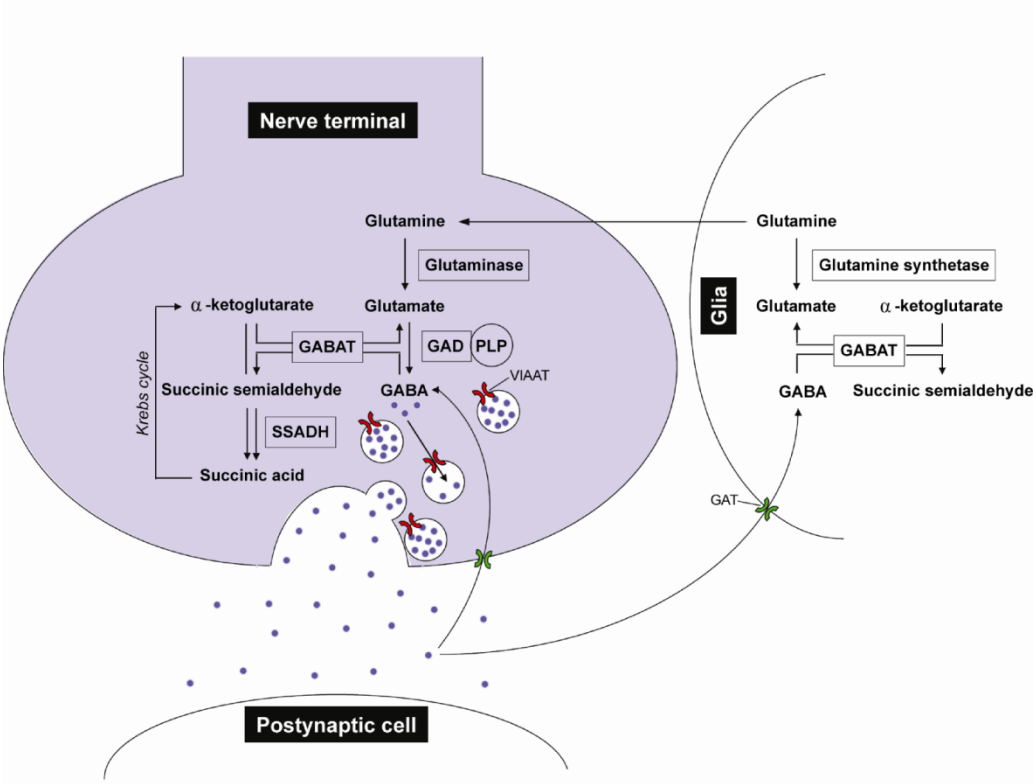
SDS	Sodium dodecyl sulphate
s.e.m	Standard error of the mean
SERT	Serotonin transporter
sEPSC	Spontaneous excitatory postsynaptic current
SG	Stratum granulare
shRNAi	Small hairpin RNA interference
siRNA	Small interference RNA
SL	Stratum lucidum
SP	Stratum pyramidale
TAE	Tris-acetate EDTA
TBS	Tris-buffered saline
TBPS	t-butylbicyclophosphorothioate
TCA	Trichloroacetic acid
TE	Tris-EDTA
TEMED	N, N, N’, N’-tetrahydroisoxazolo-pyridin-3-ol
THDOC	Trahydrodeoxycorticosterone
TM	Transmembrane
TPMA	1,2,5,6-tetrahydropyridine-4-yl methyl phosphinic acid
TrK	Tyrosine receptor kinase
TTX	Tetrodotoxin
UBA	Ubiquitin-associated
UBL	Ubiquitin-like
VIAAT	Vesicular inhibitory amino acid transporter
VDCC	Voltage-dependent calcium channels

Introduction

GABA is the major inhibitory neurotransmitter in the brain

The amino acid γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the adult mammalian central nervous system (CNS). GABA was first reported to be present in large quantities in the mammalian brain in 1950 independently by both Awapara and Roberts (Awapara et al., 1950; Roberts and Frankel, 1950), who also demonstrated its synthesis from the excitatory neurotransmitter glutamate by glutamic acid decarboxylase (GAD) (Fig 1.1). It is now estimated that at least one third of all CNS neurons utilize GABA as their primary neurotransmitter, and that most of these GABAergic neurons are interneurons and therefore uniquely able to alter the excitability of local circuits within a given brain region (Bloom and Iverson, 1971).

Interest in GABA as an inhibitory neurotransmitter was initiated by Bazemore and Florey, who demonstrated that GABA was the major component of an inhibitory factor (Factor I) isolated from mammalian brain that could mimic the effect of stimulating presynaptic inhibitory neurons at the crustacean stretch receptor and neuromuscular junction (Bazemore et al., 1957; Boistel and Fatt, 1958; Florey and McLennan, 1959; Kuffler and Edwards, 1958). Taking advantage of the numeric simplicity of the crustacean nervous system, further work carried out by Kravitz and his collaborators provided unequivocal evidence for GABA as an inhibitory neurotransmitter in invertebrates (Kravitz et al., 1963; Otsuka et al., 1966). However, it proved considerably more difficult to firmly establish its role as a neurotransmitter in the vertebrate CNS. This was



Adapted from C3 Neuropharmacology – UCL, 1998

Figure 1.1: GABA synthesis, uptake and metabolism. GABA is synthesized in nerve terminals from the amino acid glutamate in a reaction catalyzed by glutamic acid decarboxylase (GAD), requiring pyridoxal phosphate (PLP) as a cofactor. The vesicular inhibitory amino acid transporter (VIAAT) then transports GABA into synaptic vesicles, where it is stored and released in a calcium-dependent manner upon depolarization of the pre-synaptic membrane. Following release into the synaptic cleft, the actions of GABA are terminated principally by its re-uptake into GABAergic presynaptic nerve terminals and/or surrounding glia by plasma membrane GABA transporters (GATs). GABA is then metabolized by GABA-transaminase (GABAT) which, like GAD, requires PLP as a cofactor. This transamination reaction breaks down GABA to succinic semialdehyde and, in the presence of α-ketoglutarate, the GABA precursor glutamate. The enzyme succinic semialdehyde dehydrogenase (SSADH) rapidly converts succinic semialdehyde into succinic acid, which then enters the Krebs cycle.

resolved when Krnjevic and Schwartz showed that exogenous application of GABA to cerebral cortical neurons results in membrane hyperpolarization with similar reversal potentials to those observed with the endogenous ligand (Krnjevic and Schwartz, 1967). There followed an exponential growth in research activities on GABA to define all the necessary criteria of a classical neurotransmitter and its overall role in the physiology of the brain.

GABA acts on three major receptor subtypes

GABA exerts its powerful inhibitory influence by acting on three distinct classes of GABA receptors based on their electrophysiological and pharmacological properties. Ionotropic $GABA_A$ and $GABA_C$ receptors are fast acting ligand-gated chloride channels, while metabotropic $GABA_B$ receptors are coupled indirectly via guanidine nucleotide-binding proteins (G proteins) to either calcium or potassium channels to produce slow and prolonged inhibitory responses.

$GABA_A$ receptors

The $GABA_A$ receptor was the first to be identified and is responsible for mediating the majority of fast synaptic (phasic) inhibition in the brain. It is activated by muscimol, a rigid analog of GABA isolated from the hallucinogenic mushroom *Amantia muscaria* and competitively blocked by the convulsant bicuculline. In addition, $GABA_A$ receptors are subject to allosteric

modulation by a number of centrally active drugs, including benzodiazepines, barbiturates, neuroactive steroids, anesthetics and ethanol (reviewed in Sieghart et al., 2006).

The classic $GABA_A$ receptor-mediated rapid hyperpolarization of the membrane potential is attributed to the direct activation of an integral ion channel and the resultant influx of chloride along its electrochemical gradient, with the concentration-response curve exhibiting positive cooperativity consistent with the presence of at least two agonist binding sites on the receptor complex (Baumann et al., 2003). In developing neurons, $GABA_A$ receptor activation results in chloride efflux and depolarization of the cell. GABA has thus been shown to act as an excitatory neurotransmitter at this stage (Ben-Ari et al., 1989; Zhang et al., 1991). This is largely due to the fact that there is a relatively high intracellular chloride concentration in immature neurons which decreases upon the functional expression of the potassium chloride cotransporter (KCC)-2 as development proceeds, allowing GABA to become progressively inhibitory (Rivera et al., 1999).

In the cerebellum, phasic and tonic modes of $GABA_A$ receptor-mediated inhibition have been observed. This has been attributed to the presence of synaptic and extrasynaptic receptors, respectively (Brickley et al., 1996, 1999). Activation of $GABA_A$ receptors located at synaptic sites, following brief exposure to high (millimolar) concentrations of GABA (released from presynaptic vesicles), transiently moves the membrane potential away from the

spike threshold required for action potential generation and phasic inhibition of neuronal excitability. In contrast, low (submicromolar) concentrations of ambient GABA in the extracellular space can persistently activate spatially and temporally less restricted extrasynaptic receptors to generate a basal tonic inhibitory state (reviewed in Farrant and Nusser 2005).

$GABA_B$ receptors

It has now become apparent that most classical neurotransmitters, including GABA, have both rapid and modulatory effects. GABA mediates slow synaptic inhibition by acting on heterodimeric G protein-coupled $GABA_B$ receptors composed of R1 and R2 receptor subunits, which are widely expressed in the CNS and also in peripheral autonomic terminals (Bowery et al., 1987; Calver et al., 2000; Chu et al., 1990; Margeta-Mitrovic et al., 1999).

GABA acting on presynaptically located $GABA_B$ receptors inhibits neurotransmitter release, including the release of GABA in the case of autoreceptors, by depressing calcium influx through voltage-dependent calcium channels (VDCCs). The activation of postsynaptic $GABA_B$ receptors triggers the opening of inwardly rectifying potassium (Kir)-3 channels and potassium efflux, resulting in a prolonged neuronal hyperpolarization that underlies the late phase of inhibitory postsynaptic currents (IPSCs). In addition to regulating ion channel function, $GABA_B$ receptors can also inhibit adenylate cyclase activity and further decrease the cell's conductance to calcium (for a detailed review of $GABA_B$ receptor function refer to Bettler and Tiao, 2006). $GABA_B$

receptors were first discovered in the early 1980s when Bowery and others (1981) demonstrated a slow bicuculline-insensitive GABA-mediated inhibition of evoked [^{3}H]-noradrenalin release in the rat heart. It is now well established that $GABA_B$ receptors are activated by baclofen, which is a lipophilic derivative of GABA, and blocked by saclofen, neither of which have any effect on chloride conductance in central neurons (reviewed in Bonanno and Raiteri, 1993).

$GABA_C$ receptors

$GABA_C$ receptors are the newly accepted member of the GABA receptor family and are expressed predominantly in retinal neurons (Feigenspan and Bormann, 1994; Feigenspan et al., 1993; Polenzani et al., 1991). They are exclusively composed of ρ subunits that are structurally related to the $GABA_A$ receptor subunits, and as such may be viewed as a variant within the $GABA_A$ receptor family (reviewed in Bormann et al., 2000).

Although structurally homologous to the $GABA_A$ receptor, $GABA_C$ receptors are not blocked by bicuculline and do not recognize the characteristic $GABA_A$ receptor allosteric modulators. Instead, *cis*-4-aminocrotonic acid (CACA) a conformationally restricted analog of GABA is considered a selective $GABA_C$ receptor agonist (Johnston et al., 1975) and 1,2,5,6-tetrahydropyridine-4-yl methyl phosphinic acid (TPMA) a selective $GABA_C$ receptor antagonist (Ragozzino et al., 1996. Moreover, the endogenous ligand GABA is about an order of magnitude more potent at $GABA_C$ receptors, which deactivate and

desensitize more slowly than the transiently activated $GABA_A$ receptors, and therefore exhibit a more sustained response to GABA (reviewed in Bormann and Feigenspan, 1995). It is these differences between $GABA_A$ and $GABA_C$ receptors that have led the argument for the distinction of these two classes of GABA receptors (reviewed in Bormann, 2000).

Pharmacology of $GABA_A$ receptors

The pharmacology of $GABA_A$ receptors is rich and a key target for a number of clinically relevant compounds including benzodiazepines, barbiturates, neuroactive steroids, certain classes of anesthetics, and ethanol. These allosteric compounds bind to discrete sites distinct from the agonist binding site to either positively or negatively modulate GABA-gated chloride conductance (reviewed in Sieghart et al., 2006).

Benzodiazepines are a class of psychoactive drugs with varying anxiolytic, anticonvulsant, muscle relaxant, sedative, hypnotic, and amnestic properties. However, chronic use of these drugs results in tolerance and physical dependence limiting their clinical use. Classical benzodiazepine agonists, for instance diazepam (Valium™), act by increasing the affinity of $GABA_A$ receptors for GABA and by increasing the frequency of channel opening in response to GABA binding. In contrast, inverse agonists, such as β-carboline-3-carboxylate (β-CCE), decrease the frequency of channel opening and are highly anxiogenic and proconvulsant. Antagonists, such as flumazenil (Anexate™),

have no intrinsic activity but block the actions of both agonists and inverse agonists and are sometimes used to reverse the CNS depressant effects of benzodiazepine agonists in anesthesia and overdose. There has been considerable interest in the development of such partial agonists in the treatment of anxiety as bretazenil which has submaximal efficacy at the receptor and thus exhibits continued anxiolysis with reduced sedative and withdrawal side-effects (Sellers et al., 1992).

Like the classical benzodiazepines, barbiturates display a wide spectrum of effects from sedation to general anesthesia and are also effective as anxiolytics and anticonvulsants. However, barbiturates have now largely been replaced by benzodiazepines due to their increased addiction potential and risk of lethal overdose. Sedative barbiturates such as phenobarbitone (Luminal™) or the intravenous general anesthetic thiopentone (Pentothal™) potentiate responses to GABA by increasing the mean channel open time, with little effect on opening frequency. In addition, at high (anesthetic) doses barbiturates can activate $GABA_A$ receptors directly and therefore also increase the maximal response, thus contributing to their toxicity (Twyman et a., 1989).

Since the 1940s it has been recognized that the endogenous neuroactive steroids of the pregnane series such as allopregnanolone and allotetrahydrodeoxy-corticosterone (THDOC), have innate anxiolytic, anticonvulsant and sedative activity. These early studies led to the development of the general intravenous anesthetic Althesin™, which was withdrawn from human use due to rare but

serious toxicity but is still used in veterinary medicine. The active component of this drug - alphaxolone - has been shown at low concentrations to stereoselectively facilitate GABA-mediated inhibition by increasing both the frequency and duration of channel opening. However, at higher concentrations alphaxolone has also been shown to directly activate $GABA_A$ receptors in a manner inconsistent with competition for a common binding site with the barbiturates (Clarke et al., 1973). More recently, another synthetic steroid - ganaxolone - has been developed and is currently in clinical trials for the treatment of epilepsy. This drug has less sedative effects than the anesthetic steroids depicted above, but is a potent anticonvulsant that may have some advantages over older antiepileptics, mainly less development of tolerance with long term treatment (reviewed in Nohria and Giller, 2007). In addition to anesthetic steroids and barbiturates, a number of other anesthetic compounds belonging to different chemical classes, such as the inhalation anesthetics isoflurane (Aerrane™) and halothane (Fluthane™) and the intravenous anesthetics propofol (Rapinovet™) and etomidate (Amidate™), are also known to potentiate GABA-gated chloride conductance at pharmacologically relevant concentrations. However, at higher concentrations they too can directly open $GABA_A$ receptor-associated chloride channels (Banks et al., 1999; Kitamura et al., 2004). Although the majority of studies have focused on the $GABA_A$ receptor, it is evident that some anesthetics, such as ketamine (Anaket-V™) and nitrous oxide (‘laughing gas’), do not produce their effects by directly interacting with the receptor complex (reviewed in Hirota, 2006).

Similar to other $GABA_A$ receptor modulators discussed above, ethanol exhibits an array of CNS depressant effects (reviewed in Deitrich et al., 1989). Prolonged exposure to and subsequent withdrawal of ethanol are associated with marked changes in $GABA_A$ receptor subunit gene expression and subsequently the function and pharmacology of the assembled channel complexes, which have been implicated in alcohol withdrawal syndrome (Sanna et al., 2003).

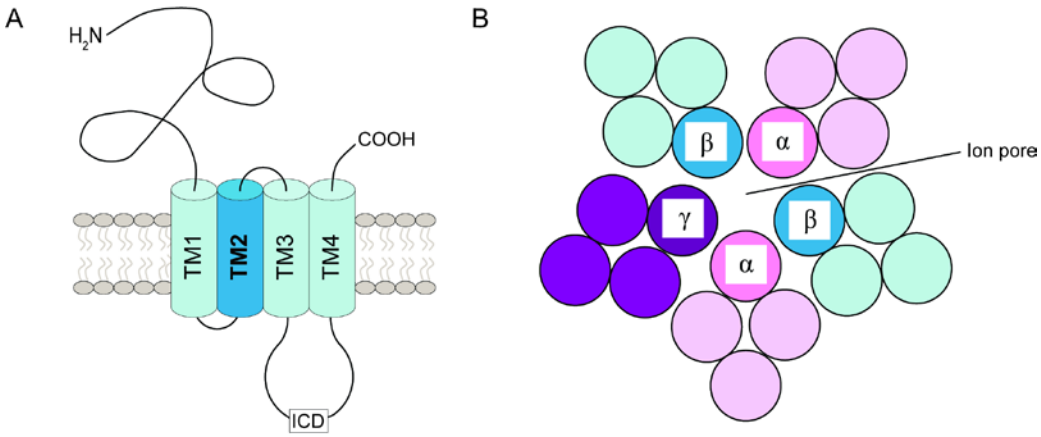
In addition, $GABA_A$ receptors are inhibited by a number of potent convulsants including t-butylbicyclophosphorothioate (TBPS) and picrotoxin, both of which noncompetitively block the receptors by binding to a site within the ion channel pore, effectively obstructing any ions from moving through the pore and stabilizing the receptor complex in an agonist-bound desensitized state (Jursky et al., 2000; Newland and Cull-Candy, 1992; Zhang et al., 1994). Finally, $GABA_A$ receptor function is also subject to regulation by divalent ions such as zinc (Smart et al., 2004; Smart et al., 1992) and changes in pH (Krishek et al., 1996).

Structure and assembly of $GABA_A$ receptors

$GABA_A$ (and $GABA_C$) receptors belong to the superfamily of cysteine-loop ligand-gated ion channels that comprises nicotinic acetylcholine (nACh) receptors, strychnine-sensitive glycine receptors, and 5-hydroxytryptamine type-3 ($5\text{-}HT_3$) receptors (reviewed in Connolly and Wafford, 2004). Members

of this receptor family are heteropentameric glycoproteins composed of homologous subunits that specifically recognize one another and assemble around an intrinsic ion pore (Unwin, 1993), which in the case of the $GABA_A$ receptor is chloride selective (reviewed in Sieghart et a., 2006).

In the mid 1980s collaborative work carried out by the groups of Barnard and Seeburg led to the cloning of the first two $GABA_A$ receptor subunits known, α and β (Schofield et al., 1987), following their successful purification from bovine brain earlier that decade (Sigel et al., 1982). Analysis of the deduced amino acid sequences of the α and β subunit cDNAs isolated by these investigators indicated that each subunit, approximately 50-60 kDa in size, consists of a large extracellular ligand binding N-terminal region and a short barely extruding C-terminus separated by four highly conserved hydrophobic transmembrane (TM)1-4 α-helices. In addition, a major cytoplasmic loop lies between TM3 and TM4 that is the most divergent part of the sequence among the subfamily (Fig 1.2). This intracellular domain (ICD) mediates the interaction with cytosolic proteins and is subject to a number of post-translational modifications. Coexpression of the α and β subunits in *Xenopus* oocytes resulted in the assembly of bicuculline-sensitive GABA-activated chloride channels that, however, lacked the characteristic benzodiazepine potentiation observed with native $GABA_A$ receptors (Angelotti and Macdonald, 1993). A novel $GABA_A$ receptor subunit (γ) that when coexpressed with α and β subunits conferred benzodiazepine sensitivity on the assembled receptor was then identified (Pritchett et al., 1989). It is now firmly established that the



Adapted from Jacob et al., 2008

Figure 1.2: $GABA_A$ receptor structure. (A) Transmembrane topology of the $GABA_A$ receptor. Each receptor subunit is composed of a large extracellular ligand binding N-terminal region that is also the site of action of various drugs followed by four hydrophobic transmembrane (TM)1-4 α-helices and a short, barely extruding C-terminus. A large intracellular domain (ICD) between TM3 and TM4 mediates the majority of protein-protein interactions and is also subject to a number of post-translational modifications. **(B)** Transverse view of the assembly of $GABA_A$ receptor subunits to form an ion channel. TM2 faces the lumen of the channel ion pore and TM4 is anchored in the lipid membrane. TM1 and TM3 interact with the neighboring subunits, respectively.

GABA binding site is located at the interface between α and β subunits (Baumann et al., 2003), whereas that of benzodiazepines lies between the α and γ subunits (Sigel and Buhr, 1997).

Nineteen $GABA_A$ receptor subunits have been cloned and sequenced from the mammalian brain thus far. These have been divided into eight classes on the basis of sequence identity (reviewed in Whiting et al., 1999): α(1-6), β(1-3), γ(1-3), δ, ε, π, θ, and ρ(1-3). Subunit isoforms within a single class share approximately 70% sequence identity but between classes this falls to 30-40%. Moreover, alternatively spliced variants of several of these subunits have been reported generating further subunit diversity and the potential for extensive molecular heterogeneity (reviewed in Sieghart et al., 1999). For example, the γ2 subunit exists in short (γ2S) and long (γ2L) forms, which differ in an eight amino acid insert in the ICD of the γ2L subunit (Whiting et al., 1990; Kofuji et al., 1991). Despite the plethora of $GABA_A$ receptor subunit isoforms, due to the selective oligomerization mediated by receptor assembly only a limited number of combinations are expressed *in vivo* (reviewed in Sieghart and Sperk, 2002; Sieghart et al., 1999). The majority of $GABA_A$ receptor subtypes in the brain are composed of α1β2γ2, followed by α2β3γ2 and α3β3γ2, with a likely stoichiometry of 2α.2β.γ, similar to that found for of nACh receptors (Chang et al., 1996; Farar et al., 1999; Knight et al., 2000; Tretter et al., 1997). To a lesser extent, δ, ε, π subunits replace the γ subunit to form benzodiazepine-insensitive receptor subtypes, whereas the θ subunit has been shown to replace the β subunit. Conversely, ρ subunits rarely coassemble with other $GABA_A$ receptor

subunits, but instead homo- as well as heterooligomerize with other ρ subunits to form the $GABA_C$ receptor (reviewed in Bormann et al., 2000).

To date, no receptor belonging to the superfamily of ligand-gated ion channels has been characterized structurally by X-ray crystallography. However, comparative modeling of the $GABA_A$ receptor based to the 4 Å resolution model of the *Torpedo* nACh receptor has for the first time provided an insight into the 3D organization of this ligand-gated ion channel (Ernst et al., 2005). The extracellular domain of each receptor subunit comprises a variable N-terminal domain and two β-folded sheets that form a twisted sandwich connected by a signature disulphide bridge. Confirming existing data, the GABA-binding sites are located in solvent-accessible pockets at the two β+α- subunit interfaces and that of benzodiazepines at the α+γ- interface (Ernst et al., 2003). The TM domain of the receptor is made up of four loosely-packed helical bundles resulting in a considerable amount of solvent-accessible space within subunits and at the subunit interfaces. The intersubunit pockets have been proposed to form a continuous groove with their extracellular counterparts, suggesting that they may play a role in the conformational mobility of the receptor but may also provide putative drug-binding sites. The intrasubunit pockets contain a number of amino acid residues that have been shown to be of key importance in the binding and/or efficacy of a number of modulatory drugs. For example, a pocket within the α1 subunit, defined by the presence of S270 in TM2 and A291 in TM3, is thought to correspond to the site of action of volatile anesthetics (Nishikawa et al., 2002). Similarly, the presence of a homologous

serine residue (S265) in the intrasubunit pocket of β1, which is replaced by an aspartic residue in the β2 and β3 subunit isoforms, is believed to account for the subtype-selectivity of etomidate and other related substances (Belelli et al., 1997). Future experiments, leading to the improvement in the accuracy of the models, will not only continue to provide a new perspective on existing data, but also pave the way for structure-based drug design.

$GABA_A$ receptor subunit expression within the brain

In addition to molecular determination of $GABA_A$ receptor assembly, subunit composition is further limited by the spatial and temporal pattern of subunit expression. *In situ* hybridization (Laurie et al., 1992; Persohn et al., 1992; Wisden et al., 1992) and immunohistochemical (Fritschy et al., 1992; Pirker et al., 2000; Sperk et al., 1997) studies have demonstrated that each one of the subunits has a distinct regional and cellular distribution within the brain. Moreover, different subunits and/or subunit combinations further dictate the subcellular localization of these ligand-gated chloride channels and determine their biophysical and pharmacological properties. Detailed knowledge of the molecular composition and the exact anatomical expression of different $GABA_A$ receptor subtypes is therefore crucial in understanding the physiological actions of GABA within the brain and for developing potentially clinically useful subtype-selective drugs that lack unwanted side-effects.

Regional and cellular distribution

The α1, β2, β3 and γ2 subunits indeed show the most prevalent expression and have been detected in numerous cell types throughout the brain (Laurie et al., 1992; Wisden et al., 1992; Pirker et al., 2000). The α2 and α3 subunits, like α1, are found in most brain regions, albeit at lower levels. Interestingly, α2 and α3 subunits are highly expressed in neonates compared to α1, suggesting that the level of expression of these subunits is developmentally regulated (Laurie et al., 1992b). The α3 subunit is the predominant receptor subtype expressed in the raphé nucleus, where only a small population of serotonergic neurons coexpress α1. The α3 subunit is also expressed in dopaminergic and noradrenergic neurons in the brainstem and shows an overlapping distribution with the θ and ε subunits, suggesting that novel $GABA_A$ receptors subtypes may regulate neuromodulatory and neruoendocrine systems in the brain (Moragues et al., 2002). In contrast, both α1 and α3 subunit immunoreactivities are present in GAD-positive neurons (Gao et al., 1993). The α4 subunit is restricted to the thalamus, striatum and molecular layer of the dentate gyrus and is the least abundant α subunit (Pirker et al., 2000; Wisden et al., 1992). The α5 subunit is enriched in the *Cornu Ammonis* (CA)1 region of the hippocampus, cerebral cortex and olfactory bulb, whereas the α6 subunit is found almost exclusively in the granular layer of the cerebellum (Laurie et al., 1992a; Pirker et al., 2000).

Despite a wide distribution of all three β subunits - notably the cerebral cortex, β1 is expressed at much lower levels than the β2 and β3 subunits (Pirker et al., 2000). In subcortical areas and the cerebellum, a higher level of expression of

one β subunit comes at the expense of another. For example, the β2 subunit is highly expressed in the thalamus compared to β1 and β3 subunits (Wisden et al., 1992; Pirker et al., 2000), whereas the β3 subunit is highly expressed in the striatum where the β2 subunit is less abundant. In the hippocampal formation, β1 and β3 subunit expression is higher than that of β2 (Wisden et al., 1992) and, in this region, β1 and β3 subunits are found mainly in the dendrites of pyramidal neurons, whereas the β2 subunit is expressed primarily in interneurons (Pirker et al., 2000). In addition, the β1 subunit is more concentrated in the CA2 than the CA1 or CA3 subfields of the hippocampus, whereas the reverse is true for β3 (Pirker et al., 2000). In the cerebellum, β2 and β3 subunits are enriched in the granular layer and the β1 subunit the molecular layer (Pirker et al., 2000).

The γ1 subunit is restricted to very few brain regions, essentially the central and medial amygdaloid nuclei, in pallidal areas, the substantia nigra pars reticulata and the inferior olive, and may replace the γ2 subunit in these areas (Pirker et al., 2000). In contrast, the γ3 subunit is distributed diffusely at low concentrations throughout the brain and constitutes a very limited number of receptors (Pirker et al., 2000). While the levels of the γ2 subunit remain relatively constant during ontogeny, that of the γ1 and γ3 subunits decreases with development (Fritschy et al., 1994). The distribution of the δ subunit parallels that of the α4 and α6 subunits and has been shown to replace the γ2 subunit in $GABA_A$ receptors containing the α4 or α6 subunits in the thalamus and granular layer of the cerebellum, respectively (Laurie et al., 1992a; Wisden et al., 1992; Pirker et al., 2000). The ε, π, θ are the least abundant $GABA_A$

receptor subunits in the brain, and currently little is known about the functional relevance of their expression.

Subcellular localization

Immunofluorescence and immunogold microscopy studies have revealed that synaptic and extrasynaptic receptors differ in their subunit composition. For example, in the cerebellum the γ2 subunit has been shown to be a component of all postsynaptic $GABA_A$ receptors, whereas the δ subunit is found almost exclusively at extrasynaptic sites (Nusser et al., 1998b). The importance of the γ2 subunit in the postsynaptic targeting of $GABA_A$ receptors is demonstrated by the profound reduction in the clustering of both $GABA_A$ receptors and the inhibitory synaptic marker gephyrin seen in γ2 subunit knockout mice (Essrich et al., 1998). Hence, $GABA_A$ receptors incorporating a γ2 subunit together with α1-3 subunits (α1-3β2/3γ2) are the predominant receptor subtypes responsible for mediating phasic inhibition. It is important to note however that these receptor subtypes are also abundant at perisynaptic and extrasynaptic sites, which is consistent with recent evidence for the dynamic mobility and rapid exchange of γ2 subunit-containing receptors between extrasynaptic and synaptic receptor pools (Jacob et al., 2005; Thomas et al., 2005).

The α1 subunit exhibits punctate and diffuse staining in the hippocampus indicating both a synaptic and extrasynaptic localization, which can in part be explained by its association with γ2 and δ subunits, respectively (Brunig et al., 2002). α1 subunit-containing $GABA_A$ receptor clusters are selectively enriched

on somatic membrane sites at GABAergic terminals that originate from parvalbumin-positive basket cells (Thomson et al., 2000; Klausberger et al., 2002). In contrast, the extensively clustered α2 subunit-containing $GABA_A$ receptors are strategically positioned at synapses on the axon initial segment (AIS) of pyramidal neurons that are innervated specifically by parvalbumin-positive chandelier cells (Brunig et al., 2002). They are also found at axo-somatic synapses at GABAergic terminals, which originate form cholecystokinin-positive basket cells (Brunig et al., 2002). The α3 subunit appears to be differentially targeted depending on its cellular distribution within the hippocampus. In pyramidal neurons it is concentrated at synaptic sites, but in a subset of hippocampal cells characterized by a round cell body and numerous short dendrites it displays a diffuse pattern of expression and fails to colocalize with gephyrin (Brunig et al., 2002). The α5 subunit is unique in that, despite its coassembly with a γ2 subunit (α5β3γ2), it is enriched at extrasynaptic sites (Brunig et al., 2002). In accordance with this finding, deletion of the α5 subunit eliminates tonic conductance in cultured hippocampal neurons (Caraiscos et al., 2004). However, there is a pool of α5 subunit-containing $GABA_A$ receptors that concentrates at GABAergic synapses on CA1 pyramidal cell dendrites (Serwanski et al., 2006). In a recent study, diazepam-sensitive IPSCs elicited by dendrite preferring interneurons in the rat neocortex was blocked by the α5 subtype-selective inverse agonist (α5IA), suggesting that α5βγ2 receptor subtypes may also play also play a role in phasic inhibition (Ali and Thomson, 2008). As depicted earlier, α4 and α6 subunits form assembled channel complexes with δ subunits (α4βδ and α6βδ) that are exclusively

extrasynaptic, accounting for tonic conductance in the thalamus and cerebellum, respectively (Nusser et al., 1998b). On the other hand, the majority of phasic signaling in the cerebellum is largely due to synaptic α6 subunit-containing receptors of the α6β2γ2 combination (Nusser et al., 1998b).

Functional significance of molecular heterogeneity

While the exact subcellular localization of different $GABA_A$ receptor subtypes undoubtedly contributes to their participation in phasic and tonic forms of signaling, this distinction alone is not sufficient to account for their differential activation. Subunit composition is a major determinant of the binding and gating properties of the ion channels, and hence the magnitude of the response following exposure to ligand. Studies using recombinant $GABA_A$ receptors have revealed that sensitivity to GABA is defined by the type of α subunit present, where extrasynaptic α6β3δ and α4β3δ subunit compositions display the highest affinities for GABA and synaptic α3β3γ2 subtypes the lowest (Bohme et al., 2004). For both αβγ and αβδ assemblies, the identity of the α subunit also affects the rates of activation, deactivation and desensitization (Bianchi et al., 2002; Caraiscos et al., 2004; Gingrich et al., 1995; Lavoie et al., 1997; McClellan and Twyman, 1999; Tia et al., 1996). Conversely, in αβ3γ2 subunit-containing receptors replacing the γ2 subunit with a δ subunit results in a dramatic reduction in single channel conductance independent of the type of α subunit present (Fisher and Macdonald, 1995), which supports the notion that GABA has a high affinity but low efficacy at δ subunit-containing extrasynaptic

receptors. The presence of a γ2 or δ subunit also influences channel kinetics; importantly αβδ receptors desensitize more slowly and less extensively than αβγ receptors (Bianchi and Macdonald, 2002; Haas and Macdonald, 1999; Saxena and Macdonald, 1996). Together the distinct biophysical properties of γ and δ subunit-containing receptors are wholly consistent with the involvement of these receptor subtypes in phasic and tonic signaling, respectively.

Differences in subunit composition between synaptic and extrasynaptic receptors are reflected in a differential modulation of phasic and tonic signaling by a number of compounds of therapeutic importance. The most frequently cited example of this is the role of the α subunit in defining receptor affinity for benzodiazepines. $GABA_A$ receptors incorporating either an α4 or α6 subunit infers insensitivity to benzodiazepines (Benson et al., 1998), as does elimination or substitution of the γ2 subunit. This difference can be attributed solely to the presence of a conserved arginine residue in α4 and α6 subunits, which in α1-3 and α5 subunits is histidine (Wieland et al., 1992). Thus benzodiazepine site ligands selective for α1-3 subunits largely influence phasic signaling, whereas those selective for α5 subunits are capable of primarily modulating tonic conductance. The diverse CNS depressant effects of benzodiazepines have been attributed to specific α subunit types of $GABA_A$ receptors (reviewed in Rudolph and Möhler, 2004). A combined molecular genetic and pharmacologic approach has revealed that α1 subunit-containing receptors mediate sedative, amnestic and, in part, the anticonvulsant actions of diazepam, whereas α2 subunits contribute to its anxiolytic and muscle-relaxant effects. Strong pharmacological

evidence for the involvement of the α3 subunit in anxiety comes from a study by Atack and colleagues (2005) showing that the α3 subtype-selective inverse agonist (α3IA) induces an anxiogenic response in rats. The α5 subunit has been implicated in learning and memory following the observation that a single point mutation (H105R), which prevents the interaction of this receptor subtype with diazepam, abolishes its memory impairing effects (Crestani et al., 2002). Moreover, the development of tolerance to the sedative actions of chronic diazepam is associated with a downregulation of α5 subunit-containing $GABA_A$ receptors, to which α5-H105R mice are resistant (Rijnsoever et al., 2004).

Following insights into $GABA_A$ receptor subtypes mediating the effects of benzodiazepines, subtype-selective drugs sharing with the classical benzodiazepines the overall high tolerability but therapeutic effects that are more selective than the classical benzodiazepines, have been developed (reviewed in Whiting, 2006). For example, zolpidem (Ambien®), which is a commonly prescribed sleep-aid, has a higher affinity for α1 subunit-containing $GABA_A$ receptors (Depoortere et al., 1986; Pritchet and Seeburg, 1990). To date, no compounds with selective binding affinity for α2 or α3 subunit-containing receptors have been described. L-838417, whilst not binding selective, exhibits significant efficacy selectivity for α2, α3 and α5 subunit-containing receptors over those of the α1 subtype (McKernan et al., 2000). In animal behavioral models, L-838417 was found to be an effective anxiolytic agent with a 30-fold separation over doses needed to elicit a sedative effect and also exhibited a reduced abuse potential compared with both classical

nonselective benzodiazepines and zolpidem (McKernan et al., 2000; Rowlett et al., 2005). Interestingly, the non-sedative anxiolytic TP-003 has significant efficacy only at α3 subunit-containing receptors, further supporting a role for this receptor subtype in mediating the anxiolytic response to benzodiazepines, (Dias et al., 2005). SL-651498, which is a full agonist at α2 and α3 subunit-containing receptors and a partial agonist at α1 and α5 subunit-containing receptors, is currently under development as a non-sedative anxiolytic for humans. Preliminary clinical trails with SL-651498 suggest similar efficacy to classical benzodiazepines as an anxiolytic, but with little or no sedation or impairment of motor skills, memory or cognitive function (de Haas et al., 2009). The α5 subtype-selective inverse agonist (α5IA) has equal binding affinity for α1, α2, α3 and α5 subtypes, but shows much higher efficacy at α5 subunit-containing receptors. It acts either as a weak partial agonist or inverse agonist at the other subtypes, with its partial agonist effect at α2 likely to be responsible for the lack of anxiogenic effects produced by this drug when compared to older α5 subtype-preferring inverse agonists such as L-655708 (Atack, 2009; Dawson et al., 2006; Navarro et al., 2002). Whether or not such a compound has efficacy in conditions associated with cognitive deficits, such as attention-deficit hyperactivity disorder, Alzheimer's disease or schizophrenia remains to be determined.

The diverse functions of GABA in the CNS are matched not just by the heterogeneity of $GABA_A$ receptors but also by the complex trafficking and clustering mechanisms that generate and maintain surface receptor populations

accessible to the transmitter at inhibitory postsynaptic specializations or extrasynaptic sites, depending on their subunit composition. The regulation of these mechanisms by $GABA_A$ receptor associated proteins is discussed below (summarized in Table 1.1).

Membrane trafficking of $GABA_A$ receptors

$GABA_A$ receptors are not static entities on the neuronal cell surface but are believed to cycle continuously between the plasma membrane and intracellular compartments (reviewed in Arancibia-Carcamo and Kittler 2009). The relative rates of receptor exo- and endocytosis are therefore key determinants in controlling the size of the postsynaptic pool accessible to GABA and GABAergic compounds and thus the strength of synaptic inhibition. Importantly, $GABA_A$ receptors have recently been reported to be inserted into and removed from the plasma membrane exclusively at extrasynaptic sites (Bogdanov et al., 2006; Thomas et al., 2005), highlighting the importance of lateral diffusion for their postsynaptic specialization.

Exocytosis of $GABA_A$ receptors to the plasma membrane

$GABA_A$ receptors can be delivered to the cell surface either as newly assembled channel complexes via a *de novo* secretory pathway, or reinserted following internalization. The oligomerization of $GABA_A$ receptor subunits into channel complexes is believed to occur in the endoplasmic reticulum (ER) and evidence

Table 1.1: $GABA_A$ receptor associated proteins

Protein	Subcellular Localization	Subunit specificity	Proposed function
GABARAP	Golgi	γ	Facilitates exocytosis
PRIP1/2	Synapses	β	Enhances cell surface stability by inhibiting PP1α mediated dephosphorylation
PLIC1	Intracellular compartments	α/β	Facilitates exocytosis by inhibiting proteasome-dependent degradation
BIG2	Intracellular compartments	β	Promotes ER exit and structural integrity of recycling endosomes
AP2	Clathrin-coated pits	β/γ	Regulates clathrin-mediated endocytosis
HAP1	Endosomes	β	Regulates post-endocytic sorting by inhibiting lysozomal degradation
GODZ	Golgi	γ	Palmitoylation
Gephyrin	Synapses	α1	Facilitates clustering/scaffolding at synaptic sites
Radixin	Extrasynaptic sites	α5	Facilitates clustering at extrasynaptic sites
GRIF1	Intracellular compartments	β2	Unknown
gC1qR	Intracellular compartments	β3	Unknown

Adapted from Arancibia-Carcamo and Moss, 2006

suggests that this assembly process plays a critical role in determining the diversity of receptor subtypes expressed on the neuronal plasma membrane. Proteins cannot exit the ER until they have achieved their correctly folded conformation, and misfolded or unassembled proteins are retrotranslocated from this organelle for degradation in the proteasome, restricting the number of subunit combinations that can access the cell surface (reviewed in Kittler et al., 2002). Following correct assembly, $GABA_A$ receptors are trafficked to the Golgi apparatus and segregated into vesicles for transport to and insertion into the plasma membrane facilitated by a number of receptor associated proteins.

Yeast two-hybrid screens using the γ2 subunit ICD as bait isolated the first known $GABA_A$ receptor associated protein (GABARAP) (Wang et al., 1999), a 17 kDa cytosolic protein belonging to the family of membrane associated proteins (MAPs) involved in membrane trafficking, including the estrogen-induced protein first isolated in guinea-pig endometrial cells (GEC)-1 (or GABRAP like-1), Golgi-associated transport enhancer of 16 kDa (GATE-16, or GABARAP like-2), Apg8L and light chain (LC)-3 subunits of MAP1. Interestingly, GABARAP knockout mice do not show differences in punctate staining of γ2 subunit-containing $GABA_A$ receptors and lack an overt behavioral phenotype (O'Sullivan et al., 2005), suggesting that GABARAP may be functionally redundant. Nevertheless, the notion that GABARAP is involved in the trafficking of $GABA_A$ receptors to the plasma membrane is supported by the finding that overexpression of GABARAP in heterologous expression systems and in cultured hippocampal neurons results in an increase in the cell

surface expression of γ2 subunit-containing $GABA_A$ receptors (Leil et al, 2004). In *Xenopus* oocytes expressing α1β2γ2 subunit-containing $GABA_A$ receptors this was accompanied by an increase in $GABA_A$ receptor-mediated synaptic inhibition, an effect requiring the γ2 subunit- and microtubule-binding motifs as well as intact polymerized microtubules (Chen et al., 2005). More recently it was demonstrated that GABARAP-mediated exocytosis of $GABA_A$ receptors is necessary for potentiation of inhibitory transmission by NMDA receptor activation, suggesting that GABARAP might have a role in the regulated delivery of $GABA_A$ receptors to the plasma membrane after activity rather than in the maintenance of basal surface receptor levels (Marsden et al., 2007).

Evidence in support of a function of GABARAP as a trafficking factor includes the identification of phospholipase C (PLC)-related catalytically inactive protein (PRIP)-1, a 130 kDa protein that is believed to competitively inhibit the binding of the γ2 subunit of $GABA_A$ receptors to GABARAP (Kanematsu et al., 2002). PRIP1 knockout mice show impairments in $GABA_A$ receptor modulation by benzodiazepines and zinc-sensitivity, indicating reduced activation of γ2 subunit-containing receptors (Kanematsu et al., 2002). These findings suggested that PRIP1 might play a role in the regulation of $GABA_A$ receptor trafficking by GABARAP, ensuring that only mature αβγ receptor complexes are delivered to the plasma membrane. In addition, PRIP1 has been shown to directly bind to the ICD of $GABA_A$ receptor β1-3 subunits, serving as an adaptor protein for the protein phosphatase (PP)-1α, and as such has been implicated in the phospho-dependent modulation of $GABA_A$ receptor functional expression (Terunuma et

al., 2004). Recently, a second PRIP isoform (PRIP2) has been identified that, like PRIP1, binds both GABARAP and PP1α, suggesting a central role for all PRIP isoforms in modulating $GABA_A$ receptor functional expression (Uji et al., 2002).

$GABA_A$ receptors also interact with the protein that links the integrin-associated protein with the cytoskeleton (Plic)-1. Plic1 is a 67 kDa protein with a ubiquitin-like (UBL) N-terminal domain and a ubiquitin-associated (UBA) C-terminal domain (Kleijnen et al., 2000). It is able to bind ubiquitin ligases and components of the proteasome and as such is thought to interfere with ubiquitin-dependent proteolysis of proteins (Kleijnen et al., 2000). Yeast two-hybrid screens and glutathione S-transferase (GST) affinity purification assays have shown that Plic1 interacts with the ICD of α1-3,6 and β1-3 (but not γ2 or δ) subunits of $GABA_A$ receptors (Bedford et al., 2001), indicating that Plic-1 function might be relevant for the majority of receptor subtypes expressed in the brain. This interaction has been demonstrated to be of significance in mediating the functional expression of $GABA_A$ receptors in human embryonic kidney (HEK-293) cells and in hippocampal slices as revealed using dominant negative peptides (Bedford et al., 2001). In a recent study by Saliba and colleagues (2008), the authors showed that Plic1 increases the accumulation of $GABA_A$ receptor β3 subunits on the cell surface in a manner independent of their rates of internalization. These findings suggested that Plic1 selectively modulates the secretory pathway. In accordance with this, Plic1 was found to significantly increase the half-life of polyubiquitinated $GABA_A$ receptor β3 subunits in the

ER and facilitate their insertion into the plasma membrane (Saliba et al., 2008). By increasing the resident times of unassembled subunits in the ER, Plic1 may also increase subunit maturation and production of heteromeric receptors. Plic1 regulation of the ubiquitin-dependent proteasomal degradation of $GABA_A$ receptors may thus provide a dynamic mechanism for regulating the efficacy of inhibitory synaptic transmission.

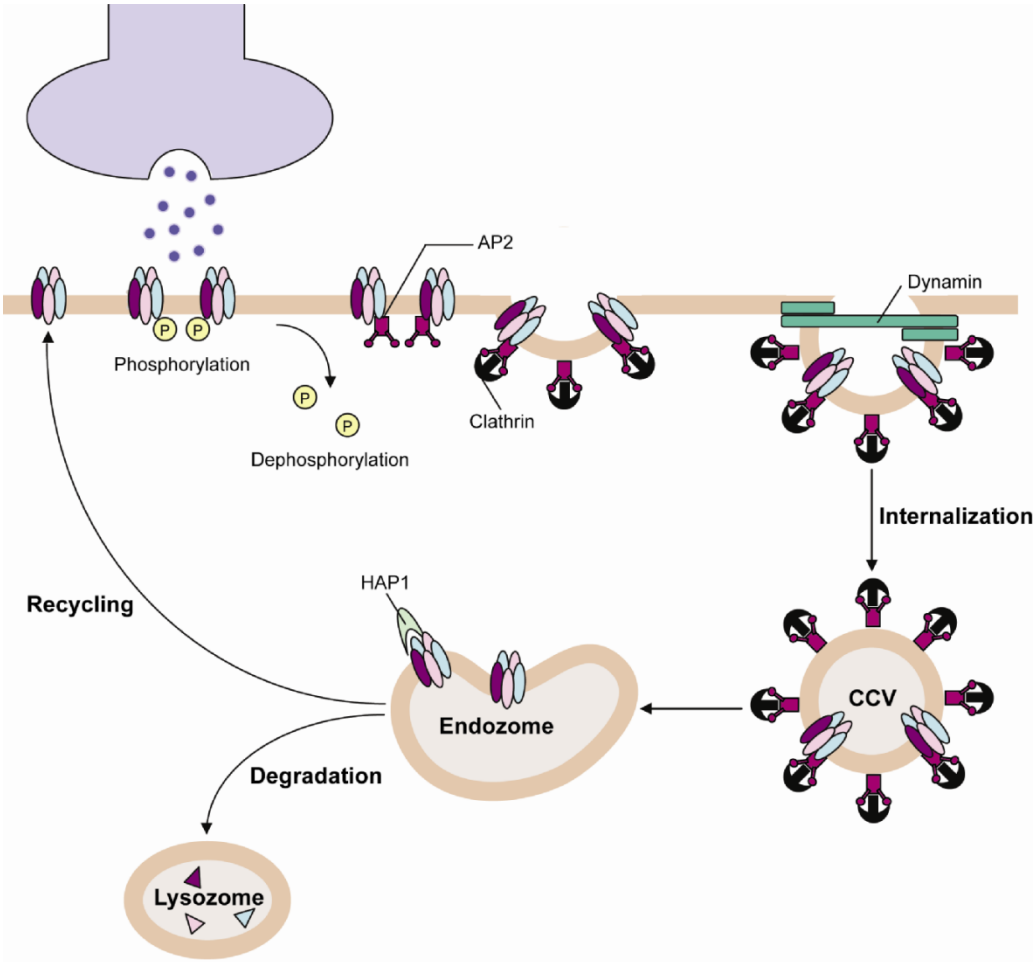
The 190 kDa brefeldin A-inhibited guanine nucleotide exchange factor (BIG)-2 was also identified as a $GABA_A$ receptor β subunit interacting protein in a yeast two-hybrid screen (Charych et al., 2004a). BIG2 is concentrated mainly in the trans-Golgi network but is also found in vesicle-like structures along dendrites and at the postsynaptic plasma membrane (Charych et al., 2004). Interestingly, coexpression of BIG2 with the $GABA_A$ receptor β3 subunit results in an increase in β3 exit from the ER, suggesting that BIG2 is involved in the post-Golgi vesicular trafficking of $GABA_A$ receptors (Charych et al., 2004). It is currently proposed that BIG2 may play a role in the transport of newly assembled $GABA_A$ receptors to the postsynaptic plasma membrane and also be involved in receptor recycling (Shen et al., 2006; Shin et al., 2004).

Other $GABA_A$ receptor associated proteins implicated in the forward trafficking of $GABA_A$ receptors include the $GABA_A$ receptor interacting factor (GRIF)-1 (Beck et al., 2002) and the multifunctional protein gC1qR (Schaerer et al., 2001). However, their functional significance remains unclear.

Endocytosis of $GABA_A$ receptors from the plasma membrane

$GABA_A$ receptors have been shown to be localized in clathrin-coated pits, suggesting that they undergo clathrin-mediated endocytosis (Fig 1.3), a process that is further dependent on dynamin for endocytic vesicle formation. The clathrin adaptor protein (AP)-2 is a central component in the formation of these vesicles, forging a link between membrane proteins and clathrin, which forms the outer layer of the coat. AP2 is a heterotetrameric complex composed of two large (~100 kDa) α and β2 subunits, a medium (50 kDa) μ2 subunit, and a small (19 kDa) σ2 subunit, commonly referred to as adaptins. The α adaptin is responsible for targeting the protein to the plasma membrane, where the β2 adaptin interacts with clathrin to trigger clathrin assembly, forming coated pits. This in turn leads to the activation of μ2 adaptin phosphorylation, inducing a conformational change in the subunit that allows the complex to directly bind to endocytic motifs in cell surface receptors, clustering the protein cargo into the assembling coated pit. Cargo is bound by the β2 and μ2 adaptins that mostly recognize a dileucine motif or the canonical tyrosine based YXXØ motif (where X denotes any amino acid and Ø a bulky hydrophobic residue) (Clague, 1998; Le Borgne and Hoflack, 1998). The σ2 adaptin is responsible for stabilizing the complex at the core by mediating subunit interactions. The AP2 complex also interacts with several accessory proteins that form essential components of the endocytic machinery (reviewed in Slepnev and De Camilli, 2000).

$GABA_A$ receptors are intimately associated with AP2 in the brain through a direct binding of the β1-3 and γ2 $GABA_A$ receptor subunits (Kittler et al.,



Adapted from Jacob et al., 2008

Figure 1.3: Clathrin-mediated endocytosis. The receptors cluster in specialized sites at the plasma membrane known as clathrin-coated pits, which invaginate and pinch off to form clathrin-coated vesicles (CCVs), a process that is dependent on dynamin. The clathrin adaptor protein (AP)-2 is a central component in the formation of these vesicles, forging a link between membrane proteins and clathrin which forms the outer layer of the coat. The vesicles subsequently lose their coat and fuse together to form an early endosome. Internalized receptors are then either subject to rapid recycling or are targeted for lysozomal degradation, an endocytic sorting decision that is regulated by the Huntingtin-associated protein (HAP)-1.

2000). In the $GABA_A$ receptor β2 subunit a dileucine AP2-β2 adaptin-binding motif ($L^{343}L^{344}$) has been identified. This motif is important for clathrin-mediated endocytosis in HEK-293 cells and in cortical slices (Herring et al., 2003, 2005). It is also present in the ICDs of receptor β1 and β3 subunits, but evidence suggests that it is not involved in the interaction of these subunits with the AP2 complex (Kittler et al., 2005). In addition, an atypical AP2-binding motif conserved within the ICD of all $GABA_A$ receptor β subunit isoforms has been identified (KTHLRRRSSQLK in the β3 subunit) (Kittler et al., 2005). This motif, which is enriched in lysine and arginine residues, also incorporates the major sites of phosphorylation for PKA and PKC within this class of receptor subunits: S409 in β1, S410 in β2 and S408/9 in β3 (Moss et al., 1992). A peptide corresponding to the AP2-μ2 adaptin-binding motif in the $GABA_A$ receptor β3 subunit binds to this complex with relatively high affinity in its nonphosphorylated state, but not when chemically phosphorylated at S408 and S409. The nonphosphorylated version of the peptide alone enhances miniature IPSC (mIPSC) amplitudes and whole-cell $GABA_A$ receptor currents in a manner that is occluded by inhibitors of dynamin (Kittler et al., 2005). More recently, a tyrosine based AP2-μ2 adaptin-binding motif in the $GABA_A$ receptor γ2 subunit ($Y^{365}GY^{367}ECL$) has been identified (Kittler et al., 2008). These tyrosine residues are principal sites for phosphorylation by Src kinase (Brandon et al., 2001; Moss et al., 1995). Utilizing nonphosphorylated and phosphorylated peptides corresponding to Y365 and Y367, the authors show that this high affinity interaction is phospho-dependent (Kittler et al., 2008). Introduction of the nonphosphorylated γ2 peptide into neurons induced a large

increase in the mIPSC amplitude that was accompanied by an increase in the number of receptors on the cell surface (Kittler et al., 2008). Interestingly, codialysis of neurons with both the nonphosphorylated β3 and γ2 subunit peptides produced an additive effect on mIPSC amplitudes (Kittler et al., 2008).

Post-endocytic $GABA_A$ receptor sorting

The fate of internalized receptors is also another determinant of surface receptor levels. Following internalization, $GABA_A$ receptors are either rapidly recycled back to the neuronal plasma membrane or, over longer time frames, are targeted for lysozomal degradation, an endocytic sorting decision that is regulated by the Huntingtin-associated protein (HAP)-1. Yeast two-hybrid screens revealed that HAP1 interacts with the $GABA_A$ receptor β1 subunit but not the α1, γ2, or δ subunits (Kittler et al., 2004). Overexpression of HAP1 in cultured neurons has been shown to increase the number of receptors on the cell surface at steady-state that correlates with a dramatic increase in the mean amplitude of mIPSCs without affecting the frequency or kinetics of these events (Kittler et al., 2004). However, whether HAP1 promotes $GABA_A$ receptor recycling or prevents their lysozomal degradation is an unresolved issue. Nevertheless, the importance of HAP1-dependent regulation of $GABA_A$ receptor trafficking is evident in a study in which selective suppression of hypothalamic HAP1 by siRNA induced a decrease in feeding behavior in mice that was attributed to reduced surface expression and activity of $GABA_A$ receptors (Sheng et al., 2006).

Postsynaptic targeting and clustering of $GABA_A$ receptors

The highly selective subcellular localization of $GABA_A$ receptor subtypes implies that subunit composition plays a major role in the postsynaptic targeting and clustering of these receptors. While the exact molecular mechanisms that govern the accumulation of $GABA_A$ receptors at inhibitory synapses are not yet fully understood, it involves a number of receptor associated proteins and cytoskeletal elements that are concentrated at postsynaptic densities (PSDs).

The inhibitory synaptic marker gephyrin is a 93 kDa subsynaptic scaffolding protein that was originally implicated in regulating the postsynaptic clustering of glycine receptors in the spinal cord by directly binding to the receptor β subunit (Meyer et al., 1995). More recently, data derived from gephyrin knockout mice and knockdown experiments using antisense oligonucleotides or shRNAi have revealed that reducing gephyrin expression also leads to an extensive loss in the punctate staining of $GABA_A$ receptors incorporating a γ2 subunit together with α2 (but not α1) subunits (Essrich et al., 1998; Jacob et al., 2005; Kneussel et al., 1999; Levi et al., 2004). This suggests the existence of both gephyrin-dependent and independent $GABA_A$ receptor clustering mechanisms. As discussed earlier, gephyrin clusters are absent in γ2 subunit knockout mice, and the remaining α and β subunit-containing receptors show diffuse staining (Essrich et al., 1998). Furthermore, transfection of γ2 subunit-deficient neurons with chimeric α2/γ2 constructs revealed that the TM4 of the γ2 subunit is sufficient for targeting these receptors to postsynaptic sites, but

that both the TM4 and ICD of the γ2 subunit were necessary for recruiting gephyrin to the synapse and rescuing GABAergic inhibitory synaptic function (Alldred et al., 2005). A role for this receptor associated protein in stabilizing previously clustered $GABA_A$ receptors at the cell surface, rather than their specialization at inhibitory synapses, has thus been proposed. Concurrent with this, postsynaptic $GABA_A$ receptors have been shown to be three times more mobile in cells where gephyrin expression has been impaired (Jacob et al., 2005). Gephyrin is believed to anchor postsynaptic $GABA_A$ receptors to the plasma membrane by cross-linking the receptor molecule to the tubulin and actin cytoskeleton. Gephyrin binds with high affinity to tubulin and as such is viewed as a *bona fide* MAP (Ramming et al., 2000). Gephyrin also mediates an indirect interaction with the cytoskeleton by binding to LC1 and LC2 of the dynein motor proteins and the microfilament associated proteins belonging to the Mena/VASP family (Fuhrmann et al., 2002). The membrane-associated protein collybistin II is a guanine-nucleotide exchange factor (GEF) that binds to and so permits the subsynaptic localization of the otherwise intracellular gephyrin, where it directly interacts with and immobilizes specific $GABA_A$ receptor subtypes (Harvey et al., 2004; Kins et al., 2000). In addition, the dystrophin glycoprotein complex (DGC) and neuroligin (NL)-2 bridge the synaptic cleft by interacting with presynaptic β-neurexin, promoting GABAergic synaptogenesis (reviewed in Tretter and Moss, 2008).

Although gephyrin is found to be concentrated at GABAergic synapses in both hippocampal and cortical neurons (where glycine receptor expression is

relatively low), in initial studies using standard biochemical methods it failed to copurify with any of the $GABA_A$ receptor subunits. Hence an intermediate linker protein was postulated, the prime candidate being GABARAP - given its ability to directly bind the γ2 subunit ICD and gephyrin. However, subsequent analysis revealed that GABARAP is not clustered at gephyrin-rich PSDs but instead is mainly localized in intracellular compartments including the ER, Golgi apparatus and, to some extent, in subsynaptic secretory vesicles (Kittler et al., 2001; Wang et al., 1999). This is consistent with a role for this protein in intracellular trafficking rather than postsynaptic clustering. In a recent study Tretter and colleagues (2008) for the first time provided evidence that gephyrin directly binds to the α2 subunit ICD *in vitro*. This interaction was blocked by low concentrations of detergent, providing a possible explanation as to why previous attempts to identify a direct association between gephyrin and $GABA_A$ receptors were unsuccessful. However, under the same conditions only very weak binding to α1, β3 and γ2 subunits was observed (Tretter et al., 2008), suggesting that the role of these subunits in the postsynaptic accumulation of $GABA_A$ receptors may be independent of the ability of these proteins to directly bind gephyrin. The 81 kDa actin-binding protein radixin, a member of the ezrin/radixin/moesin (ERM) protein family, has recently been shown to directly link the α5 subunit to the cytoskeleton via a radixin-binding motif conserved within the ICD of α1-3 and α5 subunits, differing only by two amino acids in the α2 subunit (Loebrich et al., 2006). In neurons, both depletion of radixin and replacement of the radixin actin-binding motif dramatically decreased α5 subunit-containing $GABA_A$ receptor clusters without altering total surface

expression levels. Radixin also showed limited colocalization with postsynaptic gephyrin, consistent with previous reports that $GABA_A$ receptor α5 subunits localize mainly at extrasynaptic sites (Brunig et al., 2002; Loebrich et al., 2006). However, the mechanisms responsible for the formation of radixin-dependent extrasynaptic $GABA_A$ receptor clusters remain to be elucidated.

Modulation of $GABA_A$ receptor function by post-translational modifications

The cell surface stability of $GABA_A$ receptors is further regulated by post-translational modifications such as palmitoylation, ubiquitination and phosphorylation. In addition, these modifications have been implicated in altering the biophysical and pharmacological properties of these ligand-gated ion channels (reviewed in Arancibia-Carcamo and Moss, 2009).

Palmitoylation

Palmitoylation is the covalent attachment of the saturated fatty acid palmitate to cysteine residues of a given protein by the palmitoyl transferase Golgi-specific DHHC zinc finger domain protein (GODZ). Palmitoylation enhances the hydrophobicity of proteins and contributes to their membrane association. As such it has been shown to be involved in the postsynaptic clustering and subcellular trafficking of a number of proteins, including AMPA receptors (Hayashi et al., 2005) and the neuronal scaffold proteins PSD-95 and glutamate

receptor-interacting protein (GRIP)-1 (DeSouza et al., 2002; Smotrys and Linder, 2004). It is unique in that it is the only reversible lipid modification and thus allows the cell to dynamically regulate the location of specific proteins. In a yeast two-hybrid screen, the 34 kDa protein GODZ was identified as a $GABA_A$ receptor γ2 subunit interacting protein that recognizes a 14 amino acid cysteine-rich domain conserved in the ICD of all γ1-3 subunits, N-terminal to the GABARAP binding site (Rathenberg et al., 2004). Analysis of Cys-Ala mutant γ2 constructs in transfected African green monkey kidney (COS-7) cells revealed that the γ2 subunit is palmitoylated at all four cysteines within the GODZ binding domain (Rathenberg et al., 2004). Mutation of these cysteine residues resulted in a loss of $GABA_A$ receptor clusters at the cell surface, as did drug-induced global inhibition of palmitoylation by Br-palmitate (Rathenberg et al., 2004). Likewise, disrupting GODZ function or expression levels using dominant negative or RNAi approaches results in a significant reduction in the amplitude of mIPSCs attributed to a decrease in postsynaptic $GABA_A$ receptor number (Fang et al., 2006). From these studies it is evident that palmitoylation can dynamically regulate the efficacy of neuronal inhibition by controlling the accumulation of $GABA_A$ receptors at the postsynaptic membrane, although the exact mechanism by which this is achieved is unknown.

Ubiquitination

The regulation of $GABA_A$ receptor trafficking by the ubiquitin-related protein Plic1 suggests that $GABA_A$ receptors may also be a direct target for modification by the polypeptide ubiquitin. The covalent attachment of one or

more copies of the 76-amino acid ubiquitin monomer to lysine residues of target proteins is referred to as ubiquitination. Monoubiquitination is reversible and serves as an active signal in diverse intracellular trafficking pathways, including as a trigger for endocytosis. In contrast, polyubiquitination is required for the translocation of proteins from the ER back into the cytosol, where they are degraded by the proteasome. Recently, activity-dependent polyubiquitination of $GABA_A$ receptor β3 subunits has been shown to reduce the stability of newly translated and assembled receptors in the ER via a mechanism dependent on the activity of the proteasome (Saliba et al., 2007). Coincident with a loss of cell surface expression levels, chronic blockade of neuronal activity by tetrodotoxin (TTX) treatment reduced both the amplitude and frequency of mIPSCs (Saliba et al., 2007). TTX had no effect on the enhanced functional expression of $GABA_A$ receptors incorporating β3 subunits in which all twelve lysine residues within the ICD of this subunit had been mutated to arginines ($\beta3^{K12R}$). These mutations did not alter $GABA_A$ receptor cell surface half-life or internalization rates, but significantly enhanced receptor insertion into the plasma membrane (Saliba et al., 2007).

Phosphorylation

Protein phosphorylation is fundamental to the activity of cellular signaling networks. It is achieved through protein kinase catalyzed transfer of a phosphate group to serine, threonine and/or tyrosine residues of a given protein substrate that can be reversed in a dephosphorylation reaction catalyzed by protein phosphatases. $GABA_A$ receptors are well-established phosphoproteins. Diverse

studies on $GABA_A$ receptor phosphorylation have implicated this process in altering channel gating, conductance and/or kinetics, sensitivity of the receptors to pharmacological agents, protein-protein interactions and membrane trafficking (reviewed in Kittler and Moss, 2003). Hence the coordinated activity of kinases and phosphatases plays a pivotal role in controlling neuronal excitability.

Studies in heterologous expression systems have demonstrated that $GABA_A$ receptor function, depending on the subtype analyzed, can be differentially modulated by phosphorylation of key residues within the ICDs of receptor β1-3 and γ2 subunits by a number of kinases, including cAMP-dependent protein kinase (PKA), calcium/phospholipid-dependent protein kinase (PKC), calcium/ calmodulin-dependent kinase II (CaMKII), protein kinase B (PKB - also known as Akt), cGMP-dependent protein kinase (PKG) and tyrosine kinases of the Src family (summarized in Table 1.2). This is best illustrated by the differential modulation of $GABA_A$ receptor subtypes by PKA, dependent upon the identity of the β subunit. *In vitro* studies using purified bacterially expressed GST fusion proteins combined with site directed mutagenesis revealed that the consensus motif for PKA-induced phosphorylation conserved within the ICDs of $GABA_A$ receptor β1-3 subunits is RRRXSQLK, where S is serine at position 409 in β1 and β3 subunits (McDonald and Moss, 1997; Moss et al., 1992a) and serine at position 410 in the β2 subunit (McDonald and Moss, 1997) and X represents either an alanine residue in β1 and β2 subunits or a serine residue at position 408 in the β3 subunit. However, in contrast to *in vitro* findings, analysis of

Table 1.2: $GABA_A$ receptor phosphorylation sites

Subunit	Phosphorylation site	Protein kinase		
		In vitro	Heterologous cell lines	Primary neurons
β1	S384	CaMKII		
	S409	PKA, PKC, CaMKII, PKG	PKA, PKC	
β2	S410	PKA, PKC, Akt, CaMKII, PKG	PKC, Akt	Akt
β3	S383	CaMKII		
	S408	PKC	PKA, PKC	PKA, PKC
	S409	PKA, PKC, CaMKII, PKG	PKA, PKC	PKA, PKC
γ2	S327	PKC		
	S343	PKC, CaMKII		
	S348	CaMKII		
	T350	CaMKII		
	Y365	Src	Src	
	Y367	Src	Src	

Adapted from Brandon et al., 2002

recombinant $GABA_A$ receptors in HEK-293 cells have demonstrated that PKA-induced phosphorylation of the β3 subunit occurs at both S408 and S409 (rather than at S409 alone); whereas the β2 subunit is not a substrate for this kinase in heterologous expression systems (McDonald et al., 1998). Interestingly, PKA was shown to depress GABA-activated currents in HEK-293 cells expressing β1 subunit-containing $GABA_A$ receptors, whereas in β3 subunit-expressing cells a potentiation was observed (McDonald et al., 1998). The latter was attributed to the presence of two juxtaposed serine residues (S408 and S409) in β3, as selective mutation of S408 to alanine (to structurally resemble the $GABA_A$ receptor β1 subunit at this site) converted this potentiation to a depression (McDonald et al., 1998). On the other hand, PKA had no effect on β2 subunit-expressing cells (McDonald et al., 1998), which can be accounted for by the selective recruitment of PKA to $GABA_A$ receptor β1 and β3, but not β2, subunits via the A-kinase anchoring protein (AKAP) of 79 (rat)/150 (human) kDa (Brandon et al., 2003).

AKAPs have also been shown to directly interact with PP2B and PKC in addition to PKA (Klauck et al., 1996; Colledge and Scott, 1999). However, the targeting of PP2B and PKC to $GABA_A$ receptors by AKAP remains to be investigated. Interestingly, PP2B has been shown to directly bind to the ICD of the γ2 subunit, thereby dephosphorylating the receptor and inducing long-term depression (LTD) of synaptic inhibition in the CA1 region of the hippocampus, a mechanism that is believed to contribute to learning and memory (Wang et al., 2003). PKC is recruited to the $GABA_A$ receptor via the anchoring protein

receptor for activated C kinase (RACK)-1 and, like PKA, has different effects on receptor function depending on subunit composition. In agreement with the findings *in vitro* all of the β subunit isoforms were found to be a substrate for this kinase in HEK-293 cells (Krishek et al., 1994; McDonald et al., 1998; Brandon et al., 2000). Functional studies employing site-directed mutagenesis have demonstrated a downregulation of receptor activity in response to phorbol esters mediated by PKC-induced phosphorylation of β1 S409, β2 S410, γ2S S327 and γ2L S327/343. However, the use of a constitutively active catalytic domain of PKC (PKM), produced results contradicting those obtained with phorbol esters. In transfected mouse fibroblasts PKM enhanced the response to GABA in a manner that was dependent on the phosphorylation of the β1 subunit at S409 and the γ2L subunit at S327 and S343 (Lin et al., 1996). The reason for this discrepancy remains unresolved although it has been speculated that different PKC isoforms may produce different responses (Song and Messing, 2005). In contrast, brain-derived neurotrophic factor (BDNF)-induced PKC-mediated phosphorylation of the β3 subunit has been shown to transiently increase the amplitude of mIPSCs in cultured neurons, an effect that was paralleled by an increase in $GABA_A$ receptor cell surface stability (Jovanovic et al., 2004). Furthermore, the initial enhancement observed in receptor activity was followed by a prolonged depression that was attributed to PP2A-mediated dephosphorylation of the β3 subunit (Jovanovic et al., 2004).

No adaptor protein is known for CaMKII, although the kinase is capable of coimmunoprecipitating with $GABA_A$ receptors from detergent-soluble mouse

brain extracts (Mcainish et al., unpublished data). *In vitro* studies have revealed that the serine/threonine kinase CaMKII directly phosphorylates specific residues within the ICDs of $GABA_A$ receptor β and γ2 subunits. These include S384/409 in β1, S410 in β2 and S383/409 in β3, as well as the γ2 subunit at S343 (γ2L only), S348 and T350. Interestingly, the intracellular application of purified, active CaMKII failed to modulate the function of $GABA_A$ receptors heterologously expressed in HEK-293 cells but significantly potentiated the amplitudes of whole-cell GABA-activated currents recorded from rat cultured cerebellar granule neurons and from recombinant $GABA_A$ receptors expressed in neuroblastoma-glioma hybrid (NG108-15) cells, implying the contribution of some essential neuronal factor (Houston and Smart, 2006).

In addition to binding PKC and the $GABA_A$ receptor β subunits, RACK1 also binds the tyrosine kinase Src to facilitate the phosphorylation of the γ2 subunit on Y365 and Y367 (Brandon et al., 2001; Kittler and Moss, 2003; Moss et al., 1995). Coexpression of Src with recombinant α1β1γ2 subunit-containing $GABA_A$ receptors has been shown to increase whole-cell GABA-activated currents in human embryonic kidney (A293) cells, an effect that was abolished by site-specific mutagenesis of both of these tyrosine residues to phenylalanines (Moss et al., 1995). Interestingly, mutation of these sites led to increased phosphorylation of the β1 subunit at Y384 and Y386, which exhibits relatively low stoichiometry of phosphorylation in response to Src compared to wild-type control (Moss et al., 1995). In primary neuronal cultures, intracellular application of sodium vanadate, a potent tyrosine phosphatase inhibitor,

enhanced benzodiazepine-sensitive $GABA_A$ receptor function, suggesting high endogenous tyrosine kinase and phosphatase activity under basal conditions (Moss et al., 1995).

Phosphorylation-dependent $GABA_A$ receptor cross-talk

Kinases not only modulate channel function by phosphorylating the receptor directly but also mediate the effect of extracellular stimuli such as growth factors or 'cross talk' with other neurotransmitter systems via G protein-coupled receptor activation of signal transduction systems. For example, several lines of evidence suggest that PKA is involved in the modulation of $GABA_A$ receptor function by dopamine. The activation of dopamine type-1 (D_1) receptors has been shown to enhance $GABA_A$ receptor activity via PKA-mediated phosphorylation of the β3 subunit at S408 and S409 in hippocampal slices. Interestingly, this phospho-dependent modulation was diminished in PRIP1 knockout mice that exhibited enhanced protein PP1α activity compared to wild-type controls (Terunuma et al., 2004). Conversely, inhibition of PKA activity via D_3 receptor activation causes a reduction in mIPSC amplitude in the nucleus accumbens that is attributed to an increase in the phospho-dependent endocytosis of $GABA_A$ receptor β3 subunits (Chen et al., 2006). Similarly, D_4 receptor agonists have been shown to decrease mIPSCs in globus pallidus neurons via inhibition of PKA activity (Shin et al., 2003). In addition to PKA, dopamine-mediated modulation of $GABA_A$ receptor function has been shown to involve PKC via the activation of D_2 receptors (Brandon et al., 2002).

Interestingly, D_5 receptors have been shown to bind $GABA_A$ receptors directly and modify their function independently of PKA activity (Liu et al., 2000). PKA and PKC are also involved in mediating the effects of serotonin on $GABA_A$ receptor function via G protein-coupled 5-HT receptors. Activation of postsynaptic 5-HT_2 receptors in PFC pyramidal neurons has been shown to inhibit $GABA_A$ receptor currents via a PKC-mediated pathway (Feng et al., 2001). In contrast, 5-HT_4 receptor activation modulates $GABA_A$ receptor currents bi-directionally depending on the basal PKA activation levels; elevated levels of PKA activation due to increased neuronal activity have been shown to reverse the enhancing effect of 5-HT_4 receptor activation to a depression (Cai et al., 2002).

Many drugs used to treat psychiatric disease, including antipsychotics and antidepressants, act to modulate dopaminergic and serotonergic signaling. However, their mechanism of action remains unknown. Given that the modulation of dopamine and serotonin receptor function has a common output - the modulation of $GABA_A$ receptor activity - further exploring the functional link between $GABA_A$ receptor phosphorylation and these G protein-coupled receptors may enhance our understanding of schizophrenia and depression and help to identify novel therapeutic targets with which to control these devastating conditions. The work presented in this thesis aims to describe the molecular mechanisms that may be involved in the phosphorylation-dependent functional expression of $GABA_A$ receptors and also suggest a role for serotonergic signaling in modulating $GABA_A$ receptor phosphorylation.

Methods & Materials

General materials

Chemicals and reagents

All chemicals, unless otherwise specified, were purchased from Sigma-Aldrich. Aqueous buffers and solutions were prepared in deioinized water (dH_2O). 1mM ATP (Promega, Cat# E6011) stock was prepared in dH_2O, aliquoted and stored at -20°C. [γ-^{32}P]-ATP (10mCi/ml) and EasyTag™ EXPRESS [^{35}S]-methionine protein labeling mix (11mCi/ml) were purchased from PerkinElmer (Cat# NEG002A and NEG772, respectively).

Drugs

1mM okadaic acid (Calbiochem, Cat# 459620), 0.5mM phorbol 12,13-dibutyrate (PDBu) (Calbiochem, Cat# 524390), 2.5mM calphostin C (Calbiochem, Cat# 208725) and 10mM GF-109203X (GFX) (Tocris, Cat# 0741) stocks were prepared in DMSO. 100mM serotonin-HCl (Sigma-Aldrich, Cat# H9523) and 100mM R(-)-dimethoxy-4-iodoamphetamine-HCl (DOI) (Sigma-Aldrich, Cat# D153) stocks were prepared in dH_2O. Fluoxetine-HCl was a generous gift from Wyeth Research (Princeton, NJ) and a 2mg/ml solution in dH_2O was prepared fresh on the day.

Equipment

All centrifugations were carried out in an Eppendorf model 5415R bench-top microcentrifuge, Beckman Avanti™ J-20 XP high-performance centrifuge or a

Beckman Optima™ TLX ultracentrifuge. All absorbance readings were measured using a Bio-Rad SmartSpec™ 3000 spectrophotometer and a Bio-Rad model 680 microplate reader.

Molecular biology

Constructs

A list of all DNA constructs used in this study is given in Table 2.1. Plasmids encoding $GABA_A$ receptor β subunit intracellular domains (ICDs) were cloned into pGEX-4T3, a GST gene fusion vector containing a tac promoter for chemically inducible, high level expression in any E. coli host (Brandon et al., 1999). Plasmids encoding full-length $GABA_A$ receptor α/β subunits and AP2-µ2 adaptin were cloned into pRK5, a mammalian expression vector containing a cytomegalovirus (CMV) promoter designed for high level expression of cloned genes (Haucke et al., 2000; McDonald et al., 1998).

Growth media

Growth media was made by dissolving 10g of Luria Bertani (LB) broth (Sigma-Aldrich, Cat# L3022) in 500ml dH_2O and autoclaved for sterilization. After allowing the LB medium to cool, ampicillin was added to a final concentration of 50µg/ml (LB-ampicillin medium).

Table 2.1: DNA constructs used in this study

Construct	Description
GST-β1	$GABA_A$ receptor β1 subunit ICD in pGEX-4T3
GST-β2	$GABA_A$ receptor β2 subunit ICD in pGEX-4T3
GST-β3	$GABA_A$ receptor β3 subunit ICD in pGEX-4T3
GST-$\beta3^{S408/9A}$	$GABA_A$ receptor $\beta3^{S408/9A}$ subunit ICD in pGEX-4T3
GST-$\beta3^{S408A}$	$GABA_A$ receptor $\beta3^{S408A}$ subunit ICD in pGEX-4T3
GST-$\beta3^{S409A}$	$GABA_A$ receptor $\beta3^{S409A}$ subunit ICD cloned in pGEX-4T3
α1	Full-length $GABA_A$ receptor α1 subunit in pRK5
β3	Full-length $GABA_A$ receptor β3 subunit in pRK5
$\beta3^{S408/9A}$	Full-length $GABA_A$ receptor $\beta3^{S408/9A}$ subunit in pRK5
μ2	Full-length AP2-μ2 adaptin in pRK5

Agar plates

Agar plates were made by dissolving a packet of EZMix™ LB agar powder (Sigma-Aldrich, Cat# L7533) in 500ml dH_2O, autoclaved for sterilization and equilibrated to 55°C in a water bath. Ampicillin was then added to a final concentration of 100µg/ml, mixed well and poured into sterile 100mm Petri dishes (20ml/dish). The LB-agar-ampicillin plates were allowed to solidify at room temperature and then stored at 4°C.

Bacterial strains

BL21 (DE3) pLysS competent cells, [F^- *omp*T *hsd*S_B (r_B^-, m_B^-) *dcm gal* λ(DE3) pLysS Cm^r] (Promega, Cat# L1191), were used in GST fusion protein induction and expression. XL1-Blue electrocompetent cells, *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac* [F´ *pro*AB $lacI^q$ZΔM15 Tn10 (Tet^r)] (Stratagene, Cat# 200228), were used in plasmid carriage and propagation.

Transformation of bacteria with plasmid DNA

Heat-shock

Transformation of BL21 (DE3) pLysS competent cells by heat-shock was carried out according to manufacturer's specifications. Briefly, 100µl of the *E.coli* cells were thawed on ice and mixed with 10µl of plasmid DNA (0.1-5ng/µl) in a pre-chilled BD Falcon™ polypropylene culture tube. After 30 min incubation on ice, the tube was transferred to a water bath set to 42°C for 45 s and then immediately returned on ice for 2 min. The cells were then flushed

with 900µl of pre-warmed LB medium and allowed to recover for 1 h at 37°C. Transformed cells were selected by plating on LB-agar-ampicillin plates and incubating at 37°C for 16 h or until bacterial colonies emerged.

Electroporation

Plasmid DNA was introduced into XL1-Blue electrocompetent cells by electroporation according to manufacturer's specifications. Briefly, 50µl of the *E.coli* cells were thawed on ice and mixed with 1µl of plasmid DNA (1-50ng/µl). This mix was pipetted into a pre-chilled Bio-Rad Gene Pulser® electroporation cuvette (0.1cm gap) and pulsed at 1,700V, 200Ω resistance and 25µF capacitance using the Bio-Rad Gene Pulser XCell™ electroporator. The cells were then flushed with 1ml of pre-warmed LB medium and allowed to recover at 37°C for 30 min. Transformed cells were selected by plating on LB-agar-ampicillin plates and incubating at 37°C for 16 h or until bacterial colonies emerged.

Maxi preparation of plasmid DNA

Maxi preparation of plasmid DNA was carried out according to Molecular Cloning III - Sambrook and Russell, 2001. A single bacterial colony (grown on an LB-ampicillin plate) was picked up using a pipette tip, transferred to a 15ml BD Falcon™ tube containing 3ml LB-ampicillin medium and grown for 16 h at 37°C with vigorous shaking. The bacterial culture was used to inoculate 250ml of LB-ampicillin medium and grown for a further 16 h in a 1L conical glass flask. Bacterial cells were centrifuged at 5,000 rpm for 15 min at 4°C and the

resultant pellet was resuspended in 5ml Alkaline Solution I (50mM D-glucose, 25mM Tris-Cl pH 8.0, 10mM EDTA pH 8.0). 10ml Alkaline Solution II (1% SDS, 0.2N NaOH) was then added to the solution, mixed gently and allowed to stand for 5 min. To this, 7.5ml of ice-cold Alkaline Solution III (60ml 5M K-acetate, 11.5ml glacial acetic acid, 28.5ml dH_2O) was also added, mixed gently and allowed to stand for a further 10 min on ice. The mixture was then centrifuged at 10,000rpm for 30 min at 4°C. The supernatant was removed and added to an equal volume of phenol:chloroform (1:1), mixed well and allowed to stand until separation of the two phases had occurred. The upper phase was subsequently removed and added to an equal volume of isopropanol, mixed well and allowed to stand for 10 min. The mixture was then centrifuged at 10,000rpm for 15 min and the resultant pellet was resuspended in 5ml dH_2O. 25ml absolute ethanol was added to this, mixed gently and allowed to stand for 10 min before centrifuging again. The resultant pellet was rinsed with 70% ethanol and allowed to dry. Following this, the pellet was resuspended in 3ml TE Buffer (10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0) and 8g of CsCl and 100µl 10mg/ml EtBr was added to it. The volume was bought up to 8 ml with TE Buffer, mixed well, divided into 2 Beckman Quick-Seal® tubes and then centrifuged at 100,000rpm for 16 h. Centrifugation promotes RNA to be pelleted at the bottom and the formation of two DNA bands: the upper band is linear bacterial DNA and the lower band is supercoiled plasmid DNA. The latter was collected using a 5ml syringe and wide-bore hypodermic needle. EtBr was removed from the DNA by butanol extraction: DNA was washed with water-saturated butanol a sufficient number of times to remove all EtBr

coloring. CsCl was removed from the DNA by ethanol precipitation: 4 volumes of absolute ethanol was added to the DNA together with 0.1 volumes of 3M Na-acetate, mixed well and allowed to stand for 30 min. The mixture was then centrifuged at 10,000rpm for 15 min and the resultant pellet was rinsed with 70% ethanol and allowed to dry. The purified DNA pellet was resuspended to 1mg/ml in TE Buffer and stored at 4°C. The concentration of DNA and the amount of RNA contamination in the solution was determined by reading the absorbance at 260nm (A_{260}) and 280nm (A_{280}), respectively.

The concentration of DNA was calculated using the formula (where f is the dilution factor):

[DNA] (µg/ml) = $50(A_{260})f$

The purity of the DNA solution was calculated using the ratio:

A_{260}/A_{280}

Agarose gel electrophoresis

6X DNA Loading Dye Buffer (30% glycerol, 0.25% xylene cyanol, 0.25% bromophenol blue) was added to 10ng of DNA to a final concentration of 1X DNA Loading Dye Buffer. DNA samples and a 1-kb plus DNA ladder (Life Technologies, Cat# 10787018) were then resolved by electrophoresis on a 0.9% UltraPure™ agarose gel (Invitrogen, Cat# 15510027) prepared in TAE Buffer (40mM Tris-acetate, 1mM EDTA pH 8.0) containing 2.5µl 10mg/ml EtBr/50ml of gel. Electrophoresis was carried out at 100V in TAE buffer using the Bio-

Rad Mini-Sub Cell® GT agarose gel electrophoresis system, according to manufacturer's guidelines. DNA bands were visualized using a Bio-Rad Gel Doc™ EQ UV transilluminator and Quantity-One™ analysis software.

Cell Biology

Cell line culture

Propagation

Cells derived from the African green monkey (*Cercopithecus aethiops*) kidney (COS-7) cell line (ATCC, Cat# CRL1651) were transferred to 10ml of Dulbecco's Modified Eagle Medium with F12 supplement (DMEM/F12, 1:1) (Invitrogen, Cat# 11330032), 10% heat inactivated fetal bovine serum (FBS) (Invitrogen, Cat# 26140079) and 1% penicillin/streptomycin (Invitrogen, Cat# 15140122) then grown in a 100mm culture dish at 37°C in a humidified 5% CO_2 incubator. Cells were passaged (or subcultured) 1:5 at 70-80% confluence, which was carried out by first removing the media by aspiration and washing the cells in phosphate-buffered saline (PBS). 1ml of 0.05% trypsin/0.02% EDTA (Invitrogen, Cat# 15400054) in PBS was then added to the culture dish for 1-2 min at 37°C to detach the cells. Trypsinization was blocked by resuspending the cells in 10ml culture medium, which was pipetted up and down at least 20 times to ensure efficient dissociation of the cells. 2ml of the cell suspension was then transferred to a new culture dish with 8ml of fresh culture medium.

Transfection

COS-7 cells were transiently transfected by electroporation according to Molecular Cloning III - Sambrook and Russell, 2001. Briefly, cells passaged 24 h prior to transfection were trypsinized as described above and then spun down by centrifugation at 1000rpm for 5 min. The media was removed by aspiration and the cells washed in 10ml Opti-MEM™ reduced-serum medium (Invitrogen, Cat# 11058021) and then resuspended in 200µl of Opti-MEM™. To 100µl of the cell suspension, 3µg of plasmid DNA was added and transferred to a Bio-Rad Gene Pulser® electroporation cuvette (0.2cm gap). The cells were pulsed at 110V for 20 ms using the Bio-Rad Gene Pulser XCell™ electroporator. Transfected cells were transferred to a 60mm culture dish with 4ml of culture medium maintained at 37°C in a humidified 5% CO_2 incubator for 48 h (to allow translation) prior to experimentation.

Primary neuronal culture

Rat cortical neurons were isolated as described previously in Banker and Goslin, 1988. Briefly, cortices were dissociated from embryonic day 18 (E18) Sprague-Dawley rats in 10ml of 0.25% trypsin (Invitrogen, Cat# 25090028) in HEPES buffered Hank's Balanced Salt Solution (HBSS; Invitrogen, Cat# 14065056) for 15 min at 37°C and subsequently washed 3 times in HBSS to clear cells of any further trypsin enzymatic activity. Cells were gently titurated using a fire glass polished glass Pasteur pipette and counted using a Neubeauer haemocytometer. Dissociated rat cortical neurons were seeded onto 10µg/ml poly-L-lysine (PLL) (Sigma, Cat# P1274)-coated 60mm culture dishes at a

density of 25,000 cells/mm^2 in 4ml Neurobasal™ medium (Invitrogen, Cat# 21103049) supplemented with 2% B-27 (Invitrogen, Cat# 17504044), 1% L-glutamine (Invitrogen, Cat# 25030081) and 0.6% D-(+)-glucose solution (Sigma-Aldrich, Cat# G8769). Cultured neurons were maintained at 37°C in a humidified 5% CO_2 incubator (with regular feeding) for 8-10 DIV prior to drug treatment. Following treatment, cells were washed 3 times with ice-cold PBS prior to further experimentation.

Histology

Animals

2-4 month old C57BL/6 male mice obtained from Jackson Laboratories (Bar Harbor, ME) were housed in a temperature-controlled facility with a 12 h light-dark cycle and fed standard rodent chow and water *ad libitum.* Animals were allowed to acclimatize to these local housing conditions for at least 2 weeks prior to experimentation. All procedures were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Acute PFC slice preparation

Preparation of acute PFC slices was carried out as described previously in (Svenningsson et al., 2002). Briefly, C57BL/6 mice (2-4 months old) were

deeply anesthetized with isoflurane and then decapitated at the second cervical vertebrae. Brains were carefully removed from the skull and rinsed in ice-cold artificial CSF (ACSF; 124mM NaCl, 3mM KCl, 25mM $NaHCO_3$, 2mM $CaCl_2$, 1mM NaH_2PO_4, 2mM $MgSO_4$, 10mM D-glucose) equilibrated with 95% O_2 and 5% CO_2 to yield a pH of 7.4. The PFC was then cut into 350μm slices using a Leica VT1000S vibrating microtome and allowed to recover in ACSF under constant oxygenation for 1 h at 32°C prior to drug treatment. Following treatment, PFC slices were washed 3 times with ice-cold ACSF and then stored at -80°C until assayed.

Focused microwave irradiation of the brain

C57BL/6 mice (2-4 months old) were culled by focused microwave irradiation (4.0 kW for 1.2 s) using a Muromachi Kikai small animal microwave according to manufacturer's guidelines. Brains were rapidly removed from the skull and either the PFC dissected out and stored at -80°C until assayed, or the whole-brain kept intact for immunohistochemical analysis (described below).

Biochemistry

GST fusion protein purification

Purification of GST fusion proteins was carried out as described previously in Moss et al., 1992. A single bacterial colony (grown on an LB-ampicillin plate) was picked up using a pipette tip, transferred to a 50ml BD Falcon™ tube

containing 15ml LB-ampicillin medium and grown for 16 h at 37°C with vigorous shaking. A 15ml bacterial culture was used to inoculate 1L of LB-ampicillin and grown for a further 2-3 h or until the absorbance at 600nm (A_{600}) reached 0.4-0.5. Protein expression was then induced by adding isopropylthio-β-D-galactoside (IPTG) to a final concentration of 0.2mM and by shaking vigorously for 3 h at room temperature. Bacterial protein was centrifuged at 4,200rpm for 20 min at 4°C and the resultant pellet was resuspended in 10ml Buffer A (50mM Tris-Cl pH 8.0, 10mM EDTA pH 8.0, 25% sucrose), centrifuged again and snap-frozen. The bacterial pellet was then thawed on ice and resuspended in Buffer B (10mM Tris-Cl pH.7.4, 1mM EDTA pH 8.0) with protease inhibitors (PI; 250µg/ml AEBSF, 10µg/ml antipain, 10µg/ml leupeptin and 1µg/ml pepstatin). Triton X-100 was added to a final concentration of 1% and the mixture was sonicated at full power, 5 times for 30 s each. Following sonication, 25ml of Buffer C (20mM HEPES pH 7.6, 100mM KCl, 0.2mM EDTA pH 8.0, 20% glycerol)/PI and 400µl aprotinin was added to the cell lysate and centrifuged at 35,000rpm for 30 min at 4°C. The supernatant was added to 80mg of glutathione agarose beads (pre-swollen in Buffer C/PI) and incubated for 2 h on a rotating wheel at 4°C. The beads were washed 5 times for 10 min each in Buffer C prior to elution, which was carried out once with 1ml Elution Buffer (70mM glutathione in Buffer C, pH 7.5)/PI, then twice with 0.5ml Elution Buffer/PI. Each time the beads were suspended gently in Elution Buffer for 15 min and then centrifuged at 3,000rpm for 1 min at 4°C. The collected eluant was then pooled and dialysed against 2L of Coupling Buffer (0.1M $NaHCO_3$, 0.5M NaCl, pH 8.3) for 16 h at 4°C.

Purified GST fusion protein concentrations were determined using the Bradford protein assay reagent (Bio-Rad, Cat# 5000006), according to manufacturer's specifications at A_{595}, and then made up to 1mg/ml, aliquoted and stored at -80°C.

In vitro kinase assay

In vitro kinase assays were performed essentially as described previously in Moss et al., 1992.

Phosphorylation by PKA

10µl of GST fusion proteins (1µg/µl) was combined with 4µl of 5X PKA Buffer (200mM HEPES pH7.5, 100mM $MgCl_2$) and 2µl of 1:10 diluted PKA (2,500 units) (Promega, Cat# V516A) on ice. For mock reactions, dH_2O was substituted for PKA. Reaction mixtures were pre-warmed for 1 min at 30°C prior to the addition of 4µl of 1mM ATP (to give a final concentration of 0.2mM ATP) to initiate the reaction. Reactions were incubated for a further 30 min and then terminated by the addition of an equal volume of 2X SDS Loading Dye Buffer. In addition, a parallel assay utilizing [γ-^{32}P]-ATP:ATP mix (1:9) was carried out to allow measurement of the stoichiometry of phosphorylation.

Phosphorylation by PKC

10µl of GST fusion proteins (1µg/µl) was combined with 2µl of 10X PKC Buffer (200mM HEPES pH 7.5, 100mM $MgCl_2$, 5mM $CaCl_2$, 500nM PDBu) and 4µl of PKC (10ng/µl) (Millipore, Cat# 14115) on ice. For mock reactions,

dH_2O was substituted for PKC. Reaction mixtures were pre-warmed for 1 min at 30°C prior to the addition of 4µl of 1mM ATP (to give a final concentration of 0.2mM ATP) to initiate the reaction. Reactions were incubated for a further 30 min and then terminated by the addition of an equal volume of 2X SDS Loading Dye Buffer. In addition, a parallel assay utilizing [γ-^{32}P]-ATP:ATP mix (1:9) was carried out to allow measurement of the stoichiometry of phosphorylation.

Measurement of the stoichiometry of phosphorylation

GST fusion proteins that had been subjected to an *in vitro* kinase assay in the presence of [γ-^{32}P]-ATP were spotted onto Whatman filter paper and then washed 3 times 10 min each with 12.5% TCA to remove any free [γ-^{32}P]-ATP. These were then rinsed in absolute ethanol, allowed to dry and the amount of ^{32}P incorporated into the samples measured using a liquid scintillation counter. In addition, 1% of the [γ-^{32}P]-ATP:ATP mix was also counted for ^{32}P to enable the stoichiometry of phosphorylation (mol of ^{32}P/mol of protein) to be calculated.

Antibodies

A list of all antibodies used in this study is given in Table 2.2. Total $GABA_A$ receptor β3 subunit rabbit IgG (anti-total-β3) was raised against GST-β3 (345-408) and phosphorylation sites S408- and S409-specific $GABA_A$ receptor β3 subunit rabbit IgG (anti-pS408/9-β3) against a peptide corresponding to residues 401-412 of the rat β3 subunit chemically phosphorylated at these serine

Table 2.2: Antibodies used in this study

Primary antibody (IgG)	Source	Dilution
Polyclonal rabbit anti-total-β3	Cocalico Biologicals Antisera# UCL99	1:2,000
Polyclonal rabbit anti-pS408/9-β3	Cocalico Biologicals Antisera# UP2030	1:5,000
Monoclonal mouse anti-AP2-μ2	BD Transduction Labs Cat# 611350	1:2,500
Monoclonal mouse anti-β-actin	Sigma-Aldrich Cat# A3853	1:25,000

Secondary antibody (IgG)	Source	Dilution
Polyclonal donkey HRP-anti-rabbit	Amersham Cat# NA934V	1:5,000
Polyclonal goat HRP-anti-mouse	Pierce Cat# 1858413	1:25,000
ExactaCruz™ HRP-anti-rabbit	Santa Cruz Biotechnology Cat# sc-45043	1:2,500
ExactaCruz™ HRP-anti-mouse	Santa Cruz Biotechnology Cat# sc-45039	1:2,500
Polyclonal goat biotin-anti-rabbit	Vector Laboratories Cat# BA1000	1:500

residues (pβ3-pep). The peptide (synthesized at Rockefeller University, NY, USA), was made fully immunogenic by conjugating it to Imject® Maleimide-Activated mcKLH (Pierce, Cat# 77605) according to manufacturer's specifications. Production of antisera against both antigens was carried out at Cocalico Biologicals (PA, USA) using the standard company protocol. In brief, the procedure consisted of an initial inoculation with 250µg of antigen mixed with complete Freund's adjuvant on day 0. This was followed by a booster injection with 100µg of antigen mixed with incomplete Freund's adjuvant on day 14, and two additional boosts with 50µg antigen on days 21 and 49. Two weeks after the final boost rabbits were exsanguinated and the antisera were purified using affinity columns as described below.

Antibody purification - affinity column assay

Anti-total-β3 and anti-pS408/9-β3 antibodies were purified on GST-β3 fusion protein and pβ3-pep coupled to CNBr-activated Sepharose™ 4B (GE Healthcare, Cat# 17043001) gel columns, respectively.

Preparation of affinity columns

Affinity columns were prepared according to manufacturer's guidelines. Briefly, for each column, 1g of CNBr-activated Sepharose™ 4B freeze-dried powder (~3.5ml gel volume) was swollen for 15 min in 200ml 1mM HCl added in several aliquots and then washed with 5-gel volumes of Coupling Buffer. Antigen coupling to the gel (2mg antigen/column) was carried out overnight on a rotating wheel at 4°C. Excess antigen was washed away with 5-gel volumes of

Coupling Buffer and any remaining active groups were blocked with 5-gel volumes of 100mM Tris-Cl (pH 8.0) for 2 h on a rotating wheel at 4°C. The gel was then packed into a 10ml glass column and uncoupled antigen was washed away with 3 cycles of alternating pH. Each cycle consisted of a wash with 5-gel volumes of 100mM acetate (pH 4.0) containing 0.5M NaCl followed by 100mM Tris-Cl (pH 8.0) containing 0.5M NaCl. Prior to carrying out affinity purification of antisera, the column was equilibrated to pH 7.5 by washing with 10-gel volumes of 10mM Tris-Cl (pH 7.5).

Affinity purification of antisera

Affinity purification of antisera was carried out according to Harlow and Lane, 1988. Briefly, antiserum was diluted 1:5 in 10mM Tris-Cl (pH 7.5)/PI and passed through the column 3 times. Non-specific binding was removed by washing the column with at least 20-gel volumes of 10mM Tris-Cl (pH 7.5) followed by 10mM Tris-Cl (pH 7.5) containing 0.5M NaCl and then again with 10mM Tris-Cl (pH 7.5) alone. Bound antibody was then eluted with 5-gel volumes of 100mM glycine (pH 2.5). Eluant was collected in 900µl fractions in microfuge tubes containing 100µl 1M Tris-Cl (pH 8.0) to neutralize the IgG solution immediately. The IgG solution was then pooled, concentrated and exchanged to PBS using a Centricon® Plus-20 centrifugal ultrafiltration device (Millipore, Cat# UFC2LTK08).

Purified antibody concentrations were determined by reading the absorbance at 280nm (A_{280}) and using the extinction coefficient:

A_{280} 1.0 = 0.8mg/ml IgG

Antibodies were made up to 1mg/ml, NaN_3 added to a final concentration of 0.02%, aliquoted and stored at -80°C.

Preparation of cell and tissue extracts

Unless otherwise stated, COS-7 cells and/or rat cortical neurons were lysed in 30µl of SDS Buffer (1% SDS, 1mM EDTA, 50mM NaF), collected using a cell scraper, transferred to a microfuge tube and, following a 1:10 dilution in Triton Buffer (1% Triton X-100, 20mM Tris-Cl pH 8.0, 150mM NaCl, 10mM NaF, 2mM Na_3VO_4, 10mM $Na_4P_2O_7$, 5mM EDTA pH 8.0)/PI, briefly sonicated. Mouse PFC slices and/or tissue was briefly sonicated in 100µl of SDS Buffer and then 1:5 diluted in Triton Buffer/PI. Following lysis, all samples were centrifuged at 13,200 rpm for 15 min at 4°C and supernatants or extracts collected. Protein concentrations were determined using the Micro-BCA™ protein assay kit (Pierce, Cat# 23235), according to manufacturer's specifications at A_{570}, and the volumes adjusted with lysis buffer.

SDS-PAGE

2X or 5X SDS-polyacrylamide gel electrophoresis (PAGE) Loading Dye Buffer was added to all protein samples to a final concentration of 1X SDS-PAGE Loading Dye Buffer (50mM Tris-Cl pH 8.0/2% SDS/10% glycerol/0.1M DTT/ 0.1% bromophenol blue) and boiled for 5 min at 100°C. Protein samples with

the appropriate low-range (Bio-Rad, Cat# 1610304) or high-range (Bio-Rad, Cat# 1610303) protein molecular weight marker were then resolved by electrophoresis on either an 8% (for cell and tissue extracts) or 10% (for GST fusion proteins) SDS-PAGE gel (for preparation of SDS-PAGE gels see Table 2.3). Electrophoresis was carried out at 100V in SDS Running Buffer (25mM Tris-base, 250mM glycine, 0.1% SDS) using the Bio-Rad Mini-PROTEAN® 3 gel electrophoresis system, according to the manufacturer's guidelines.

Coomassie stain of SDS-PAGE gels

Visualization of proteins resolved by SDS-PAGE was achieved by incubating the gel in Coomassie Stain (0.25% brilliant blue R/30% methanol/10% glacial acetic acid) for 5 min with agitation. The gel was rinsed with dH_2O and incubated in Destain (30% methanol/10% glacial acetic acid) for 15 min with agitation and then replaced with fresh Destain overnight. The following day the gel was incubated in 10% glycerol for 15 min before drying for 1-2 h at 80°C on a Bio-Rad model 583 gel dryer, according to the manufacturer's guidelines.

Electro-transfer of SDS-PAGE gels

After proteins were resolved by SDS-PAGE they were electro-transferred onto Hybond™-C Extra nitrocellulose membrane (GE Healthcare, Cat# RPN303E) for 16 h at 30mA in Transfer Buffer (25mM Tris-Cl, 192mM glycine, 0.037% SDS and 20% methanol) in a 4°C cold-room using the Bio-Rad Mini Trans-Blot® electrophoretic transfer system, according to manufacturer's instructions.

Table 2.3: Preparation of SDS-PAGE gels

	Resolving gel		Stacking gel
	8%	**10%**	**5%**
dH_2O	4.6	4.0	1.4
30% acrylamide mix	2.7	3.3	0.33
1.5M Tris-Cl (pH 8.8)	2.5	2.5	-
1M Tris-Cl (pH6.8)	-	-	0.25
10% SDS	0.1	0.1	0.02
10% APS	0.1	0.1	0.02
TEMED	0.006	0.004	0.002
	Volume (ml)		

Adapted from Molecular Cloning III - Sambrook and Russell, 2001

Western blotting

Following electro-transfer of proteins, nitrocellulose membranes were briefly immersed in Ponceau Stain (0.1% ponceau S, 5% acetic acid), rinsed with dH_2O and the positions of the molecular weight marker indicated in pencil. Membranes were then blocked with 5% milk in Tris-buffered saline (TBS) containing 0.1% Tween®-20 (TBS-T) for 1 h. Next, membranes were incubated with primary antibody diluted to its workable concentration in blocking buffer for 2 h. Excess antibody was removed by washing membranes 3 times for 5 min each wash in TBS-T. The membrane was then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted to its workable concentration in blocking buffer for 1 h. Again, excess antibody was removed by washing membranes 3 times for 10 min each wash in TBS-T. It is important to note that each of the above incubations and washes were carried out with agitation. After discarding the last wash, SuperSignal® West Dura Substrate (Pierce, Cat# 34075) was applied to the blots for 5 min to allow development of chemiluminscence, and visualized using the Fujifilm LAS-3000 imaging system and Multi Gauge™ analysis software.

Dot blot assay

1-3µl of pβ3-pep and β3-pep (0.1µg/µl) were dotted onto nitrocellulose membranes that had been pre-soaked in Transfer Buffer and subject to immunoblotting with crude antiserum diluted 1:1000, as described below.

Overlay assay

GST fusion proteins separated by SDS-PAGE (1µg protein/lane) and electro-transferred onto nitrocellulose membranes were subsequently blocked with 5% BSA in TBS-T for 1 h and then overlaid with 50µl of *in vitro* translated ^{35}S-AP2-µ2 adaptin reaction mixture (TNT® T7 Quick Coupled Transcription/Translation System; Promega, Cat# TM045) in blocking buffer for 2 h. Excess radiolabeled protein was removed by washing membranes 3 times for 10 min each in TBS-T. Bound material was captured by exposing membranes to a phosphorimage screen (Bio-Rad, Cat# 1707843) and then visualized using the Bio-Rad Molecular Imager™ FX phosphorimager and Quantity-One™ analysis software.

Immunoprecipitation assay

Immunoprecipitation assays were carried out using the appropriate ExactaCruz™ rabbit (Santa Cruz biotechnology, Cat# sc-45043) and/or mouse (Santa Cruz biotechnology, Cat# sc-45039) immunoprecipitation/western blotting reagents, according to manufacturer's guidelines. Briefly, 3µg of anti-total-β3 antibody was incubated with 40µl of suspended (25% v/v) ExactaCruz™ rabbit immunoprecipitation matrix and 500µl of PBS for 1 h on a rotating wheel at 4°C. Following this, the anti-total-β3 antibody-immunoprecipitation matrix complex was pelleted by centrifugation at 3,000rpm for 1 min at 4°C. The supernatant was then carefully aspirated and discarded and the pellet washed 2 times with 500µl of Triton Buffer to remove

excess antibody. After discarding the final wash, detergent-soluble whole-cell extracts (prepared from rat cortical neurons, 8-10 DIV, lysed in 300µl of Triton Buffer/PI alone) were transferred to the pelleted matrix and incubated for 2 h at 4°C. Following this, immunoprecipitated material was pelleted by centrifugation at 3,000rpm for 1 min at 4°C and the supernatant carefully aspirated and discarded. The pellet was then washed 2 times with 500µl of high salt (500mM NaCl) Triton Buffer followed by 2 times with Triton Buffer to remove nonspecific binding. After discarding the final wash, 40µl of 2X SDS Loading Dye Buffer was added to the pelleted matrix and boiled for 5 min at 100°C. Samples were then resolved by SDS-PAGE, electro-transferred onto nitrocellulose membrane and subject to western blotting with anti-total-β3, anti-pS408/9-β3 and anti-AP2-µ2 antibodies and the appropriate HRP-conjugated ExactaCruz™ anti-rabbit (Santa Cruz Biotechnology, Cat# sc-45043) and/or anti-mouse (Santa Cruz Biotechnology, Cat# sc-45039) secondary antibodies.

Biotinylation cell surface assay

Biotinylation of cell surface proteins was carried out as described in Fairfax et al., 2004. Briefly, rat cortical neurons (8-10 DIV) were incubated with 1mg/ml EZ-link™ Sulfo-NHS-Biotin (Pierce, Cat# 21217) dissolved in ice-cold PBS containing Ca^{2+} and Mg^{2+} (PBS-CM) (Invitrogen, Cat# 14287072) for 15 min at 4°C to label surface fractions. Un-reacted biotin was then quenched by washing cells twice with Quenching Buffer (50mM glycine, 0.1% BSA in PBS-CM) and twice with PBS-CM alone. Cells were then lysed and whole-cell extracts incubated with 40µl of suspended (50% v/v) UltraLink® immobilized

NeutrAvidin™ beads (Pierce, Cat# 53150) and 500µl of Triton Buffer/PI for 2 h on a rotating wheel at 4°C to pull down biotinylated surface protein fractions. Following this, biotinylated material was pelleted by centrifugation at 3,000rpm for 1 min at 4°C. The supernatant was then carefully aspirated and discarded and the pellet washed 2 times with 500µl of high salt (500mM NaCl) Triton Buffer followed by 2 times with Triton Buffer to remove nonspecific binding. After discarding the final wash, 20µl of 2X SDS Loading Dye Buffer was added to the pelleted matrix and boiled for 5 min at 100°C. Samples were then resolved by SDS-PAGE, electro-transferred onto nitrocellulose membrane and subject to western blotting as described above.

Immunohistochemistry

HRP staining of paraffin-embedded brain sections

Preparation and HRP staining of paraffin-embedded brain sections was carried out essentially as described previously in (Chi and Chandy, 2007).

Preparation of paraffin-embedded brain sections

Brains from mice culled by microwave irradiation were fixed in 10% formalin for 16 h, washed in dH_2O for 30 min and then dehydrated in increasing alcohol grade washes: 70% ethanol (x1), 95% ethanol (x1) and absolute ethanol (x3) for 1 h each. Brains were then cleared with 3 washes in xylene for 1 h each and immersed in 3 changes of melted paraffin for 1 h each in a 60°C oven to allow

paraffin to penetrate deep into the cellular structure of the tissue. Paraffin-embedded brains were allowed to rapidly cool to form paraffin wax blocks and the PFC serially sectioned at a thickness of 6µm using a Leica SM2000R vibrating microtome. Sections were briefly immersed in a water bath set to 50°C and then mounted onto positively charged Superfrost® Plus microscope slides (Thermo scientific, Cat# 4951+).

Antigen retrieval

In order to improve the sensitivity and specificity of the immunohistochemical analysis, antigen retrieval was performed. Paraffin-embedded brain sections were first deparaffinized in a 50°C slide warmer for 10 min followed by 2 washes in xylene for 2 min each. Next, sections were rehydrated in decreasing alcohol grade washes: absolute ethanol (x2), 95% ethanol (x2) and 70% ethanol (x2) for 2 min each followed by 2 washes in dH_2O for 2 min each. Finally, antigen retrieval was performed by placing slides in a Coplin jar containing 100mmM Na-citrate (pH 8.5) and microwaving at maximum power for approximately 2 min or until buffer came to a boil. Slides were left in the boiling buffer for a further 30 min and then rinsed 2 times with dH_2O followed by 2 times with PBS.

HRP staining

In order to minimize volume of antibody required for labeling a liquid-repellent slide marker pen was first used to mark around the brain sections. After allowing the liquid-repellent to dry for a few seconds, sections were blocked in

10% goat serum (Vector Laboratories, Cat# S-1000)/1% BSA (Vector Laboratories, Cat# SP5050) diluted in PBS containing 0.3% Tween®-20 (PBS-T). The sections were then incubated with either anti-pS408/9-β3 or anti-total-β3 antibodies (1:50 diluted in blocking buffer) for 1 h. In addition, a no-primary antibody control was included for each experiment. All of the sections were then washed 5 times in PBS-T and then incubated with biotin-conjugated anti-rabbit secondary (1:500 diluted in blocking buffer) for 1 h. Sections were washed 3 times with PBS-T followed by 2 times with PBS alone and then developed utilizing HRP-conjugated avidin (Vectastain® ABC kit; Vector Labs, Cat# PK6100) with diaminiobenzidine (DAB) (Vector Labs, Ca# SK41000) as substrate, according to manufacturer's specifications. The reaction was stopped by washing the sections 5 times with ice-cold tap water. For control experiments sections were also counterstained with 10% cresyl violet, a basic synthetic dye that binds acidic components such as RNA-rich ribosomes, nuclei, and nucleoli, for 1 min and then washed with tap water until the water was clear. After allowing the sections to dry overnight, they were dehydrated in increasing alcohol grade washes: 70% ethanol (x2), 95% ethanol (x2) and absolute ethanol (x2) for 2 min each. Sections were then cleared with 2 washes in xylene for 2 min each and mounted under coverslips with permount (Fisher, Cat# SP15100).

Light microscopy

Images of HRP-stained brain sections were taken using an Olympus BXJ1 research microscope (10X objective) equipped with an Olympus DP70 digital

camera. Images were captures as .tif files and edited using Corel Photo-Paint® X3 software to enable background subtraction, contrast/brightness adjustments and cropping.

Results I

Development and characterization of phosphorylation sites S408/9-specific

$GABA_A$ receptor β3 subunit antibody

Introduction

Protein phosphorylation is one of the most extensively studied and ubiquitous mechanisms used by neurons to regulate signaling pathways. It is achieved through protein kinase catalyzed transfer of a phosphate group to serine, threonine and/or tyrosine residues of a given protein substrate that can be reversed in a dephosphorylation reaction catalyzed by protein phosphatases. Like other post-translation modifications, phosphorylation can modulate protein function by inducing a conformational change that can in turn render the protein active or inactive, alter its ability to interact with other proteins, determine its subcellular localization, and even target it for degradation. Identification and quantification of changes in the phosphorylation state of specific proteins is thus crucial in defining the function of a given protein and the consequences of its reversible phosphorylation. In many cases, a protein can be phosphorylated on multiple sites, which can act independently or synergistically when phosphorylated simultaneously. It is therefore important to examine the level of phosphorylation for individual sites on a given protein in addition to looking at the overall level of phosphorylation.

Until recently, the investigation of protein phosphorylation in intact cells and tissue was limited to methods employing pre-incubating cells with radioactive phosphate to label intracellular ATP pools that would become incorporated in the protein upon phosphorylation. Although this method continues to be very valuable, it consists of a number of complex and laborious steps when analyzing

the specific site of protein phosphorylation. It is also difficult to ascertain the spatiotemporal distribution of the phosphoprotein, since all subsequent biochemical analyses using labeled proteins require the cells to be lysed first. The advent of antibodies directed against proteins phosphorylated at a specific site has made it possible to study protein phosphorylation *in situ*, allowing for a qualitative as well as quantitative analysis of protein phosphorylation, and provides a relatively simple preparation afforded by immunochemical methodology.

$GABA_A$ receptors are well-established phosphoproteins. Emerging evidence indicates that the function of $GABA_A$ receptors is subject to dynamic modulation by phosphorylation. The β1-3 subunit ICDs are of particular interest in this context as they contain conserved serine residues (S409 in β1, S410 in β2 and S408/9 in β3) that can be differentially phosphorylated by a number or kinases in purified and recombinant receptor preparations, including PKA and PKC (Brandon et al., 2002; Krishek et al., 1994; McDonald et al., 1998; Moss et al., 1992). However, the cell signaling molecules responsible for regulating the phosphorylation state of native $GABA_A$ receptors remains elusive.

Recent studies using the phosphorylation sites S408- and S409-specific $GABA_A$ receptor β3 subunit antibody (anti-pS408/9-β3) have begun to delineate neuronal signaling pathways that modulate fast synaptic inhibition by regulating $GABA_A$ receptor β3 subunit phosphorylation and dephosphorylation. For example, the activation of tyrosine receptor kinase (Trk)-B neurotrophin

receptors has been shown to induce PKC-mediated phosphorylation of the β3 subunit, leading to a transient increase in the amplitude of mIPSCs in both cortical and hippocampal neurons, an effect that was paralleled by an increase in $GABA_A$ receptor cell surface stability (Jovanovic et al., 2004). The initial enhancement observed in receptor activity was followed by a prolonged depression that was attributed to PP2A-mediated dephosphorylation of the β3 subunit (Jovanovic et al., 2004). In addition, the activation of D_1 receptors has been shown to enhance $GABA_A$ receptor activity via PKA-mediated phosphorylation of the β3 subunit at S408 and S409 in hippocampal slices (Terunuma et al., 2004). Interestingly, this phospho-dependent modulation was diminished in PRIP-1 knockout mice that exhibited enhanced PP1α activity compared to wild-type controls (Terunuma et al., 2004).

Whilst these studies clearly demonstrate that the anti-pS408/9-β3 antibody is an invaluable tool in examining the function of the β3 subunit and the significance of its interchangeable state of phosphorylation, they do not address the degree of phosphorylation *in vivo*. This is of critical importance as treating isolated neural cells or tissue with various drugs may not yield the same results as dosing animals, given the complex infrastructure of the brain. However, a caveat in measuring the *in vivo* phosphorylation state of a given protein is ongoing activity of phosphatases postmortem following classical methods of euthanasia such as cervical dislocation, which can potentially mask the effects of a drug. Focused microwave irradiation is a relatively new method of sacrificing rodents. This irradiation rapidly heats the brain to >90°C in less than

1 s and thereby immediately destroys all enzyme activity at the time of death, preserving the *in vivo* phosphorylation state of proteins (O'Callaghan and Sriram 2004) and levels of various neurotransmitters (Butcher et al., 1976; Ishikawa et al., 1982; Katsura et al., 1992) and energy metabolites (Delaney and Geiger, 1996; Guattari 1989) that otherwise would fluctuate widely as a result of postmortem delay.

In this chapter, I report the development of anti-pS408/9-β3 (newly raised in our laboratory) to elucidate the role of serotonergic signaling in $GABA_A$ receptor β3 subunit phosphorylation and functional expression. After confirming the phospho- and site-specificity of this antibody by means of western blotting, I evaluate the use of focused microwave irradiation to measure the *in vivo* phosphorylation state of the $GABA_A$ receptor β3 subunit. I go on to address the application of this antibody in immunohistochemistry using paraffin-embedded brain sections from animals fixed by focused microwave irradiation.

Results

Design and production of anti-pS408/9-β3 antibody

Rabbit anti-pS408/9-β3 antibody was raised against a chemically phosphorylated synthetic peptide corresponding to a short region (12 amino acids) of the $GABA_A$ receptor β3 subunit phosphorylated at S408 and S409 (pβ3-pep):

K - T - H - L - R - R - R - S(PO_4) - S(PO_4) - Q - L – K

Small peptides usually elicit only a very poor immune response themselves, and therefore a cysteine residue was also introduced at the N-terminus of the peptide to enable conjugation to a carrier protein, keyhole limpet haemocyanin (KLH), which is inherently antigenic. This cysteine residue also allows the peptide to be coupled to an affinity column, which was subsequently used to purify the antibody following production. In addition, a nonphosphorylated version of the otherwise identical peptide (β3-pep) was also synthesized to later aid in antibody characterization. All peptide synthesis was carried out at the Rockefeller University (NY, USA).

The antigenic pβ3-pep was used to immunize 3 rabbits: UP-2028, UP-2029 and UP-2030 at Cocalico Biologicals (PA, USA), using the standard company protocol. In brief, the procedure consisted of an initial inoculation with 250µg of antigen mixed with complete Freund's adjuvant on day 0. This was followed by a booster injection with 100µg of antigen mixed with incomplete Freund's adjuvant on day 14, and two additional boosts with 50µg antigen on days 21 and

49. Two weeks after the final boost rabbits were exsanguinated and the serum collected.

Characterization of anti-pS408/9-β3 antibody in western blotting

Exsanguinations collected from animals UP-2028, UP-2029 and UP-2030 were screened for antisera by dot blot analysis which, unlike traditional western blotting techniques, eliminates the need to run a gel or having to do an electro-transfer and is therefore relatively quick and easy to perform. Nitrocellulose membranes were dotted with increasing concentrations (0.1-0.3µg) of pβ3-pep and β3-pep and analyzed by immunoblotting. Antisera from all 3 animals displayed strong immunoreactivity against the immunizing pβ3-pep, but not the otherwise identical nonphosphorylated β3-pep (Fig 3.1). Given that a dot blot assay is reliable only for a 'yes-or-no' answer, the antisera were subjected to a second, more stringent antibody screening.

To further scrutinize if UP-2028, UP2029 and UP-2030 antisera specifically recognize the β3 subunit in its phosphorylated state, β3 subunit ICDs were expressed as GST fusion proteins and subject to an *in vitro* phosphorylation assay either in the absence or presence of purified PKC. Mock-phosphorylated and phosphorylated proteins were then separated by SDS-PAGE and, following electro-transfer onto nitrocellulose membranes, probed with the antisera. Immunoblotting with UP-2028, UP-2039 and UP-2030 resulted in a band at the predicted molecular mass of GST-β3 (~46 kDa) when phosphorylated by PKC, with no detectable (UP-2029 and UP-2030) or weak (UP-2028)

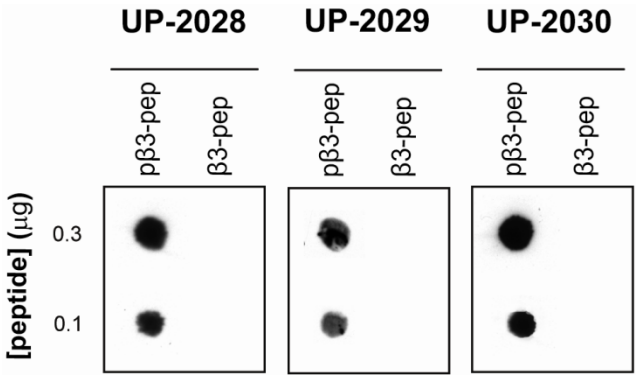


Figure 3.1: Dot blot assay. UP-2028, UP-2029 and UP-2038 specifically recognize phospho- but not nonphospho-S408/9-β3 peptide. 0.1-0.3μg of immunizing phospho-S408/9-β3 peptide (pβ3-pep) and, the otherwise identical, nonphospho-S408/9-β3 peptide (β3-pep) was dotted onto nitrocellulose membranes and immunoblotted with UP-2028, UP-2029 and UP-2030 antisera.

immunoreactivity in the absence of kinase (Fig 3.2). Given that UP-2030 demonstrated the greatest selectivity for the phosphorylated state of GST-β3 compared to the other antisera examined, it was selected for purification by affinity column assay.

To verify if the affinity-purified anti-pS408/9-β3 antibody still comprised high affinity and phospho-specificity for the target protein, GST-β3 fusion proteins were again subject to an *in vitro* phosphorylation assay by PKC and analyzed by western blotting. Anti-pS408/9-β3 displayed robust immunoreactivity for phosphorylated but not mock-phosphorylated proteins. To ensure that this discrimination was not due to unequal protein loading membranes were also probed with anti-total-β3. In addition, phosphorylation of GST-β3 was confirmed by performing a parallel kinase assay with [γ-^{32}P]-ATP. Phosphorimage analysis of proteins separated by SDS-PAGE revealed a ^{32}P-labeled band corresponding to GST-β3 when this fusion protein was incubated in the presence, but not in the absence, of kinase (Fig 3.3).

The ability of anti-pS408/9-β3 to recognize the full length of the $GABA_A$ receptor β3 subunit phosphorylated at S408 and S409 was analyzed next in COS-7 cells cotransfected with α1 and either β3 or $\beta3^{S408/9A}$. Cells were treated with either vehicle or 1μM PDBu in the presence of 1μM okadaic acid for 30 min and lysed. Equal amounts of protein extracts were then separated by SDS-PAGE and subject to western blot analysis. Immunoblotting with anti-pS408/9-β3 demonstrated that basal phosphorylation of α1/β3 subunit-containing

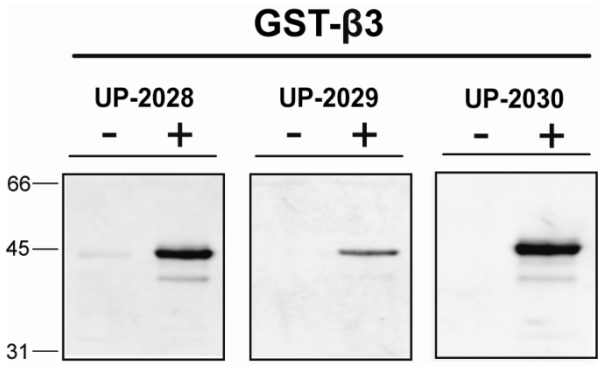


Figure 3.2: UP-2028, UP-2029 and UP-2030 antisera specifically recognize GST-β3 phosphorylated *in vitro* by PKC. GST-β3 fusion proteins were subjected to an *in vitro* phosphorylation assay either in the absence (-) or presence (+) of purified PKC. Proteins (1µg/lane) were resolved by SDS-PAGE then electro-transferred onto nitrocellulose membranes and immunoblotted with UP-2028, UP-2029 and UP-2030 antisera.

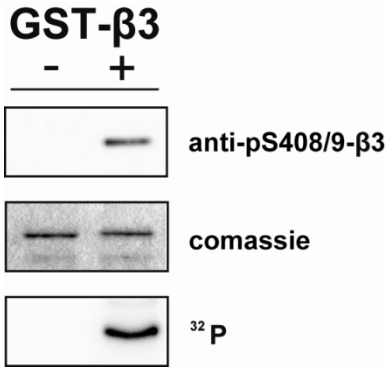


Figure 3.3: Affinity purified anti-pS408/9-β3 antibody specifically recognizes GST-β3 phosphorylated in vitro by PKC. GST-β3 fusion proteins were subjected to an in vitro phosphorylation assay either in the absence (-) or presence (+) of purified PKC. Proteins (1µg/lane) were resolved by SDS-PAGE then electro-transferred onto nitrocellulose membranes and immunoblotted with affinity purified anti-pS408/9-β3 and anti-total-β3 antibodies. The autoradiograph confirms phosphorylation of GST-β3 in a parallel assay utilizing [γ-^{32}P]-ATP.

recombinant receptors is enhanced following activation of PKC and simultaneous inhibition of PP1/PP2A activity respectively, whereas this immunoreactivity (~53 kDa) was absent from cells cotransfected with $\beta3^{S408/9A}$ (Fig 3.4) and mock transfected controls (data not shown). To confirm that reduced expression of $\beta3^{S408/9A}$ was not the basis for this lack of recognition, membranes were also probed with anti-total-β3, which revealed no significant difference in total expression.

To determine if anti-pS408/9-β3 antibody could be used to examine phosphorylation of native $GABA_A$ receptor β3 subunits, rat cortical neurons were treated with 1μM okadaic acid for increasing time periods and whole-cell extracts analyzed by western blotting. At time 0, immunoblotting with anti-pS408/9-β3 identified a band at ~58 kDa that was significantly enhanced following okadaic acid treatment for 10 (195.5 ± 19.0%; $p<0.01$), 20 (263.0 ± 24.9%; $p<0.001$) and 30 (363.3 ± 17.6%; $p<0.001$) min, and also resulted in the emergence of another possibly hyperphosphorylated band, the migration of which was slowed with increasing exposure time (~73 kDa at 30 min). Interestingly, immunoblotting with anti-total-β3 also identified a band at ~58 kDa that was significantly enhanced following okadaic acid treatment for 10 (176.8 ± 6.7%; $p<0.05$), 20 (347.2 ± 26.5%; $p<0.001$) and 30 (573.5% ± 30.5%; $p<0.001$) min, in addition to a band at ~53 kDa which remained unchanged (Fig 3.5). Given that treatment with 1μM okadaic acid for 30 min resulted in the greatest increase in the basal phosphorylation of the β3 subunit, this concentration and time point was used in subsequent experiments to further

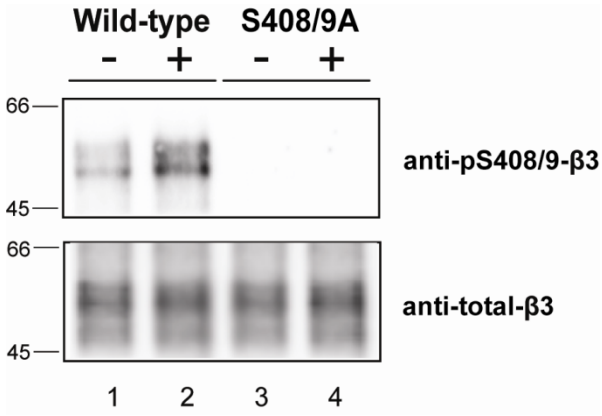


Figure 3.4: Anti-pS408/9-β3 antibody specifically recognizes $GABA_A$ receptor β3 subunit in its phosphorylated state in COS-7 cells. COS-7 cells coexpressing α1 with either wild-type (lanes 1 and 2) or S408/9A mutant (lanes 3 and 4) β3 subunit-containing recombinant $GABA_A$ receptors were treated with either vehicle (-) or 1μM PDBu (a PKC activator) in the presence of 1μM okadaic acid (a PP1/PP2A inhibitor) (+) for 30 min and subsequently lysed with detergent. Whole-cell extracts (1μg protein/lane) were then subjected to western blotting with anti-pS408/9-β3 and anti-total-β3 antibodies.

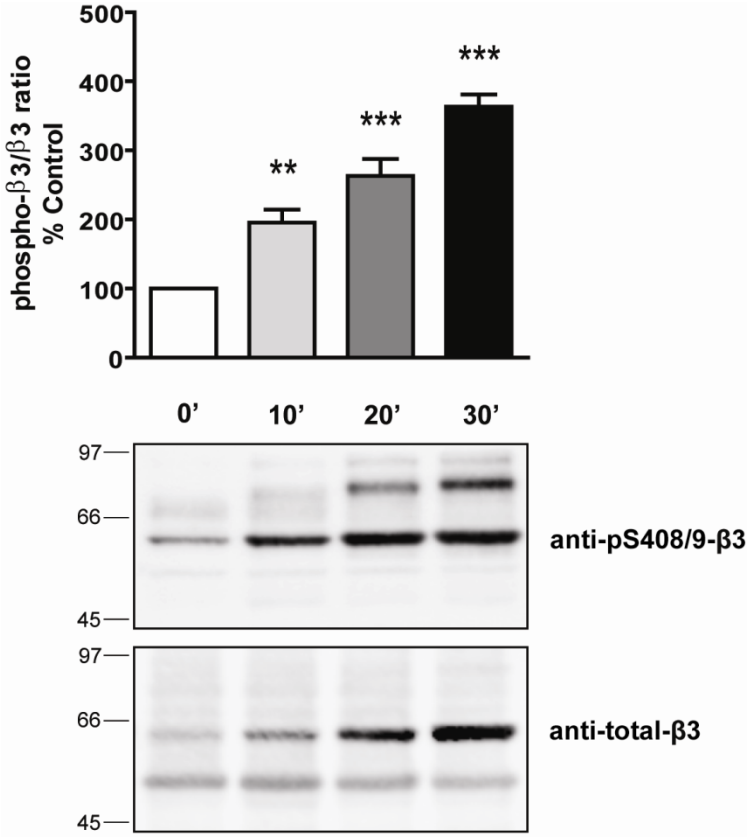


Figure 3.5: Inhibiting PP1/PP2A activity increases anti-pS408/9-β3 immunoreactivity in cultured neurons. Western blot analysis of detergent-soluble whole-cell extracts (30µg protein/lane) prepared from rat cortical neurons treated with the PP1/PP2A inhibitor okadaic acid (1µM) for the times indicated using anti-pS408/9-β3 and anti-total-β3 antibodies. Histogram shows the proportion of $GABA_A$ receptor β3 subunits phosphorylated expressed as a percentage of 0 min treatment time (**p<0.01, ***p<0.001; one-way ANOVA followed by Newman-Keuls multiple comparison test).

evaluate if these slower migrating bands correspond to phosphorylation of the $GABA_A$ receptor β3 subunit at S408 and S409.

As before, immunoblotting whole cell extracts prepared from rat cortical neurons with anti-pS408/9-β3 identified a band at ~58 kDa that was enhanced following okadaic acid treatment and also resulted in the emergence of another band at ~73 kDa. This immunodetection was prevented when the primary antibody was pre-absorbed with molar excess of pβ3-pep but not β3-pep (Fig 3.6). In addition, anti-pS408/9-β3 immunoreactive bands were absent in membranes pre-treated with λ-PPa, an Mn^{2+}-dependent protein phosphatase with activity towards phosphorylated serine, threonine and tyrosine residues. Similarly, immunoreactivity of anti-total-β3 for the slow migrating phosphorylated state of β3 (~58 kDa), but not for dephosphorylated β3 (~53 kDa), was abolished under these treatment conditions. In fact, the anti-total-β3 antibody signal for dephosphorylated β3 was amplified, probably due to freeing of antibody available for binding to this region (Fig 3.7).

Like the immunoreactivity observed in rat cortical neurons, anti-pS408/9-β3 signal was amplified following okadaic treatment at both ~58 and ~73 kDa in mouse PFC slices. However, by contrast the ~73 kDa band was much more prominent than was the barely visible ~58 kDa band, which was absent from vehicle-treated controls and not detected by the anti-total-β3 antibody. This immunodetection was again abolished in the presence of pβ3-pep (Fig 3.8) and dramatically reduced following dephosphorylation by λ-PPa (Fig 3.9),

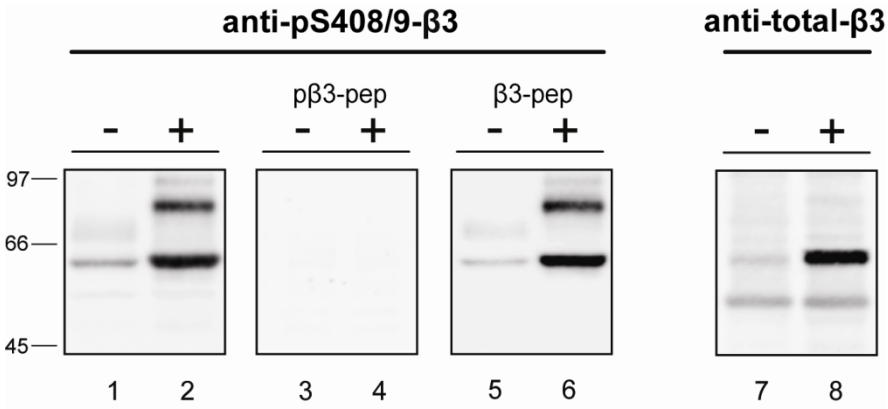


Figure 3.6: Phospho- but not nonphospho-S408/9-β3 peptide blocks immunodetection of phosphorylated $GABA_A$ receptor β3 subunit by anti-pS408/9-β3 antibody in cultured neurons. Western blot analysis of detergent-soluble whole-cell extracts (30µg protein/lane) prepared from rat cortical neurons treated with either vehicle (-) or 1µM okadaic acid (+) for 30 min using anti-pS408/9-β3 antibody either alone (lanes 1 and 2) or pre-incubated with 500 molar excess of chemically phosphorylated (pβ3-pep, lanes 3 and 4) and nonphosphorylated (β3-pep, lanes 5 and 6) S408/9 β3 synthetic peptides. Lanes 7 and 8 indicate total β3.

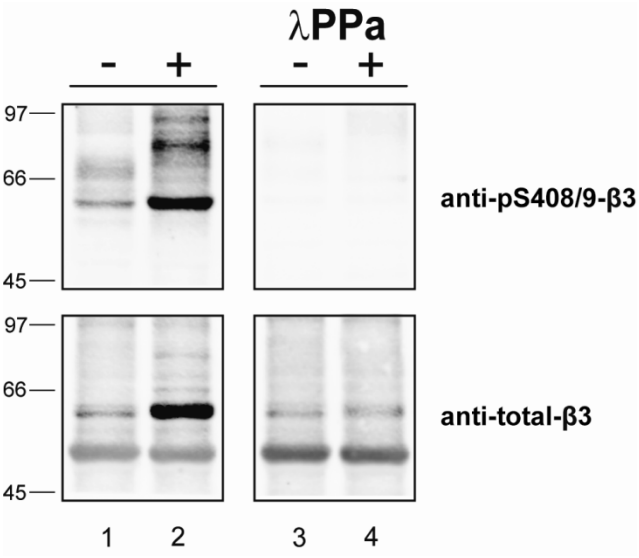


Figure 3.7: λ-PPa treatment blocks immunodetection of phosphorylated $GABA_A$ receptor β3 subunit by anti-pS408/9-β3 and anti-total-β3 antibodies in cultured neurons. Western blot analysis of detergent-soluble whole-cell extracts (30μg protein/lane) prepared from rat cortical neurons treated with either vehicle (-) or 1μM okadaic acid (+) for 30 min. Membranes were incubated either in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of λ-PPa and then probed with anti-pS408/9-β3 and anti-total-β3 antibodies.

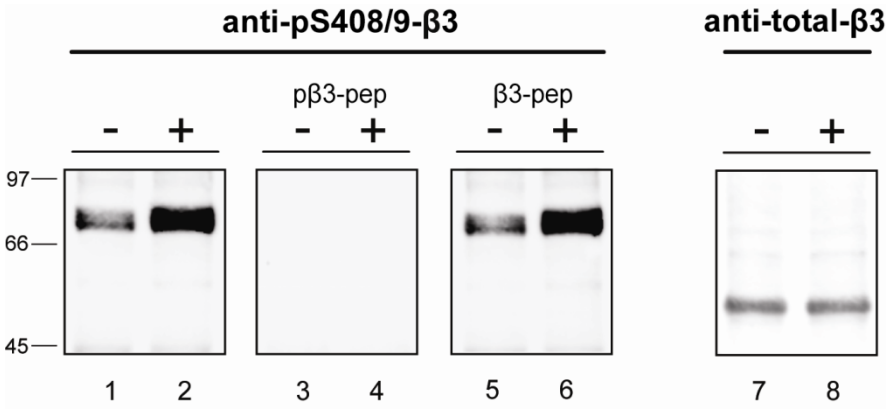


Figure 3.8: Phospho- but not nonphospho-S408/9-β3 peptide blocks immunodetection of phosphorylated $GABA_A$ receptor β3 subunit by anti-pS408/9-β3 antibody in brain slices. Western blot analysis of detergent-soluble brain extracts (30µg protein/lane) prepared from C57BL/6 mouse PFC slices treated with either vehicle (-) or 1µM okadaic acid (+) for 30 min, using anti- pS408/9-β3 antibody either alone (lanes 1 and 2) or pre-incubated with 500 molar excess of chemically phosphorylated (pβ3-pep, lanes 3 and 4) and nonphosphorylated (β3-pep, lanes 5 and 6) S408/9 β3 synthetic peptides. Lanes 7 and 8 indicate total β3.

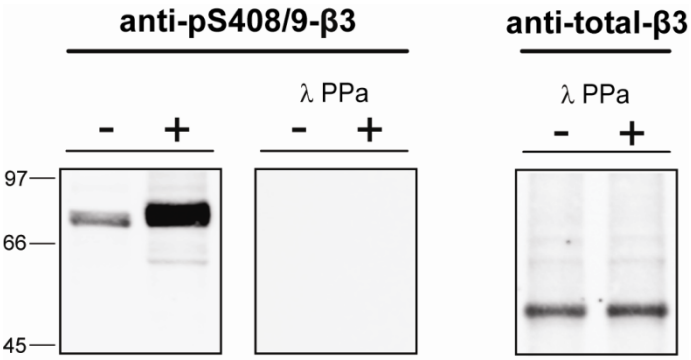


Figure 3.9: λ-PPa treatment blocks immunodetection of phosphorylated $GABA_A$ receptor β3 subunit by anti-pS408/9-β3 antibody in brain slices. Western blot analysis of detergent soluble brain extracts (30µg protein/lane) prepared from C57BL/6 mouse PFC slices treated with either vehicle (-) or 1µM okadaic acid (+) for 30 min. Membranes were incubated either in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of λ-PPa and then probed with anti-pS408/9-β3 and anti-total-β3 antibodies.

confirming that these slower migrating bands correspond to the $GABA_A$ receptor β3 subunit phosphorylated at S408 and S409.

Focused microwave irradiation to measure *in vivo* $GABA_A$ receptor β3 subunit phosphorylation in the brain

To assess if rapid heat inactivation of enzymes by focused microwave irradiation sacrifice can prevent the loss of *in vivo* levels of $GABA_A$ receptor β3 subunit phosphorylation due to postmortem delay, brain samples from animals sacrificed by either cervical dislocation or focused microwave irradiation were analyzed by western blotting. The results show significantly ($p<0.001$) higher (244.2% ± 7.6%) levels of basal phosphorylation in microwaved samples compared to those from animals sacrificed by cervical dislocation, as detected by anti-pS408/9-β3, while immunoblotting with the anti-total-β3 antibody revealed that total receptor subunit expression was unaffected by the mode of euthanasia (Fig 3.10). Moreover, instant fixation by focused microwave irradiation reduced animal-animal variability seen with sacrifice by cervical dislocation, likely due to small but significant differences in the time interval between sacrificing the animal and sonication of brain tissue in denaturing lysis buffer (Fig 3.11).

Characterization of anti-pS408/9-β3 antibody in immunohistochemistry

Having established that focused microwave irradiation of the brain delivers the highest and most consistent levels of *in vivo* $GABA_A$ receptor β3 subunit

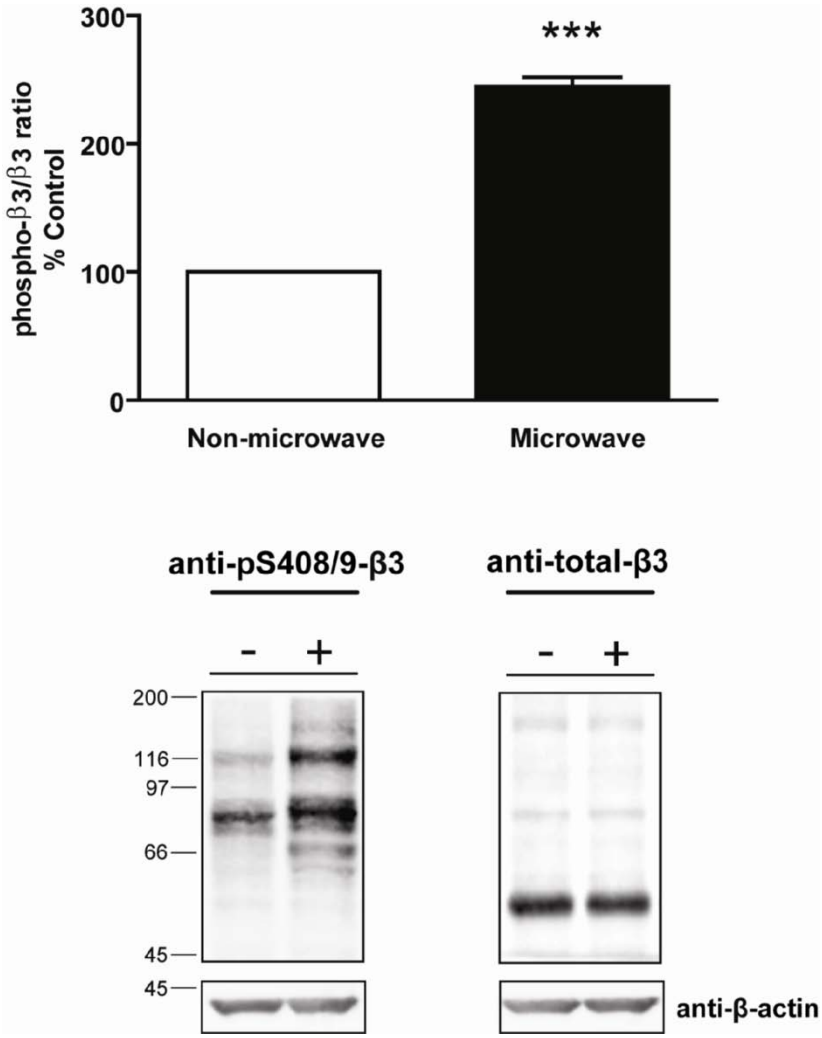


Figure 3.10: Focused microwave irradiation of the brain preserves *in vivo* $GABA_A$ receptor β3 subunit phosphorylation. C57BL/6 mice were culled either by cervical dislocation (-) or by focused microwave irradiation (+). Brains were rapidly removed from the skull, dissected on ice and immediately frozen in liquid nitrogen prior to lysis. Detergent-soluble PFC extracts (30µg protein/lane) were then subjected to western blotting with anti-pS408/9-β3 and anti-total-β3 antibodies. Histogram shows the proportion of $GABA_A$ receptor β3 subunits phosphorylated expressed as a percentage of non-microwave control (***$p<0.001$; Students *t* test).

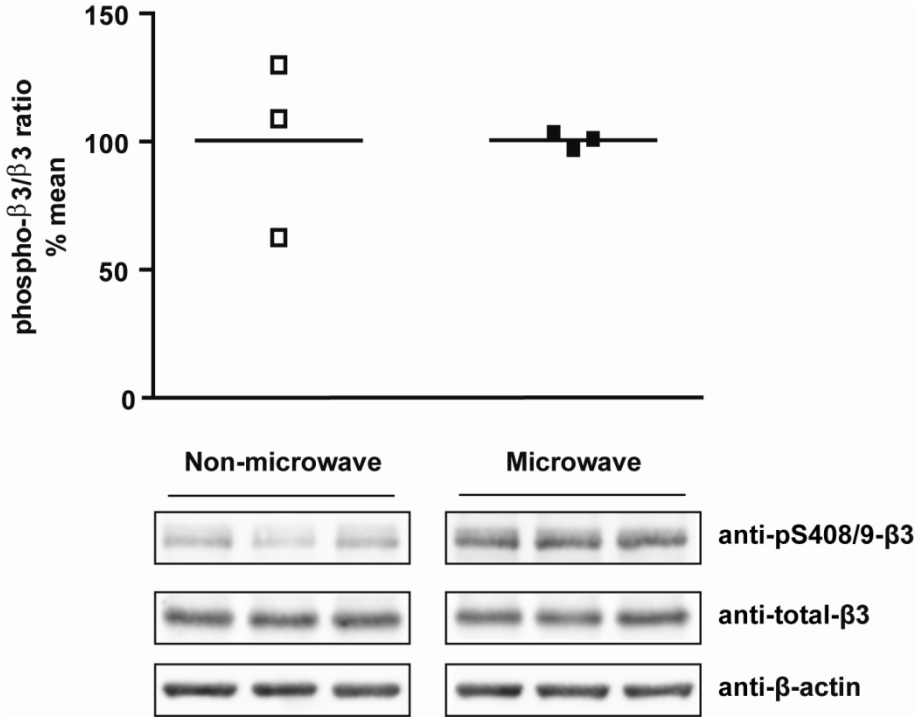


Figure 3.11: Focused microwave irradiation minimizes *in vivo* $GABA_A$ receptor β3 subunit phosphorylation animal-animal variability in the brain. C57BL/6 mice were culled either by cervical dislocation (□) or by focused microwave irradiation (■). Brains were rapidly removed from the skull, dissected on ice and immediately frozen in liquid nitrogen prior to lysis. Detergent-soluble PFC extracts (30µg protein/lane) were then subjected to western blotting with anti-pS408/9-β3 and anti-total-β3 antibodies. Scatter plot shows the proportion of $GABA_A$ receptor β3 subunits phosphorylated expressed as a percentage of non-microwave and microwave mean values (***$p<0.001$; Students *t* test).

phosphorylation at S408 and S409, this method of fixation was adopted for subsequent immunohistochemical analysis of this phosphoprotein. HRP staining of paraffin-embedded brain sections with either anti-pS408/9-β3 or anti-total-β3 antibodies demonstrated that, *in vivo,* the $GABA_A$ receptor β3 subunit is basally phosphorylated at S408 and S409 in the PFC (Fig 3.12). Counterstaining with cresyl violet, a basic synthetic dye that binds acidic components such as RNA-rich ribosomes, nuclei, and nucleoli demonstrates that the HRP staining observed in the presence of these antibodies is neuronal-specific. No HRP staining was observed in the absence of primary antibody (Fig 3.12), confirming the validity of this method for measuring the regional and cellular distribution this phosphoprotein.

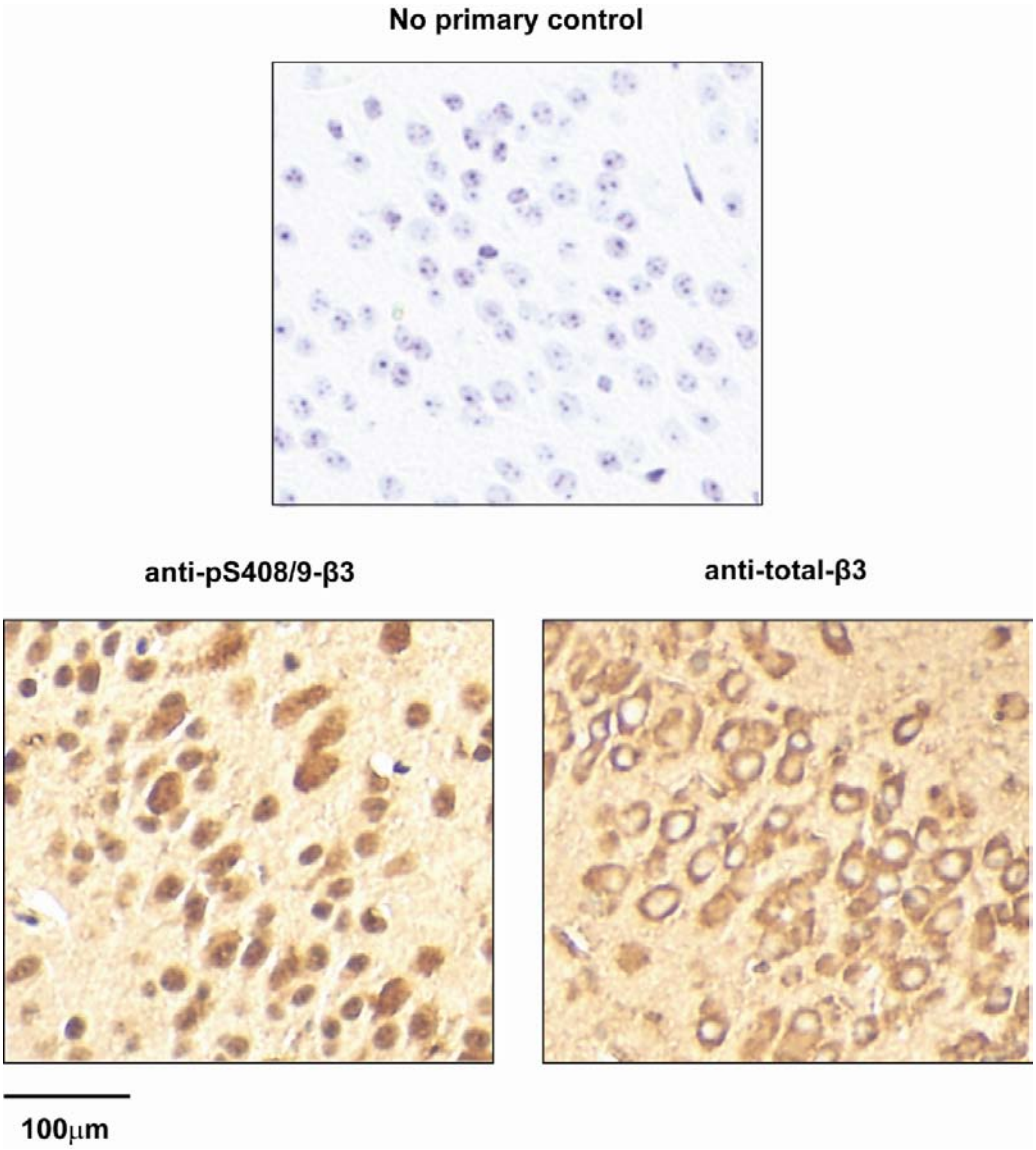


Figure 3.12: HRP staining of paraffin-embedded brain sections from mice fixed by focused microwave irradiation. Paraffin-embedded PFC sections from C57BL/6 mice culled by focused microwave irradiation were stained either in the absence (upper panel) or presence (lower panels) of primary antibody (brown), counterstained with cresyl violet (purple) and then visualized using HRP-conjugated secondary antibody coupled to light microscopy.

Discussion

In this chapter, I report the development of a polyclonal antibody directed against the $GABA_A$ receptor β3 subunit phosphorylated at sites S408 and S409, anti-pS408/9-β3, and its use in characterizing $GABA_A$ receptor phosphorylation in the brain. Whilst this antibody has been previously described in our laboratory (Jovanovic et al., 2004), it is imperative to ensure the phospho- and site-specificity of every new batch of antibody made. Initial screening identified positive antisera from all 3 animals tested. These antisera displayed strong affinity and highly selective immunoreactivity for the immunizing pβ3-pep but not the otherwise identical nonphosphorylated β3-pep. Subsequent screening using GST-β3 fusion proteins either mock phosphorylated or phosphorylated *in vitro* by PKC revealed that UP-2030 comprised the highest titer compared to the other antisera examined, and was therefore selected for purification and subject to further screening.

Affinity purified anti-pS408/9-β3 displayed robust and selective immunoreactivity for GST-β3 phosphorylated *in vitro* by PKC, confirming the recovery of high affinity phospho-specific antibody following purification. In addition, anti-pS408/9-β3 specifically recognized the phosphorylated state of full length wild-type, but not S408/9A mutant, α1/β3 subunit-containing $GABA_A$ receptors expressed in COS-7 cells, further demonstrating the site-specificity of the antibody. Consistent with previous studies, the β3 subunit was shown to be basally phosphorylated at S408 and S409 (McDonald et al., 1998)

and was enhanced following activation of PKC in the presence of a PP1/PP2A inhibitor.

However, given that it is difficult and often misleading to correlate results derived from *in vitro* assay systems, using purified and recombinant receptors along with those from isolated cell and tissue preparations, the ability of anti-pS408/9-β3 to specifically recognize native $GABA_A$ receptor β3 subunits phosphorylated at S408 and S409 in cultured neurons and brain slices was also determined. Inhibiting endogenous PP1/PP2A activity in both rat cortical neurons and mouse PFC slices dramatically enhanced basal phosphorylation levels and also slowed the migration of this receptor subunit on a denaturing SDS-PAGE gel, as detected by immunoblotting with anti-pS408/9-β3. This finding was confirmed using a competition assay in which the primary antibody was pre-absorbed with molar excess of either pβ3-pep or β3-pep, where the latter failed to block this immunodetection. Moreover, pre-treating the blots with λ-PPa, which releases phosphate groups from phosphorylated serine, threonine and tyrosine residues, also reduced the signal for the slow migrating phosphorylated form of the β3 subunit by both the anti-pS408/9-β3 and anti-total-β3 antibodies.

Together these results demonstrate that the anti-pS408/9-β3 antibody specifically recognizes the $GABA_A$ receptor β3 subunit phosphorylated at S408 and S409, and that phosphorylation reduces the electrophoretic mobility of this protein. The slowed migration of this phospho-protein may be explained by the

presence of SDS-resistant domains which are unfolded as a result of phosphorylation. However, further experiments using urea gradient-PAGE to monitor the denaturant-induced unfolding of this phosphoprotein is necessary to examine this phenomenon.

Having characterized the phospho- and site-specificity of anti-pS408/9-β3 antibody, the use of focused microwave irradiation in measuring *in vivo* $GABA_A$ receptor β3 subunit phosphorylation in the brain was determined by western blotting. Consistent with previous reports, this mode of sacrifice delivered the highest and most consistent levels of phosphorylation when compared with cervical dislocation (Hebert and O'Callaghan, 2000; Hossain et al., 1994; Jope et al., 1991; Mobley and Gonzalez, 1991; O'Callaghan and Sriram, 2004; O'Callaghan et al., 1998; Snyder et al., 2000), confirming that focused microwave irradiation of the brain preserves *in vivo* $GABA_A$ receptor β3 subunit phosphorylation. Conversely, a dramatic loss and variability in the phosphorylation state of this receptor subunit was seen following sacrifice by cervical dislocation, despite every attempt made to minimize the time-interval between sacrificing the animal and sample preparation. These results indicate that classical methods of euthanasia will not prove suitable for most *in vivo* phosphoprotein analysis, and that rapid heat inactivation of enzymes by focused microwave irradiation of the brain may be required to achieve biologically meaningful data. Given the marked regional and cellular distribution of any given phosphoprotein, the application of anti-pS408/9-β3 in immunohistochemistry was also confirmed in the brains of animals fixed by

focused microwave irradiation. However, it will also be interesting to address the practicality of this antibody in immunofluorescence staining, which would enable costaining with other proteins and/or markers of interest.

Results II

Phospho-dependent modulation of $GABA_A$ receptor β3 subunit functional expression

Introduction

Altering the number of postsynaptic $GABA_A$ receptors accessible to neurotransmitter represent one of the most powerful mechanisms used by neurons to regulate the efficacy of inhibitory synaptic transmission (Kittler et al., 2000; Kittler and Moss, 2003; Nusser et al., 1998a, 1997). Given the critical role of these receptors in controlling neuronal excitability and animal behavior, it is unsurprising that the molecular mechanisms underlying the expression and stability of cell surface $GABA_A$ receptor populations have been an active area of research for the past decade.

$GABA_A$ receptors have been shown to be localized in clathrin-coated pits suggesting that they undergo classical clathrin-mediated endocytosis, a process that is further dependent on dynamin for endocytic vesicle formation. AP2 is a central component in the formation of these vesicles, forming a link between membrane proteins and clathrin which forms the outer layer of the coat. Internalized receptors are then either subjected to rapid recycling or are targeted for lysozomal degradation, an endocytic sorting decision that is regulated by HAP1 (Kittler et al., 2004). Changes in the rates of receptor endocytosis and/or endocytic sorting thus play significant roles in the postsynaptic modulation of $GABA_A$ receptor-mediated synaptic inhibition.

Given the pivotal role of the AP2 complex in clathrin-mediated endocytosis, a possible approach to alter the rate of receptor internalization would be to

modulate the interaction between the AP2 complex and endocytic motifs in cell surface receptors that destine the receptor to be internalized. An atypical AP2-binding motif conserved within the ICD of all $GABA_A$ receptor β subunit isoforms has recently been identified (KTHLRRRSSQLK in the β3 subunit) (Kittler et al., 2005). This motif, which is enriched in lysine and arginine residues, also incorporates the major sites of phosphorylation for PKA and PKC within this class of receptor subunits - S409 in β1, S410 in β2 and S408/9 in β3 (Moss et al., 1992; Krishek et al., 1994; Brandon et al., 2002). *In vitro* binding studies have revealed that the μ2 adaptin of the AP2 complex was able to bind the β3 subunit in its dephosphorylated state but not when phosphorylated by PKA or PKC. This phospho-dependent interaction was also found to modulate mIPSC amplitudes and whole-cell $GABA_A$ receptor currents in a manner that was occluded by inhibitors of dynamin (Kittler et al., 2005).

Although these findings provide evidence of an interaction between the μ2 adaptin of the AP2 complex and the $GABA_A$ receptor β3 subunit that is negatively regulated by phosphorylation *in vitro*, in this chapter the effect of $GABA_A$ receptor β3 subunit phosphorylation on AP2-μ2 adaptin binding is also addressed in cultured neurons. Utilizing an overlay assay with radiolabeled AP2-μ2 adaptin, I first confirm and extend previous *in vitro* binding studies to show that binding to this complex is dependent on the phosphorylation state of $GABA_A$ receptor β1, β2 and β3 subunit isoforms and that, in the case of the β3 subunit, S409, but not S408, is critical for this phospho-dependent interaction. I then go on to demonstrate that coimmunoprecipitation of these proteins is

greatly reduced in cultured neurons treated with okadaic acid, a potent inhibitor of S408 and S409 dephosphorylation (Jovanovic et al., 2004). Finally, I show that this reduction in coimmunoprecipitation is accompanied by a corresponding increase in the number of biotin-labeled $GABA_A$ receptor β3 subunits on the cell surface.

Results

AP2-μ2 adaptin binding to $GABA_A$ receptor β subunits is negatively regulated by phosphorylation *in vitro*

Recent studies have demonstrated that a basic-rich motif localized within the ICD of all $GABA_A$ receptor β subunit isoforms is capable of directly interacting with the μ2 adaptin of the AP2 complex *in vitro*. Interestingly, this binding region overlaps with conserved serine residues that are known substrates for several serine/threonine kinases, including PKA and PKC and, in the case of the β3 subunit, phosphorylation of S408 and S409 has been shown to block this interaction *in vitro* (Kittler et al., 2005).

To determine if AP2-μ2 adaptin binding to $GABA_A$ receptor β1 and β2 subunits is also negatively regulated by phosphorylation, GST fusion protein constructs encoding ICDs of the β1, β2 and β3 subunits were subjected to an *in vitro* phosphorylation assay by PKA. Mock-phosphorylated and phosphorylated proteins (1, 3 and 10μg/lane) were then separated by SDS-PAGE and, following electro-transfer onto nitrocellulose membranes, overlayed with [^{35}S]-methionine-labeled AP2-μ2 adaptin (^{35}S-μ2). Bound radiolabeled protein was detected by phosphorimaging and normalized to mock-phosphorylated coomassie stain. ^{35}S-μ2 binding was expressed as a percentage of 'maximal' binding to 10μg mock-phosphorylated controls and the resultant binding curves used to derive the apparent dissociation constants (K_d, concentration of fusion protein giving rise to half-maximal binding) as a measure of relative binding

affinities for mock-phosphorylated and phosphorylated GST-β1, β2 and β3 fusion proteins in this assay (Fig 4.1). Given that K_d has molar units, the following conversion was used:

$$\frac{\left(\frac{\text{Mass}}{\text{Molecular weight}}\right)}{\text{Volume}} = \text{Molarity}$$

Where: Volume = 10µl; Molecular weight = 43, 42 and 45 kDa for GST-β1, β2 and β3 fusion proteins, respectively.

It was found that ^{35}S-µ2 binding affinity of GST-β1 (K_d = 3.1µM) was ~1.6-fold lower and that of GST-β2 (K_d = 3.5µM) ~1.7-fold lower when phosphorylated *in vitro* by PKA (K_d = 5.0µM and 6.0µM, respectively). In contrast, a ~5.2-fold decrease in binding affinity was observed for phospho-GST-β3 (K_d = 20.2µM) compared to mock-phosphorylated control (K_d = 3.9µM). Further analysis of ^{35}S-µ2 binding within the linear range (i.e. to 1µg of fusion proteins) revealed a significant ($p<0.001$) decrease in binding to the phosphorylated state of GST-β1 (41.8 ± 6.4%), β2 (37.8 ± 5.2%) and GST-β3 (6.3 ± 1.3%) fusion proteins, expressed as a percentage of mock-phosphorylated controls (Fig 4.2). In this study, the stoichiometry of phosphorylation achieved was much higher for GST-β3 (0.7mol/mol) than for GST-β1 (0.3mol/mol) and β2 (0.4mol/mol), as measured in a parallel *in vitro* kinase assay utilizing [γ-^{32}P]-ATP (see methods). This may in part explain the more dramatic effect seen with the phosphorylated state of GST-β3 compared to the other β subunit isoforms.

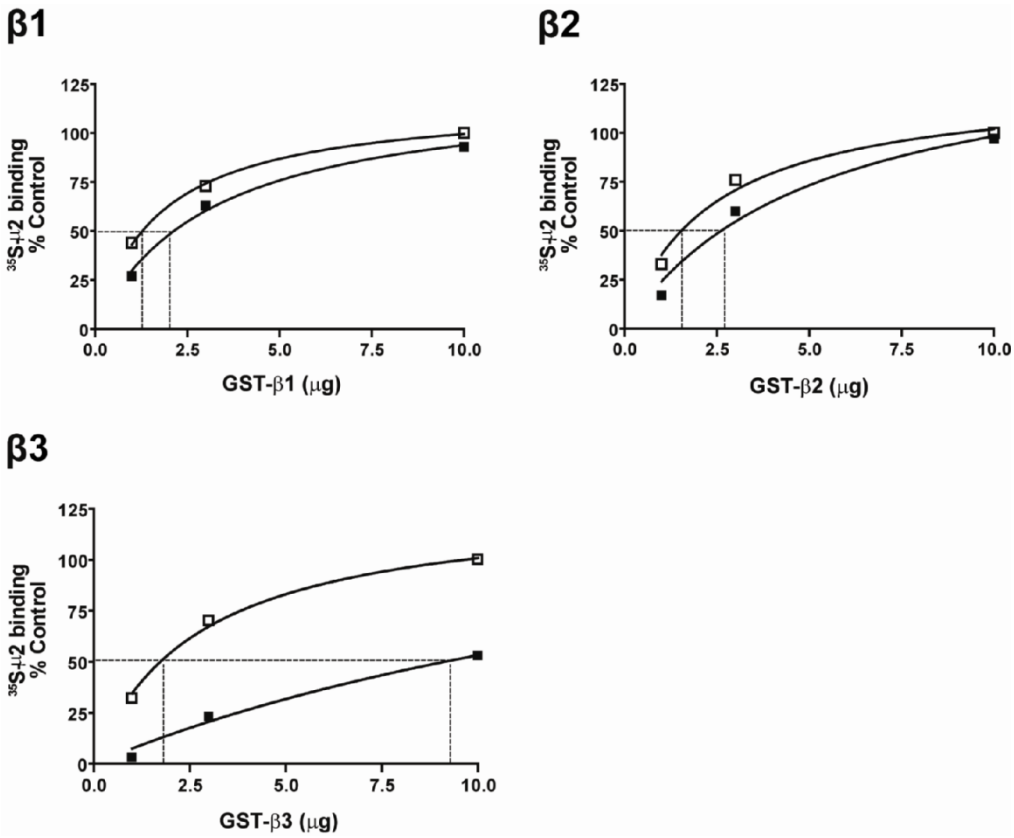


Figure 4.1: PKA-induced phosphorylation of GST-β1, β2 and β3 fusion proteins shifts ^{35}S-μ2 binding curves to the right *in vitro*. GST-β1, β2 and β3 fusion proteins were subjected to an *in vitro* phosphorylation assay either in the absence (□) or presence (■) of purified PKA. Mock-phosphorylated and phosphorylated proteins (1, 3 or 10μg/lane) were separated by SDS-PAGE and, following electro-transfer onto nitrocellulose membranes, overlayed with *in vitro* translated ^{35}S-μ2. Bound material was visualized by phosphorimaging and normalized to mock-phosphorylated coomassie stain. Binding curves show ^{35}S-μ2 binding to fusion proteins expressed as a percentage of 10μg mock-phosphorylated controls, set to a value of 100%. Micrograms of fusion protein giving rise to half-maximal binding (dashed line) were subsequently used to calculate K_d (see text).

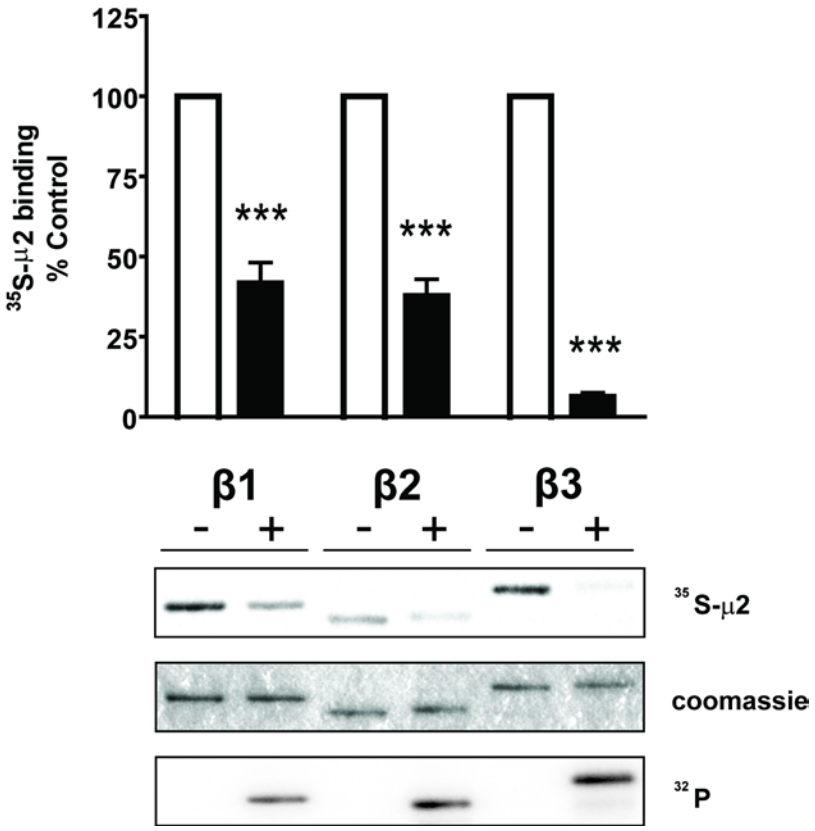


Figure 4.2: PKA-induced phosphorylation decreases ^{35}S-μ2 binding to GST-β1, β2 and β3 fusion proteins *in vitro*. GST-β1, β2 and β3 fusion proteins were subjected to *in vitro* phosphorylation either in the absence (-) or presence (+) of purified PKA. Mock-phosphorylated and phosphorylated proteins (1μg/lane) were separated by SDS-PAGE then electro-transferred onto nitrocellulose membrane and overlayed with *in vitro* translated ^{35}S-μ2. Bound material was visualized by phosphorimaging (upper panel) and normalized to coomassie stain (middle panel). The stoichiometry of phosphorylation was measured in a parallel assay utilizing [γ-^{32}P]-ATP (lower panel). Histogram shows ^{35}S-μ2 binding to fusion proteins expressed as a percentage of mock-phosphorylated controls (***$p<0.001$; one-way ANOVA followed by a Newman-Keuls multiple comparison test).

S409 (but not S408) of the $GABA_A$ receptor β3 subunit is critical for AP2-μ2 adaptin binding *in vitro*

In the previous study, phosphorylation by PKA was shown to completely abolish AP2-μ2 adaptin binding to GST-β3. Given that S409, but not S408, is a substrate for this kinase *in vitro* (McDonald and Moss, 1997), it was speculated that this interaction was dependent on the presence of S409 alone. To pinpoint the serine residue(s) critical for binding to the μ2 adaptin of the AP2 complex, β3 subunit ICDs bearing various serine-to-alanine mutations (S408/9A, S408A and S409A) were constructed by site-directed mutagenesis and expressed as GST fusion proteins. Again utilizing an overlay assay, GST-β3 wild-type and serine-alanine mutants were examined for their ability to bind ^{35}S-μ2. Phosphorimage analysis revealed significantly ($p<0.001$) decreased ^{35}S-μ2 binding in the S408/9A mutant (58.0 ± 5.6% compared to wild-type control). Interestingly, mutating only the serine at position 409 to an alanine was sufficient to mimic this effect (63.7 ± 2.1% compared to wild-type control). Conversely, the S408A mutant exhibited a significant ($p<0.001$) increase in bound ^{35}S-μ2 (112.5 ± 1.1% compared to wild-type control) (Fig 4.3), indicating that not only is the serine at position 408 not required for AP2-μ2 adaptin binding *in vitro*, but that this interaction is more pronounced when this residue is mutated to an alanine.

I went on to repeat this experiment using GST-β3 wild-type and serine-to-alanine mutant fusion proteins that had been subjected to *in vitro* phosphorylation by PKC, a kinase that is known to phosphorylate both S408

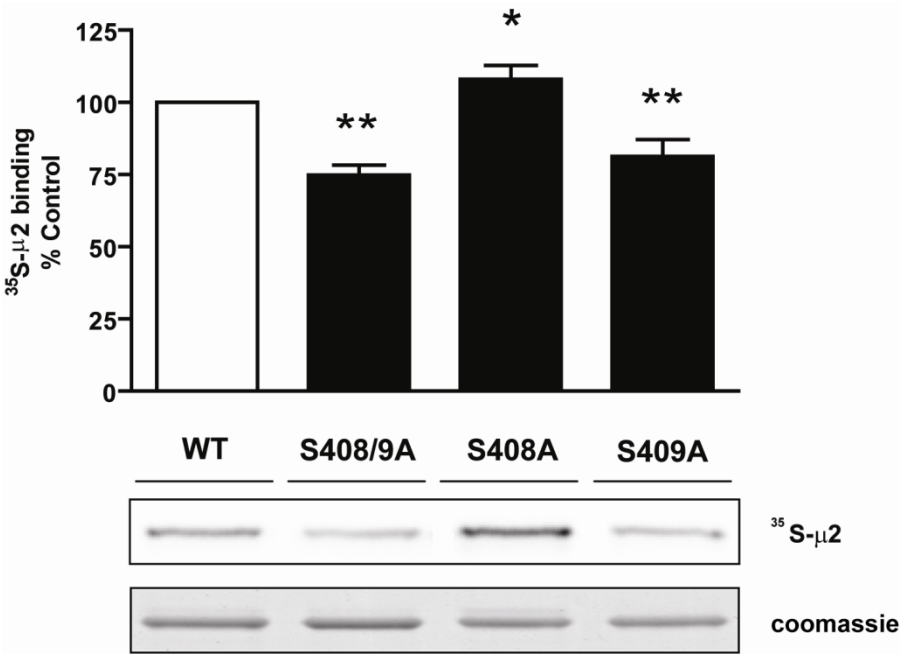


Figure 4.3: Selective mutation of S409 but not S408 decreases ^{35}S-μ2 binding to GST-β3 fusion proteins *in vitro*. GST-β3 wild-type, S408/9A, S408A and S409A mutant fusion proteins were separated by SDS-PAGE (1μg protein/lane) and, following electro-transfer onto nitrocellulose membranes, overlayed with *in vitro* translated ^{35}S-μ2. Bound material was visualized by autoradiography (top panel) and normalized to coomassie stain (bottom panel). Histogram shows ^{35}S-μ2 binding to fusion proteins expressed as a percentage of GST-β3 wild-type control (*p<0.05, **p<0.01, ***p<0.001; one-way ANOVA followed by a Newman-Keuls multiple comparison test).

and S409 (McDonald and Moss, 1997). In agreement with the findings from the previous study, ^{35}S-μ2 binding to wild-type GST-β3 fusion protein was significantly ($p<0.001$) reduced by phosphorylation (64.9 ± 2.8% compared to mock-phosphorylated control. The smaller effect observed in this experiment, may in part be explained by the lower level of phosphorylation stoichiometry observed in this experiment (0.4mol/mol). Phosphorylating only S409 (i.e. in the S408A mutant) also significantly ($p<0.05$) blocked this interaction (65.5 ± 10.3% compared to mock-phosphorylated control), while subjecting the S409A mutant to phosphorylation did not further influence the reduction in binding seen as a result of this mutation (94.4 ± 7.5% compared to mock-phosphorylated control). This difference is not likely due to differences in the stoichiometry of phosphorylation as these were relatively similar (0.4 and 0.3mol/mol, respectively). No difference in binding was observed in the S408/9A mutant (99.8 ± 6.3% compared to mock-phosphorylated control) in which both sites of serine phosphorylation had been replaced with alanine residues (Fig 4.4). This is consistent with the background level of phosphorylation stoichiometry seen with this mutant (0.07mol/mol). Together these findings indicate that it is the serine at position 409 and not that at 408 of β3 that is critical for AP2-μ2 adaptin binding *in vitro,* and that this interaction is negatively regulated by phosphorylation.

AP2-μ2 adaptin binding to the $GABA_A$ receptor β3 subunit is negatively regulated by phosphorylation in cultured neurons

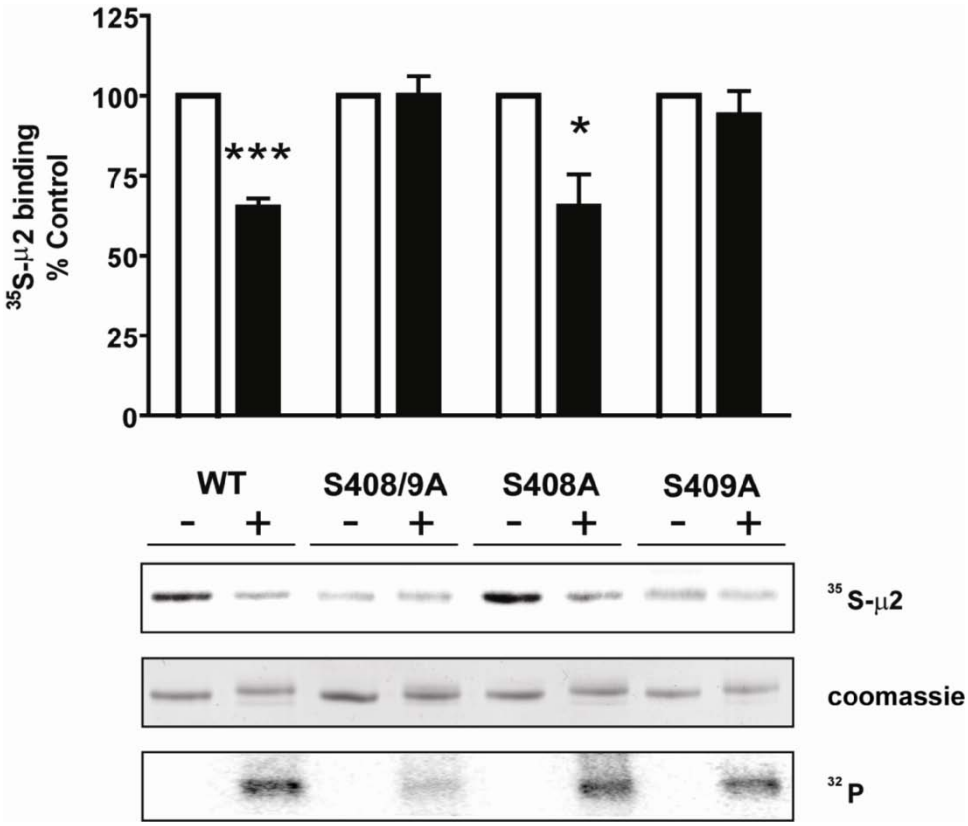


Figure 4.4: Selective PKC-induced phosphorylation of S409 but not S408 decreases ^{35}S-μ2 binding to GST-β3 fusion proteins *in vitro*. GST-β3 wild-type, S408/9A, S408A and S409A mutant fusion proteins were subjected to an *in vitro* phosphorylation assay either in the absence (-) or presence (+) of purified PKC. Mock-phosphorylated and phosphorylated proteins (1μg/lane) were separated by SDS-PAGE then electro-transferred onto nitrocellulose membrane and overlayed with *in vitro* translated ^{35}S-μ2. Bound material was visualized by autoradiography (upper panel) and normalized to coomassie stain (middle panel). The stoichiometry of phosphorylation was measured in a parallel assay utilizing [γ-^{32}P]-ATP (lower panel). Histogram shows ^{35}S-μ2 binding to fusion proteins expressed as a percentage of mock-phosphorylated controls (***$p<0.001$; one-way ANOVA followed by a Newman-Keuls multiple comparison test).

In order to examine if binding of AP2-μ2 adaptin to the $GABA_A$ receptor β3 subunit is dependent on the phosphorylation state of native receptor protein, rat cortical neurons were treated with either vehicle or 1μM okadaic acid for 30 min and lysed under non-denaturing conditions to preserve protein-protein interactions. $GABA_A$ receptor β3 subunits were then immunoprecipitated with anti-total-β3 antibodies and analyzed by immunoblotting with antibodies against AP2-μ2 adaptin. After controlling for recovery of $GABA_A$ receptor β3 subunits, okadaic acid significantly ($p<0.001$) decreased the level of associated AP2-μ2 adaptin (43.0 ± 0.7% of vehicle-treated control) without affecting total expression levels. This effect was paralleled by a significant ($p<0.01$) increase in the phosphorylation state of immunoprecipitated protein, as detected by immunoblotting with anti-pS408/9-β3 (215.3 ± 10.3% of vehicle-treated control) (Fig 4.5).

Phosphorylation enhances the cell surface stability of $GABA_A$ receptor β3 subunits in cultured neurons

A reduction in the binding of AP2-μ2 adaptin would be predicted to lead to an accumulation of $GABA_A$ receptor β3 subunits on the cell surface, attributed to a decrease in their endocytosis from the plasma membrane. To test this, rat cortical neurons treated with either vehicle or okadaic acid were incubated with NHS-Biotin to label surface fractions. Following cell lysis, biotinylated proteins were purified on avidin and analyzed by western blotting. After normalizing to total fractions, okadaic acid significantly increased the proportion of $GABA_A$ receptor β3 subunits on the cell surface (167 ± 17% of vehicle-treated control;

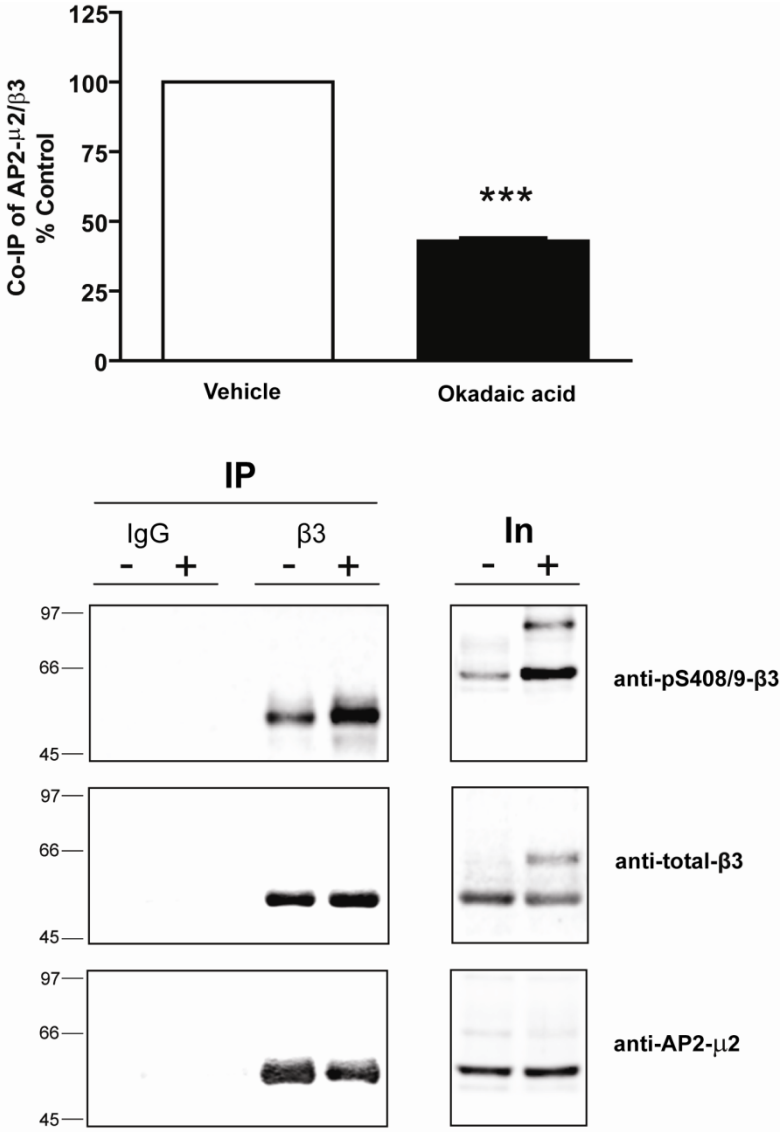


Figure 4.5: Inhibiting PP1/PP2A activity decreases coimmunoprecipitation of AP2-μ2 adaptin with $GABA_A$ receptor β3 subunits in cultured neurons. Rat cortical neurons were treated with either vehicle (-) or 1μM okadaic acid (+) for 30 min and detergent-soluble whole-cell extracts immunoprecipitated with either control IgG or anti-total-β3 antibodies. Immunoprecipitated (IP) and total (10% input; In) fractions were then analyzed by immunoblotting. Histogram shows co-IP of AP2-μ2 adaptin with $GABA_A$ receptor β3 subunits expressed as a percentage of vehicle-treated control (***$p<0.001$; Student's *t* test).

$p<0.001$). Under the same conditions, immunoblotting with anti-pS408/9-β3 revealed a concomitant increase in the phosphorylation state of cell surface protein (229 ± 26% of vehicle-treated control) (Fig 4.6).

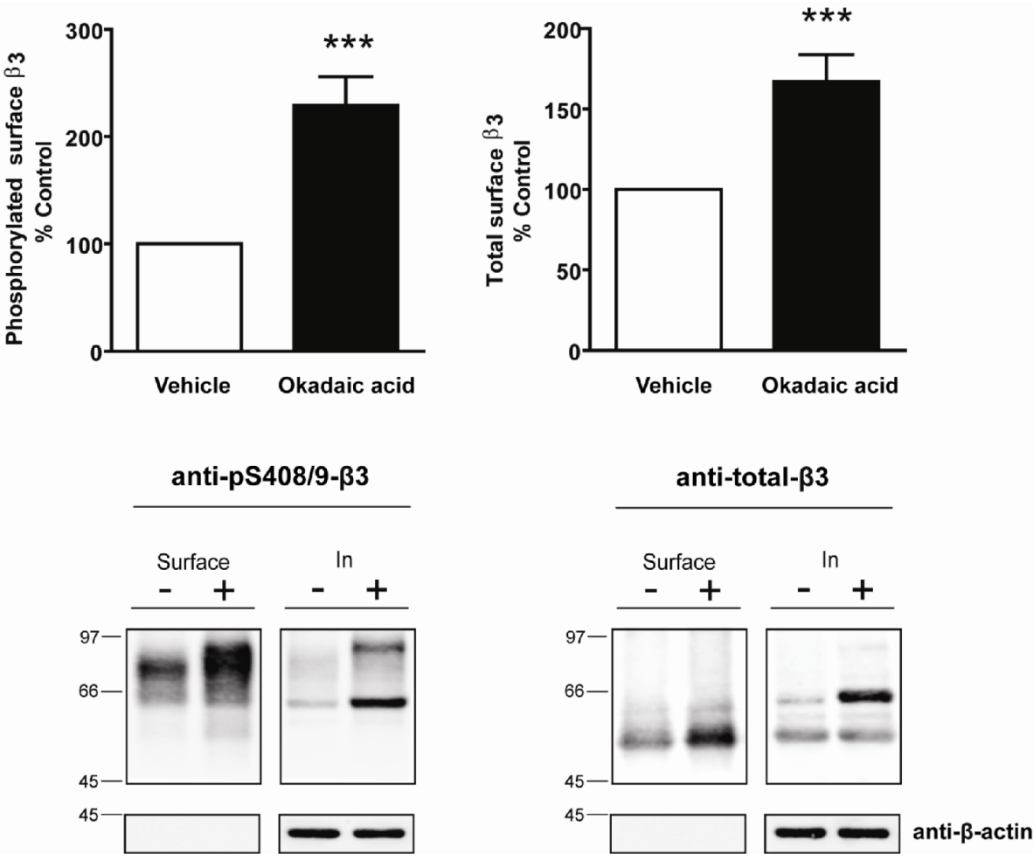


Figure 4.6: Inhibiting PP1/PP2A activity increases biotin-labeled $GABA_A$ receptor β3 subunits on the surface in cultured neurons. Rat cortical neurons treated with either vehicle (-) or 1μM okadaic acid (+) for 30 min were labeled with NHS-Biotin and detergent-soluble whole-cell extracts purified on avidin. Surface and 10% of input (In) fractions were then analyzed by immunoblotting. Histograms show the phosphorylated and total $GABA_A$ receptor β3 subunit levels on the surface normalized to input and expressed as a percentage of vehicle-treated control (*$p<0.001$; Student's *t* test).

Discussion

Inhibitory synaptic transmission is reliant upon the accumulation of $GABA_A$ receptors at postsynaptic sites, a process that is regulated by their relative rates of exo- and endocytosis (reviewed in Jacob et al., 2008). At steady-state significant rates of $GABA_A$ receptor endocytosis is apparent in heterologous and neuronal systems and is believed to occur predominantly via a clathrin-mediated pathway, with approximately 25% of β3 subunit-containing receptors being internalized within 30 minutes (Kittler et al., 2004). Recruitment of $GABA_A$ receptors into endocytic vesicles is facilitated via the interactions of receptor β1-3 and γ2 subunit ICDs with the μ2 adaptin of the AP2 complex. In the case of the β3 subunit, *in vitro* binding studies have revealed that this interaction is negatively regulated by phosphorylation of S408 and S409, which lie within the basic-rich AP2-μ2 adaptin-binding motif (Kittler et al., 2008, 2005, 2001).

In this chapter, the μ2 adaptin of the AP2 complex was shown to preferentially bind to $GABA_A$ receptor β1 , β2 as well as β3 subunit ICDs in their dephosphorylated state *in vitro* and in cultured neurons. The reduction in binding observed following okadaic acid treatment of cultured neurons was accompanied by an increase in the cell surface stability of endogenous $GABA_A$ receptor β3 subunits. Furthermore, S409, but not S408, of the $GABA_A$ receptor β3 subunit was shown to be critical for this phospho-dependent interaction. In contrast, selectively mutating the S408 to an alanine (to structurally resemble

$GABA_A$ receptor β1 and β2 subunits at this site) resulted in an increase in affinity of the β3 subunit for the AP2-μ2 adaptin.

To directly visualize the effect of S408/9A mutation on membrane trafficking, fluorescent $GABA_A$ receptor β3 subunits capable of interacting with bungaratoxin were subsequently employed in confocal imaging studies. Importantly these modifications have previously been shown to be functionally silent (Bogdanov et al., 2006). In agreement with the *in vitro* binding data, hippocampal neurons expressing pHBBS-$\beta3^{S408/9A}$ subunits exhibited reduced endocytosis leading to enhanced accumulation at the cell surface at both synaptic and extrasynaptic sites (Jacob et al., 2009). This finding is not unexpected given that $GABA_A$ receptors have recently been reported to be inserted into and removed from the plasma membrane exclusively at extrasynaptic sites and to diffuse laterally to access postsynaptic sites, where they are tethered by interactions with the subsynaptic cytoskeleton (Bogdanov et al., 2006). Despite the dramatic increase in the density of postsynaptic $GABA_A$ receptors, no significant alterations in the size of the presynapse were observed (Jacob et al., 2009), consistent with prior studies showing little evidence of presynaptic compensation with changes in postsynaptic $GABA_A$ receptor number (Jacob et al., 2005; Kneussel et al., 2001, 1999; Levi et al., 2004). Intriguingly, while analyzing the cell surface distributions of wild-type and S408/9A mutant pHBBS-tagged β3 subunit-containing $GABA_A$ receptors, an apparent increase in long, filopodia-like spines on neurons expressing the latter was noted. This deficit in spine maturity was reversed by pharmacological

blockade of $GABA_A$ receptors. Therefore, regulating the efficacy of synaptic inhibition by modulating $GABA_A$ receptor membrane trafficking may play a critical role in regulating spinogenesis and, thus, synaptic plasticity.

Consistent with this, increased postsynaptic accumulation of pHBBS-tagged β3 subunit-containing $GABA_A$ receptors in response to S408/9A mutation was shown to concomitantly enhance both the amplitude and frequency of mIPSCs (Jacob et al., 2009). A possible explanation for the observed increase in the frequency of events is the recruitment of mIPSCs previously below the threshold of detection. A decrease in the rise time of mIPSCs was also observed in these cultures, indicating that in addition to altering $GABA_A$ receptor endocytosis, mutation (or modification by phosphorylation) of S408/9 may also modulate channel kinetics (Jacob et al,2009). In a study by McDonald and colleagues (1998) phosphorylation of recombinant $GABA_A$ receptor β3 subunits at S408 and S409 by PKA was shown to enhance GABA-activated currents in HEK-293 cells. Interestingly, selectively mutating S408 to alanine switched this potentiation into a depression, comparable to that of β1 subunit-containing receptors that are phosphorylated solely on S409 (reviewed in Moss and Smart, 1996; Smart, 1997). In contrast, β2 subunit-containing receptors were not affected by PKA, in line with the inability of this kinase to phosphorylate full-length $GABA_A$ receptor β2 subunits *in vivo*. It was further reported that the phospho-dependent modulation of β1 and β3 subunit-containing receptors was most likely due to altered channel gating and/or conductance as no accompanying changes in cell surface number were observed. Whilst these

findings contradict those from this study, in which phosphorylation of $GABA_A$ receptor β1 and β3 subunits at S409 would be predicted to increase the efficacy of synaptic inhibition by virtue of increased cell surface stability, they support the notion that S408 and S409 of the β3 subunit have opposing roles in the phospho-dependent modulation of $GABA_A$ receptor function, offering a molecular mechanism to regulate the efficacy of neuronal inhibition in a synapse-specific manner.

Results III

Serotonergic modulation of $GABA_A$ receptor β3 subunit phosphorylation and cell surface stability

Introduction

The serotonergic system is involved in the regulation of diverse psychophysiological processes including emotion, perception and cognition (reviewed in Buhot, 1997; Davidson et al., 2000 and Manji et al., 2001). To date, fifteen serotonin receptor subtypes have been identified. These have been divided into seven different subclasses on the basis of conserved structures and signaling mechanisms (reviewed in Martin et al., 1998): 5-HT_{1A-F} and 5-HT_{5A-B} receptor subtypes couple to $G_{i/o}$ proteins to inhibit adenylate cyclase; 5-HT_{2A-C} receptors couple to $G_{q/11}$ proteins to stimulate the hydrolysis of phospholipids; 5-HT_4, 5-HT_6 and 5-HT_7 receptor subtypes couple to G_s proteins to stimulate adenylate cyclase, and 5-HT_3 receptors are ligand-gated sodium/calcium channels (Martin et al., 1998).

Several lines of electrophysiological evidence indicate that serotonin receptors are involved in the complex regulation of GABAergic inhibitory transmission in the PFC. Activation of postsynaptic 5-HT_2 receptors in cultured PFC pyramidal neurons has been shown to inhibit $GABA_A$ receptor currents via a PKC-mediated pathway (Feng et al., 2001). In contrast, 5-HT_4 receptor activation modulates $GABA_A$ receptor currents bi-directionally depending on the basal PKA activation levels; elevated levels of PKA activation due to increased neuronal activity have been shown to reverse the enhancing effect of 5-HT_4 receptor activation to a depression (Cai et al., 2002). The involvement of these kinases in the serotonergic modulation of $GABA_A$ receptor function suggests

that phosphorylation of $GABA_A$ receptors subunits may be the molecular mechanism underlying this phenomenon. As modifications in serotonergic signaling have long been implicated in the etiology of mood disorders and their alleviation, such functional cross-talk may play a critical role in controlling the efficacy of synaptic inhibition under both normal and pathological conditions. Moreover, this potential mechanism may underpin the antidepressant and/or anxiolytic effects of selective SSRIs such as fluoxetine (Prozac™).

In this chapter, using anti-pS408/9-β3 antibody I show that serotonin treatment of rat cortical neurons significantly increases the phosphorylation state of the $GABA_A$ receptor β3 subunit in a manner that is dependent upon the activity of PKC. The involvement of this kinase suggests that 5-HT_2 receptors may be the receptor subtype responsible for mediating this effect. Therefore, using 5-HT_2 receptor subtype-specific compounds in acute PFC slices, I further elucidate the role that this receptor subtype plays in mediating the serotonergic modulation of $GABA_A$ receptor β3 subunit phosphorylation. In order to address the role of serotonergic signaling *in vivo*, fluoxetine-treated mice were culled by focused microwave irradiation and analyzed by western blotting. To further scrutinize the regional and cellular distribution of fluoxetine-induced increase in $GABA_A$ receptor β3 subunit phosphorylation, paraffin-embedded brain sections from mice culled by focused microwave irradiation were examined by immunohistochemistry.

Results

Serotonin enhances $GABA_A$ receptor β3 subunit phosphorylation via a PKC-mediated pathway in cultured neurons

To examine the effect of serotonin on $GABA_A$ receptor β3 subunit phosphorylation, rat cortical neurons were treated with 100μM serotonin for varying times and detergent-soluble whole-cell extracts analyzed by western blotting. At time 0, immunoblotting with anti-pS408/9-β3 identified a band at ~58 kDa that was significantly enhanced following serotonin treatment for 5 (151.8 ± 10.2%; $p<0.01$), 15 (178.1 ± 17.3%; $p<0.001$) and 30 (145.6 ± 9.3%; $p<0.01$) min. As seen previously (see Fig 3.5), immunoblotting with anti-total-β3 also identified a likely-phosphorylated band at ~58 kDa. However, unlike that seen with okadaic acid, serotonin treatment did not significantly increase this immunoreactivity, possibly due to a comparatively lower stoichiometry of phosphorylation. Immunodetection at ~53 kDa by anti-total-β3 (the predicted molecular mass of the $GABA_A$ receptor β3 subunit) remained unchanged at all time points tested, indicating no change in total β3 subunit expression under these treatment conditions (Fig 5.1).

SSRIs such as fluoxetine increase the availability of serotonin at the synapse by blocking reuptake of this neurotransmitter. Whilst there was a trend towards an increase (118.2 ± 5.2%) in the proportion of β3 subunits phosphorylated in rat cortical neurons treated with 10μM fluoxetine alone for 12 min, this was not

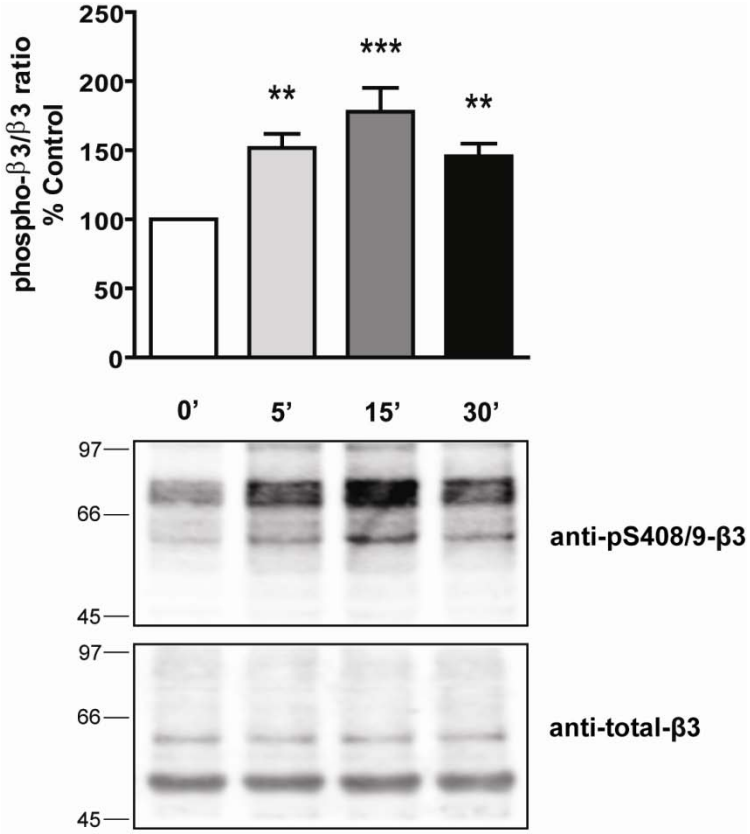


Figure 5.1: Serotonin increases $GABA_A$ receptor β3 subunit phosphorylation in cultured neurons. Western blot analysis of detergent-soluble whole-cell extracts (30µg protein/lane) prepared from rat cortical neurons treated with serotonin (100µM) for the times indicated, using anti-pS408/9-β3 and anti-total-β3 antibodies. Histogram shows the proportion of $GABA_A$ receptor β3 subunits phosphorylated expressed as a percentage of 0 min treatment time (**p<0.01, ***p<0.001; one-way ANOVA followed by Newman-Keuls multiple comparison test).

found to be significant ($p>0.05$) (Fig 5.2). However, pre-treating neurons with 10μM of fluoxetine for 2 min before adding 100μM serotonin in the presence of fluoxetine for a further 10 min resulted in a significant ($p<0.001$) increase ($159.3 \pm 17.6\%$) in the proportion of β3 subunits phosphorylated as compared to vehicle-treated control and significantly ($p<0.05$) potentiated the effect of serotonin alone (144.8 ± 8.7; $p<0.01$) (Fig 5.2).

Interestingly, treatment with 100μM serotonin for 10 min also significantly ($p<0.001$) increased ($255.7 \pm 11.5\%$) the phosphorylation state of PKC compared to vehicle-treated control, as detected by immunoblotting with an antibody directed against PKC-α phosphorylated at S657 (anti-pPKC) (Fig 5.3). Under the same conditions, immunoblotting with anti-PKC revealed no change in the total expression of PKC-α, β, γ (Fig 5.3), indicating a selective increase in the activation of this kinase. To determine if serotonin-induced increase in $GABA_A$ receptor β3 subunit phosphorylation is PKC-dependent, rat cortical neurons were treated with serotonin either in the absence or presence of calphostin C, a potent inhibitor of PKC. Treatment with 100μM serotonin alone for 10 min resulted in a significant ($p<0.001$) increase ($144.8 \pm 8.7\%$) in the proportion of β3 subunits phosphorylated as compared to vehicle-treated control. This effect was blocked in the presence of and following 20 min pre-treatment with 2.5μM calphostin C and, similar to treatment with calphostin C alone ($49.5 \pm 0.5\%$; $p<0.01$), induced a significant decrease in basal phosphorylation levels ($56.0 \pm 0.0\%$; $p<0.01$) (Fig 5.4).

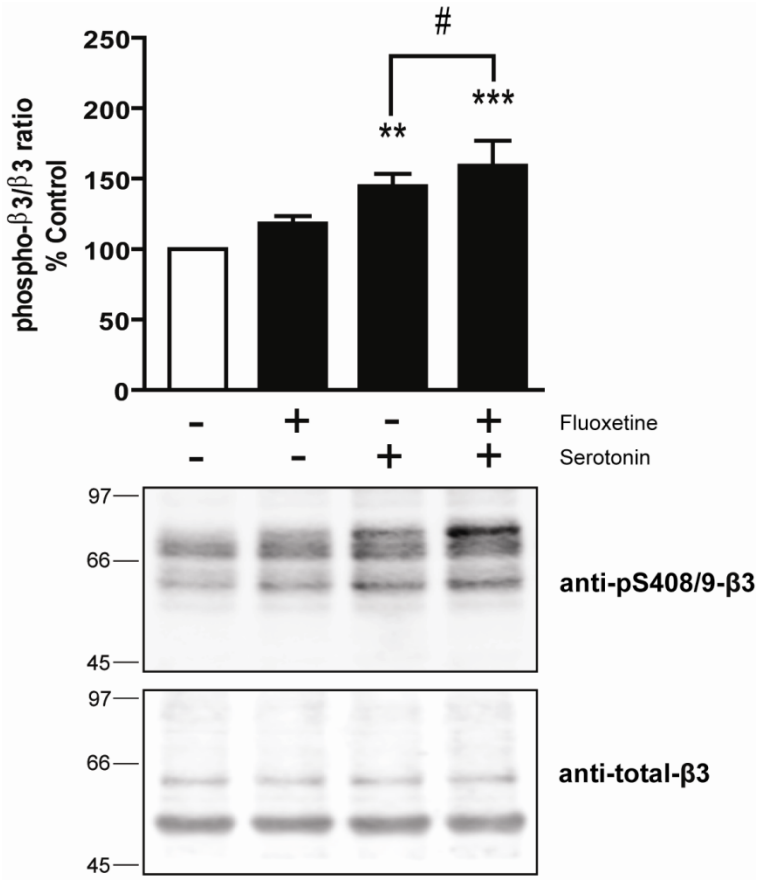


Figure 5.2: Serotonin-induced increase in $GABA_A$ receptor β3 subunit phosphorylation is potentiated by fluoxetine in cultured neurons. Western blot analysis of detergent-soluble whole-cell extracts (30µg protein/lane) prepared from rat cortical neurons treated with either vehicle for 12 min (-/-), 10µM fluoxetine for 12 min (+/-), 100µM serotonin for 10 min (-/+), or pre-treated with fluoxetine for 2 min and then, in the presence of this SSRI, treated with serotonin for a further 10 min (+/+). Histogram shows the proportion of $GABA_A$ receptor β3 subunits phosphorylated expressed as a percentage of vehicle-treated control (**p<0.01, ***p<0.001; one-way ANOVA followed by Newman-Keuls multiple comparison test. $^{\#}$p<0.05; Student's *t* test).

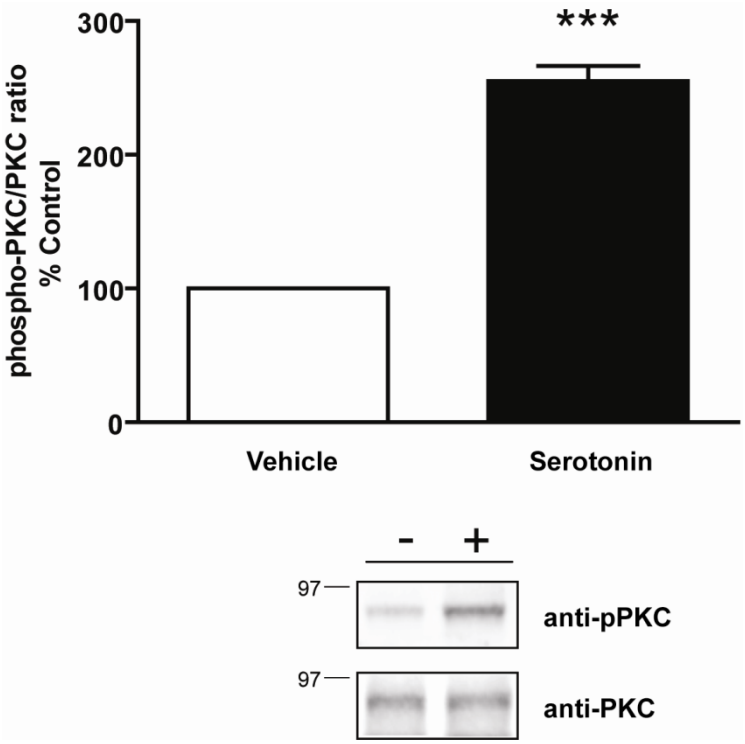


Figure 5.3: Serotonin increases PKC phosphorylation in cultured neurons. Western blot analysis of detergent-soluble whole-cell extracts (30µg protein/lane) prepared from rat cortical neurons treated with either vehicle (-) or 100µM serotonin (+) for 10 min using anti-pPKC and anti-total-PKC antibodies. Histogram shows the proportion of PKC phosphorylated expressed as a percentage of vehicle control (***$p<0.001$; Student's *t* test).

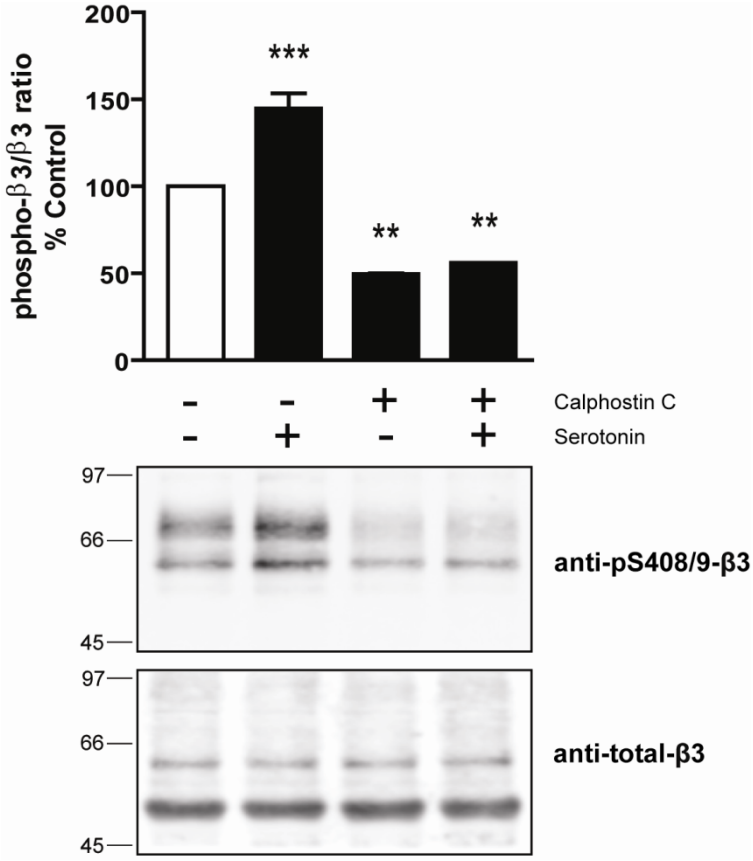


Figure 5.4: Serotonin-induced increase in $GABA_A$ receptor β3 subunit phosphorylation is blocked by calphostin C in cultured neurons. Western blot analysis of detergent-soluble whole-cell extracts (30µg protein/lane) prepared from rat cortical neurons treated with either vehicle for 30 min (-/-), 100µM serotonin for 10 min (-/+), 2.5µM calphostin C for 30 min (+/-), or pre-treated with calphostin C for 20 min and then, in the presence of this PKC inhibitor, treated with serotonin for a further 10 min (+/+). Histogram shows the proportion of $GABA_A$ receptor β3 subunits phosphorylated expressed as a percentage of vehicle-treated control (**p<0.01, ***p<0.001; one-way ANOVA followed by Newman-Keuls multiple comparison test).

Serotonin enhances $GABA_A$ receptor β3 subunit cell surface stability in cultured neurons

To examine if serotonin-induced increase in $GABA_A$ receptor β3 subunit phosphorylation translates to an increase in receptor accumulation on the plasma membrane, rat cortical neurons treated with either vehicle or 100μM serotonin for 10 min were subject to a cell surface biotinylation assay and detergent-soluble whole-cell extracts analyzed by western blotting. After normalizing to total fractions, serotonin significantly ($p<0.05$) increased the proportion of $GABA_A$ receptor β3 subunits on the cell surface (130.3 ± 6.4%) of vehicle-treated control (Fig 5.5).

5-HT_2 receptors modulate PKC-mediated $GABA_A$ receptor β3 subunit in brain slices

The involvement of PKC in the serotonergic modulation of $GABA_A$ receptor β3 subunit phosphorylation suggests that 5-HT_2 receptors may be the receptor subtype responsible for mediating this effect. To test this, acute PFC slices were treated with 1, 10 and 100μM of the $5HT_2$ receptor agonist DOI for varying time periods and detergent-soluble extracts analyzed by western blotting. Analysis of the results at the 0 and 15 min time points revealed a significant increase in β3 subunit phosphorylation with 1 and 10μM DOI (190.2 ± 16.5%; $p<0.01$ and 182.3 ± 21.9%; $p<0.001$, respectively), whereas a significant ($p<0.01$) decrease (33.5 ± 9.7%) was observed with 100μM DOI (Fig 5.6). The effect of treatment with 10μM DOI for 15 min was blocked in the presence

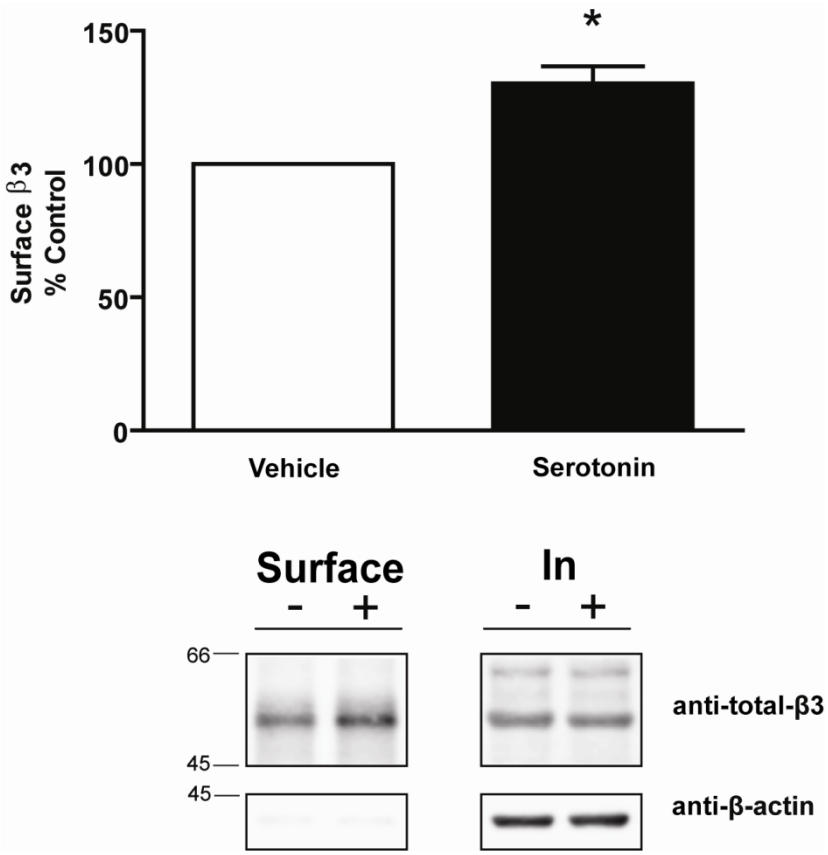


Figure 5.5: Serotonin increases biotin-labeled $GABA_A$ receptor β3 subunits on the surface in cultured neurons. Rat cortical neurons treated with either vehicle (-) or 100μM serotonin (+) for 10 min were labeled with NHS-Biotin and detergent-soluble whole-cell extracts purified on avidin. Surface and 10% of input (In) fractions were then analyzed by immunoblotting. Histograms show the total $GABA_A$ receptor β3 subunit levels on the surface normalized to input and expressed as a percentage of vehicle-treated control (*$p<0.05$; Student's *t* test).

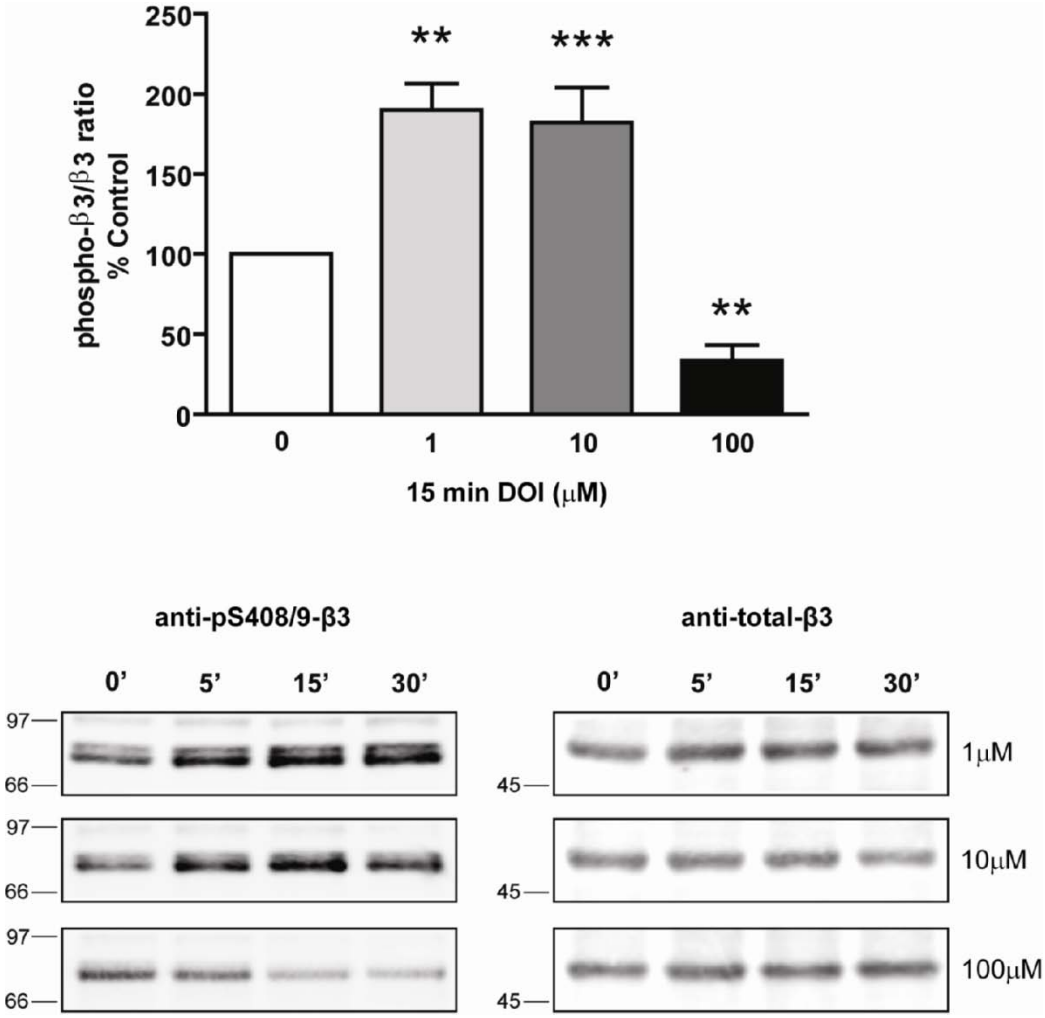


Figure 5.6: DOI increases $GABA_A$ receptor β3 subunit phosphorylation in brain slices. Western blot analysis of detergent-soluble brain extracts (30μg protein/lane) prepared from C57BL/6 mouse PFC slices treated with 1, 10 or 100μM DOI for the times indicated using anti-pS408/9-β3 and anti-total-β3 antibodies. Histogram shows the proportion of $GABA_A$ receptor β3 subunits phosphorylated in response to 15 min treatment with DOI (1, 10 and 100μM) expressed as a percentage of vehicle-treated (0μM DOI) control (**p<0.01, ***p<0.001; one-way ANOVA followed by Newman-Keuls multiple comparison test).

of and following 15 min pre-treatment with 10μM GFX (a potent inhibitor of PKC) and, similar to GFX alone (36 ± 8.7%; $p<0.01$), induced a significant decrease in basal phosphorylation levels (27.8 ± 7.9%; $p<0.05$) (Fig 5.7). Taken together, these findings suggest that serotonergic modulation of PKC-mediated $GABA_A$ receptor β3 subunit phosphorylation and cell surface stability is in part dependent upon the activation of $5HT_2$ receptors.

Fluoxetine enhances $GABA_A$ receptor β3 subunit phosphorylation in a region-specific manner *in vivo*

In order to address the role of serotonergic signaling *in vivo*, mice were injected intraperitonealy (i.p.) with 20mg/kg fluoxetine and then culled 1 h later by focused microwave irradiation. This dose of fluoxetine was chosen on the basis that previous behavioral studies have demonstrated a reduction in the time spent immobile in the tail-suspension test (Svenningsson et al., 2007), an accepted measure of antidepressant efficacy. Western blot analysis of detergent-soluble PFC extracts revealed a significant ($p<0.001$) increase (175.8 ± 11.0%) in the proportion of $GABA_A$ receptor β3 subunits phosphorylated compared to vehicle-treated control without altering total subunit expression (Fig 5.8). Furthermore, immunohistochemical analysis of paraffin-embedded PFC sections revealed that the $GABA_A$ receptor β3 subunit is basally phosphorylated in certain limbic regions of the PFC. Phosphorylation of the β3 subunit was enhanced following fluoxetine treatment in the anterior cingulate cortex, but not the endopiriform cortex, above its already high level of basal phosphorylation, whereas total subunit expression remained unchanged (Fig 5.9). In addition,

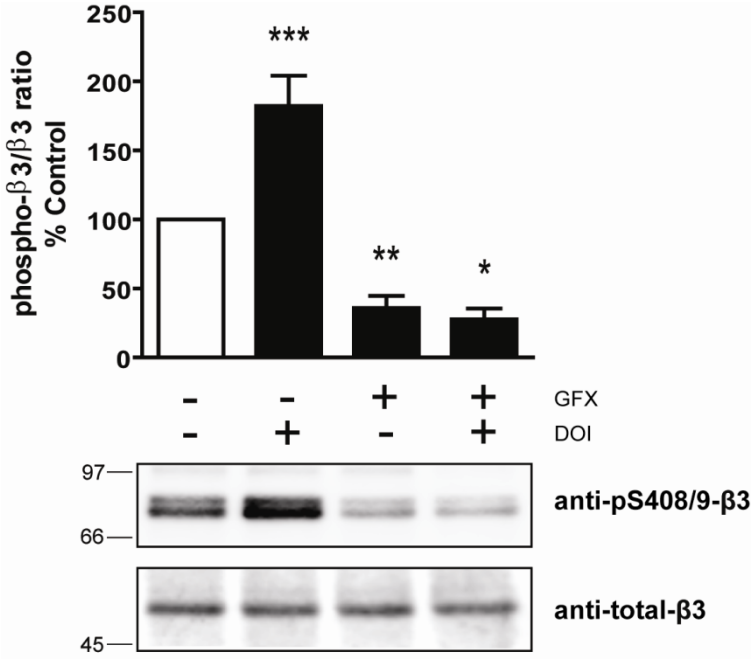


Figure 5.7: DOI-induced increase in $GABA_A$ receptor β3 subunit phosphorylation is blocked by GFX in brain slices. Western blot analysis of detergent-soluble brain extracts (30µg protein/lane) prepared from C57BL/6 mouse PFC slices treated with either vehicle (-/-), 10µM DOI for 15 min (-/+), 10µM GFX for 30 min (+/-), or pre-treated with GFX for 15 min and then, in the presence of this PKC inhibitor, treated with DOI for a further 15 min (+/+). Histogram shows the proportion of $GABA_A$ receptor β3 subunits phosphorylated expressed as a percentage of vehicle-treated control (**p<0.01, ***p<0.001; one-way ANOVA followed by Newman-Keuls multiple comparison test).

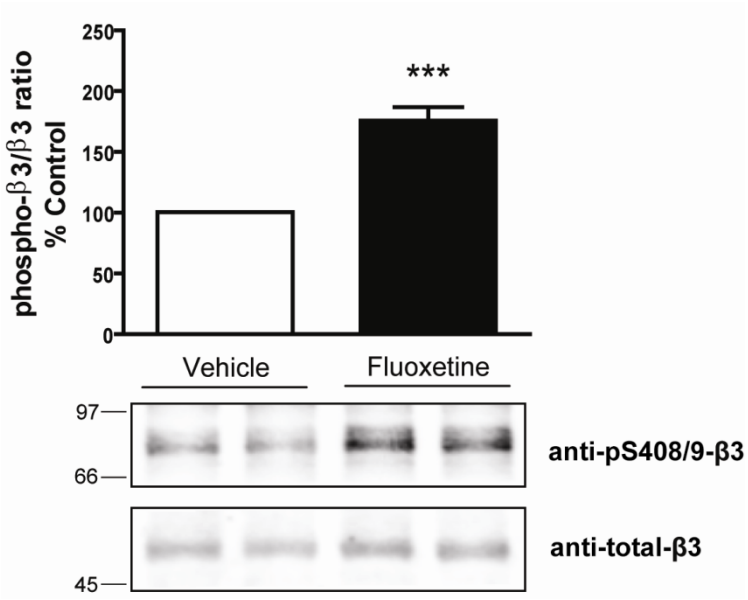


Figure 5.8: Fluoxetine treatment increases $GABA_A$ receptor β3 subunit phosphorylation *in vivo*. C57BL/6 mice were dosed with either vehicle or 20mg/kg fluoxetine i.p. and then culled 1 h later by focused microwave irradiation. Detergent-soluble PFC extracts (30µg protein/lane) were then subjected to western blotting with anti-pS408/9-β3 and anti-total-β3 antibodies. Histogram shows the proportion of $GABA_A$ receptor β3 subunits phosphorylated expressed as a percentage of vehicle-treated control (***$p<0.001$; Students *t* test).

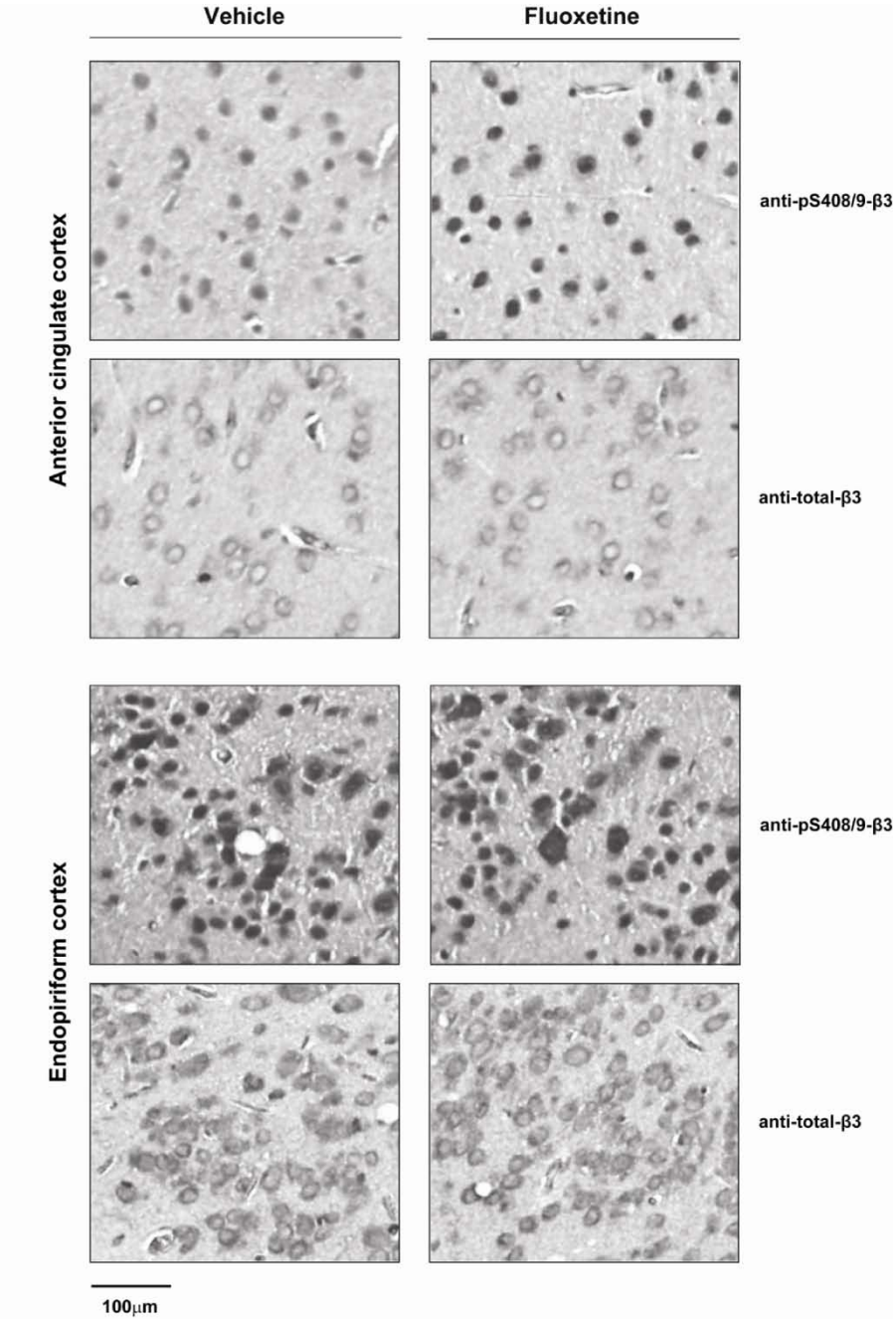


Figure 5.9: Regional and cellular distribution of fluoxetine-induced increase in $GABA_A$ receptor β3 subunit phosphorylation in the PFC. C57BL/6 mice were dosed with either vehicle or 20mg/kg fluoxetine i.p. and then culled 1 h later by focused microwave irradiation. Paraffin-embedded PFC sections were then stained with anti-pS408/9-β3 and anti-total-β3 antibodies and visualized using HRP-conjugated secondary antibody coupled to light microscopy.

immunostaining hippocampal sections with anti-pS408/9-β3 demonstrated that the $GABA_A$ receptor β3 subunit is basally phosphorylated mainly in the granular layer of the dentate gyrus and the pyramidal cell layer of CA1-CA3 subfields (Fig 5.10). Consistent with this, high levels of anti-total-β3 immunoreactivity was observed in these brain regions (Fig 5.10). Interestingly, fluoxetine treatment specifically increased anti-pS408/9-β3 staining in the dentate gyrus and CA3, but not CA1, hippocampal subfields, whereas no change in anti-total-β3 antibody staining was detected under the same conditions (Fig 5.10).

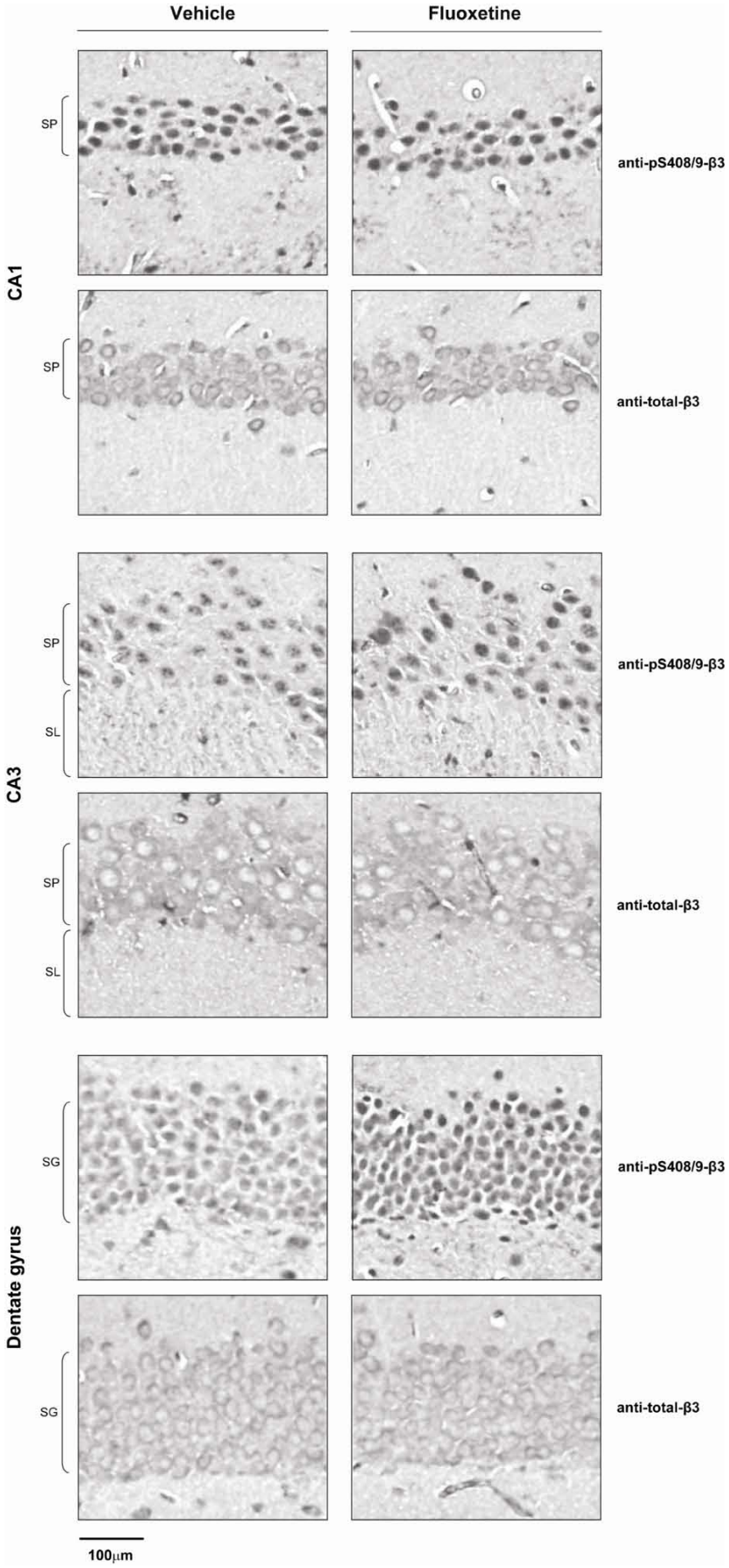

Vehicle
Fluoxetine
CA1
SP
anti-pS408/9-β3
SP
anti-total-β3
CA3
SP
SL
anti-pS408/9-β3
SP
SL
anti-total-β3
Dentate gyrus
SG
anti-pS408/9-β3
SG
anti-total-β3
100μm

Figure 5.10: Regional and cellular distribution of fluoxetine-induced increase in $GABA_A$ receptor β3 subunit phosphorylation in the hippocampal formation. C57BL/6 mice were dosed with either vehicle or 20mg/kg fluoxetine i.p. and then culled 1 h later by focused microwave irradiation. Paraffin-embedded hippocampal sections of were then stained with anti-pS408/9-β3 and anti-total-β3 antibodies and visualized using HRP-conjugated secondary antibody coupled to light microscopy.

Discussion

In this chapter serotonin treatment of rat cortical neurons was shown to significantly enhance the phosphorylation state of the $GABA_A$ receptor β3 subunit at S408/9. This effect was significantly potentiated by the SSRI fluoxetine. SSRIs act selectively at the serotonin transporter (SERT) to inhibit reuptake and subsequent degradation of serotonin in the presynaptic cleft. However, the absence of seronotonergic neurons in these cultures indicates fluoxetine action independent of its role in serotonergic signaling. In addition to its actions as an SSRI, fluoxetine has been reported to directly interact with the $GABA_A$ receptor complex (Derry et al., 2007; Goren et al., 2007; Robinson et al., 2003; Tunnicliff et al., 1999) that may account for the observed effects of fluoxetine on the phosphorylation of the receptor β3 subunit at S408/9.

Interestingly, serotonin treatment concomitantly enhanced the phosphorylation of PKC-α, indicating an increase in the activity of this kinase following 5-HT receptor activation. In accordance with this, serotonin-induced increase in $GABA_A$ receptor β3 subunit phosphorylation was blocked in the presence of and following pre-treatment with calphostin C, a potent inhibitor of PKC. The involvement of PKC in the serotonergic modulation of $GABA_A$ receptor β3 subunit phosphorylation points towards the $5\text{-}HT_2$ receptor as the receptor subtype responsible for mediating this effect. Activation of $G_{q/11}$ protein-coupled $5\text{-}HT_2$ receptors stimulates the phospholipase C (PLC)-β isoform and the release of ionsitol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) through the hydrolysis of phospholipids, which can in turn activate PKC.

However, serotonin can also activate PKA via G_s protein-coupled 5-HT_4, 5-HT_6 and 5-HT_7 receptors. Further experiments are thus necessary to elucidate the role these signaling pathways play in regulating serotonin-induced S408/9 phosphorylation and the stability of $GABA_A$ receptor β subunits on the cell surface.

The role of serotonergic signaling in $GABA_A$ receptor phosphorylation was also addressed *in vivo* in this chapter. Acute systemic administration of fluoxetine was shown to significantly enhance the phosphorylation state of the $GABA_A$ receptor β3 subunit at S408/9 in mice subjected to focused microwave irradiation of the brain in a region-specific manner. In the PFC, this increase was localized to the anterior cingulate cortex, an important neural substrate for the integration of emotional, cognitive and physiological information as well as the coordination of responses to anticipated stimuli. An increase in receptor phosphorylation was also observed in the dentate gyrus and the CA3 subfield of the hippocampus, which plays important roles in long-term memory and spatial navigation. Chronic SSRI treatment has been shown to increase adult neurogenesis and synaptic plasticity in the dentate gyrus in a time-course that parallels the therapeutic time-lag associated with antidepressant therapy (Malberg et al., 2000; Santarelli et al., 2003; Wang et al., 2008); however, the effects of SSRIs on cognitive function are complex and contradictory (Li et al., 2008; Majlessi and Naghdi, 2002; Valluzzi and Chan, 2007).

Discussion

Summary

S408/9 phosphorylation enhances the functional expression of β3 subunit-containing $GABA_A$ receptors

Activity-dependent changes in the number of postsynaptic $GABA_A$ receptors accessible to neurotransmitter represent a potent mechanism underlying the functional plasticity of GABAergic synapses (Kittler and Moss, 2003; Kittler et al., 2000; Nusser et al., 1998a; 1997). Moreover, changes in the trafficking of $GABA_A$ receptors to and from postsynaptic membrane sites may contribute to the etiology and/or manifestation of a wide range of neurological and psychiatric disorders. The major focus of this thesis was thus to detail the cellular and molecular mechanisms that control the expression and stability of cell surface $GABA_A$ receptor populations at inhibitory synapses.

Recent experimental observations have suggested that the trafficking of $GABA_A$ receptors is subject to dynamic modulation by phosphorylation and that the β subunits play a key role in this phospho-dependent regulation (Kittler et al., 2005). In this study, phosphorylation of conserved serine residues (S409 in β1, S410 in β2 and S408/9 in β3) by either PKA or PKC was found to reduce [^{35}S]-methionine-labeled AP2-μ2 adaptin binding to GST fusion proteins encoding the ICDs of $GABA_A$ receptor β1-3 subunit isoforms *in vitro*. Moreover, mutation of S408/9 to alanines was shown to mimic the effects of phosphorylation and decrease binding of the β3 subunit to the μ2 adaptin of the AP2 complex. In cultured neurons, okadaic acid (a potent inhibitor of S408/9

dephosphorylation) was shown to dramatically reduce the amount of AP2-μ2 adaptin coimmunoprecipitated with $GABA_A$ receptor β3 subunits and, in parallel, enhance the stability of biotin-labeled receptors at the cell surface. Furthermore, $GABA_A$ receptors incorporating pHBBS-$\beta3^{S408/9A}$ subunits have been shown to exhibit increases in the number and size of inhibitory synapses together with enhanced synaptic inhibition, a phenomenon that was found to reduce spine maturity (Jacob et al., 2009). As β subunits are essential components of all $GABA_A$ receptor subtypes, these results suggest that phosphorylation may be a universal mechanism used by all neurons to control neuronal excitation, synaptic plasticity and animal behavior.

Modulation of $GABA_A$ receptor phosphorylation by serotonergic signaling may underlie the therapeutic efficacy of SSRIs

Serotonergic pathways arising from the raphé nuclei in the brainstem innervate a wide range of structures thought to be involved in the pathogenesis of mood disorders such as depression, including the PFC, hippocampus and amygdala. Interestingly, in depressed patients and in animal models reduced GABA levels have been reported in the plasma, CSF and various brain regions including the PFC and hippocampus. Increased benzodiazepine-sensitive $GABA_A$ receptor binding sites were also documented in the post-mortem brains of depressed suicide victims (Cheetham et al., 1988; Manchon et a., 1987), which could possibly be the result of an adaptive mechanism to overcome the decrease in GABA activity.

Given that recent studies have implicated PKA and PKC in the mediation of serotonergic modulation of $GABA_A$ receptor activity in the PFC, it was speculated that phosphorylation of $GABA_A$ receptor β subunits may underlie this regulation. To address this, a phospho-specific antibody directed against β3 at S408/9 was developed. Immunoblotting with anti-pS408/9-β3 demonstrated a PKC-dependent increase in the phosphorylation state of $GABA_A$ receptor β3 subunits following enhanced 5-HT_2 receptor activation *ex vivo.*

The role of serotonergic signaling in $GABA_A$ receptor β3 subunit phosphorylation at S408/9 was examined *in vivo* by utilizing focused microwave irradiation, which prevents rapid dephosphorylation of proteins as a result of postmortem delay associated with classical methods of sacrifice (O'Callaghan and Sriram 2004). Consistent with this, focused microwave irradiation delivered the highest and most consistent levels of basal S408/9 phosphorylation when compared with cervical dislocation in western blot analysis. This was enhanced following acute systemic fluoxetine administration at a dose correlating with antidepressant efficacy in accepted behavioral paradigms. Given the marked regional and cellular distribution of any given phosphoprotein, microwaved brains were also subject to immunohistochemical analysis. In the PFC, fluoxetine treatment was shown to increase S408/9 phosphorylation in the anterior cingulate cortex, but not the endopiriform cortex above its already high level of basal phosphorylation. Results from neuroimaging studies have implicated the anterior cingulate cortex in depression and anxiety disorders (Mayberg et al., 2000; Brody et al., 2001) and

the perception of pain (Rainville et al., 1997, Ventafridda et al., 2003). Since SSRI binding to SERT is particularly high in this brain region, this may explain the effectiveness of SSRIs in alleviating mood and pain disorders.

The hippocampal formation is also a known target for antidepressant treatment. Acute systemic fluoxetine was shown to specifically enhance S408/9 phosphorylation in the granular layer of the dentate gyrus and the pyramidal cell layer of the CA3 subfield. Chronic SSRI treatment has been shown to increase adult neurogenesis in the dentate gyrus, which is thought to contribute to the delayed onset of therapeutic efficacy (Malberg et al., 2000; Santarelli et al., 2003; Wang et al., 2008). Both newborn and existing granule cells of the dentate gyrus give rise to axons that form the mossy fiber pathway that project to CA3 pyramidal cells, a brain region that has been shown to be critically involved in the acquisition and memory consolidation of spatial information (Stupien et al., 2003). Interestingly, $GABA_A$ receptor $\gamma 2$ subunit $Y^{365/7F}$+/- knockin mice have been shown to have severe impairments in spatial object recognition that is paralleled by a specific increase in the efficacy of synaptic inhibition and reduced spine density in CA3 pyramidal cells (Tretter et al., unpublished data). This is reminiscent of animal models of Down syndrome, in which enhanced levels of hippocampal neuronal inhibition are believed to contribute to cognitive deficits (Fernandez et al., 2007). A recent study showed that fluoxetine rescued impaired neurogenesis in the dentate gyrus of Ts65Dn mouse model for Down syndrome; however, the authors did not determine if the mice had any changes in cognitive behavior (Clark et al., 2006).

Together, the results presented herein suggest that the phospho-dependent increase in $GABA_A$ receptor functional expression may underlie the therapeutic action of SSRIs. However, additional experiments are necessary to further establish this.

Future directions

In this thesis, the molecular mechanisms underlying the phospho-dependent changes in the stability and function of postsynaptic $GABA_A$ receptor β3 subunits were described. Furthermore, a role for serotonergic signaling in modulating β3 subunit S408/9 phosphorylation and functional expression suggested as a possible mechanism of antidepressant action.

Currently, the most widely prescribed antidepressants are SSRIs such as fluoxetine (Prozac^{TM}). However, the clinical benefit of these and other classes of antidepressants on the market is observed only 2-3 weeks following initiation of treatment, during which time an initial increase in anxiety that has been linked to an increased likelihood of suicide has been reported. It would thus be of significant therapeutic benefit to investigate the effects of acute versus chronic SSRI treatment.

The generation of knockin mice is a strategy often used to investigate the role of disease-causing mutations. The introduction of point mutations into the mouse genome that mimic or prevent phosphorylation at S408/9 of the $GABA_A$

receptor β3 subunit combined with biochemical, electrophysiological and behavioral studies might not only confirm the sites of kinase activity *in vivo* but also provide key insights into how prolonged changes in the efficacy of fast synaptic inhibition impacts behavior. Furthermore, performing these studies in fluoxetine-treated mice would aid in deciphering the role that these phosphorylation sites play in the therapeutic action of SSRIs.

Elucidation of the signal transduction pathway engaged in modulation of $GABA_A$ receptor phosphorylation and function following chronic antidepressant treatment raises the possibility that intracellular signaling components could be potential targets for novel pharmacological agents with a faster onset of antidepressant action.

References

Ali AB, Thomson AM (2008) Synaptic alpha 5 subunit-containing GABAA receptors mediate IPSPs elicited by dendrite-preferring cells in rat neocortex. Cereb Cortex 18:1260-1271.

Alldred MJ, Mulder-Rosi J, Lingenfelter SE, Chen G, Luscher B (2005) Distinct gamma2 subunit domains mediate clustering and synaptic function of postsynaptic GABAA receptors and gephyrin. J Neurosci 25:594-603.

Angelotti TP, Macdonald RL (1993) Assembly of GABAA receptor subunits: alpha 1 beta 1 and alpha 1 beta 1 gamma 2S subunits produce unique ion channels with dissimilar single-channel properties. J Neurosci 13:1429-1440.

Arancibia-Carcamo IL, Moss SJ (2006) Molecular organization and assembly of the central inhibitory postsynapse. Results Probl Cell Differ 43:25-47.

Arancibia-Carcamo IL, Kittler JT (2009) Regulation of GABA(A) receptor membrane trafficking and synaptic localization. Pharmacol Ther 123:17-31.

Atack JR (2009) Preclinical and clinical pharmacology of the GABA(A) receptor alpha5 subtype-selective inverse agonist alpha5IA. Pharmacol Ther.

Atack JR, Hutson PH, Collinson N, Marshall G, Bentley G, Moyes C, Cook SM, Collins I, Wafford K, McKernan RM, Dawson GR (2005) Anxiogenic properties of an inverse agonist selective for alpha3 subunit-containing GABA A receptors. Br J Pharmacol 144:357-366.

Awapara J, Landua AJ, Fuerst R, Seale B (1950) Free gamma-aminobutyric acid in brain. J Biol Chem 187:35-39.

Banker G, Goslin K (1988) Developments in neuronal cell culture. Nature 336:185-186.

Banks MI, Pearce RA (1999) Dual actions of volatile anesthetics on GABA(A) IPSCs: dissociation of blocking and prolonging effects. Anesthesiology 90:120-134.

Basemore AW, Elliot KA, Florey E (1957) Isolation of factor I. J Neurochem 1:334-339.

Baumann SW, Baur R, Sigel E (2002) Forced subunit assembly in alpha1beta2gamma2 GABAA receptors. Insight into the absolute arrangement. J Biol Chem 277:46020-46025.

Baumann SW, Baur R, Sigel E (2003) Individual properties of the two functional agonist sites in GABA(A) receptors. J Neurosci 23:11158-11166.

Beck M, Brickley K, Wilkinson HL, Sharma S, Smith M, Chazot PL, Pollard S, Stephenson FA (2002) Identification, molecular cloning, and characterization of a novel GABAA receptor-associated protein, GRIF-1. J Biol Chem 277:30079-30090.

Bedford FK, Kittler JT, Muller E, Thomas P, Uren JM, Merlo D, Wisden W, Triller A, Smart TG, Moss SJ (2001) GABA(A) receptor cell surface number and subunit stability are regulated by the ubiquitin-like protein Plic-1. Nat Neurosci 4:908-916.

Belelli D, Lambert JJ, Peters JA, Wafford K, Whiting PJ (1997) The interaction of the general anesthetic etomidate with the gamma-aminobutyric acid type A receptor is influenced by a single amino acid. Proc Natl Acad Sci U S A 94:11031-11036.

Ben-Ari Y, Cherubini E, Corradetti R, Gaiarsa JL (1989) Giant synaptic potentials in immature rat CA3 hippocampal neurones. J Physiol 416:303-325.

Benson JA, Low K, Keist R, Mohler H, Rudolph U (1998) Pharmacology of recombinant gamma-aminobutyric acidA receptors rendered diazepam-insensitive by point-mutated alpha-subunits. FEBS Lett 431:400-404.

Bettler B, Tiao JY (2006) Molecular diversity, trafficking and subcellular localization of GABAB receptors. Pharmacol Ther 110:533-543.

Bianchi MT, Macdonald RL (2002) Slow phases of GABA(A) receptor desensitization: structural determinants and possible relevance for synaptic function. J Physiol 544:3-18.

Bianchi MT, Haas KF, Macdonald RL (2002) Alpha1 and alpha6 subunits specify distinct desensitization, deactivation and neurosteroid

modulation of GABA(A) receptors containing the delta subunit. Neuropharmacology 43:492-502.

Bloom FE, Iversen LL (1971) Localizing 3H-GABA in nerve terminals of rat cerebral cortex by electron microscopic autoradiography. Nature 229:628-630.

Bogdanov Y, Michels G, Armstrong-Gold C, Haydon PG, Lindstrom J, Pangalos M, Moss SJ (2006) Synaptic GABAA receptors are directly recruited from their extrasynaptic counterparts. EMBO J 25:4381-4389.

Bohme I, Rabe H, Luddens H (2004) Four amino acids in the alpha subunits determine the gamma-aminobutyric acid sensitivities of GABAA receptor subtypes. J Biol Chem 279:35193-35200.

Boistel J, Fatt P (1958) Membrane permeability change during inhibitory transmitter action in crustacean muscle. J Physiol 144:176-191.

Bonanno G, Raiteri M (1993) gamma-Aminobutyric acid (GABA) autoreceptors in rat cerebral cortex and spinal cord represent pharmacologically distinct subtypes of the GABAB receptor. J Pharmacol Exp Ther 265:765-770.

Bormann J (2000) The 'ABC' of GABA receptors. Trends Pharmacol Sci 21:16-19.

Bormann J, Feigenspan A (1995) GABAC receptors. Trends Neurosci 18:515-519.

Bowery NG (1987) Glycine-binding sites and NMDA receptors in brain. Nature 326:338.

Bowery NG, Doble A, Hill DR, Hudson AL, Shaw JS, Turnbull MJ, Warrington R (1981) Bicuculline-insensitive GABA receptors on peripheral autonomic nerve terminals. Eur J Pharmacol 71:53-70.

Brandon NJ, Jovanovic JN, Smart TG, Moss SJ (2002) Receptor for activated C kinase-1 facilitates protein kinase C-dependent phosphorylation and functional modulation of GABA(A) receptors with the activation of G-protein-coupled receptors. J Neurosci 22:6353-6361.

Brandon NJ, Delmas P, Hill J, Smart TG, Moss SJ (2001) Constitutive tyrosine phosphorylation of the GABA(A) receptor gamma 2 subunit in rat brain. Neuropharmacology 41:745-752.

Brandon NJ, Uren JM, Kittler JT, Wang H, Olsen R, Parker PJ, Moss SJ (1999) Subunit-specific association of protein kinase C and the receptor for activated C kinase with GABA type A receptors. J Neurosci 19:9228-9234.

Brandon NJ, Jovanovic JN, Colledge M, Kittler JT, Brandon JM, Scott JD, Moss SJ (2003) A-kinase anchoring protein 79/150 facilitates the phosphorylation of GABA(A) receptors by cAMP-dependent protein kinase via selective interaction with receptor beta subunits. Mol Cell Neurosci 22:87-97.

Brandon NJ, Delmas P, Kittler JT, McDonald BJ, Sieghart W, Brown DA, Smart TG, Moss SJ (2000) GABAA receptor phosphorylation and functional modulation in cortical neurons by a protein kinase C-dependent pathway. J Biol Chem 275:38856-38862.

Brickley SG, Cull-Candy SG, Farrant M (1996) Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABAA receptors. J Physiol 497 (Pt 3):753-759.

Brickley SG, Cull-Candy SG, Farrant M (1999) Single-channel properties of synaptic and extrasynaptic GABAA receptors suggest differential targeting of receptor subtypes. J Neurosci 19:2960-2973.

Brunig I, Scotti E, Sidler C, Fritschy JM (2002) Intact sorting, targeting, and clustering of gamma-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. J Comp Neurol 443:43-55.

Buhot MC (1997) Serotonin receptors in cognitive behaviors. Curr Opin Neurobiol 7:243-254.

Butcher SH, Butcher LL, Harms MS, Jendent DJ (1976) Fast fixation of brain in situ by high intensity microwave irradiation: application to neurochemical studies. J Microw Power 11:61-65.

Cai X, Flores-Hernandez J, Feng J, Yan Z (2002) Activity-dependent bidirectional regulation of GABA(A) receptor channels by the 5-HT(4) receptor-mediated signalling in rat prefrontal cortical pyramidal neurons. J Physiol 540:743-759.

Calver AR, Medhurst AD, Robbins MJ, Charles KJ, Evans ML, Harrison DC, Stammers M, Hughes SA, Hervieu G, Couve A, Moss SJ, Middlemiss DN, Pangalos MN (2000) The expression of GABA(B1) and GABA(B2) receptor subunits in the cNS differs from that in peripheral tissues. Neuroscience 100:155-170.

Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by alpha5 subunit-containing gamma-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 101:3662-3667.

Chang Y, Wang R, Barot S, Weiss DS (1996) Stoichiometry of a recombinant GABAA receptor. J Neurosci 16:5415-5424.

Charych EI, Yu W, Miralles CP, Serwanski DR, Li X, Rubio M, De Blas AL (2004) The brefeldin A-inhibited GDP/GTP exchange factor 2, a protein involved in vesicular trafficking, interacts with the beta subunits of the GABA receptors. J Neurochem 90:173-189.

Cheetham SC, Crompton MR, Katona CL, Parker SJ, Horton RW (1988) Brain GABAA/benzodiazepine binding sites and glutamic acid decarboxylase activity in depressed suicide victims. Brain Res 460:114-123.

Chen G, Kittler JT, Moss SJ, Yan Z (2006) Dopamine D3 receptors regulate GABAA receptor function through a phospho-dependent endocytosis mechanism in nucleus accumbens. J Neurosci 26:2513-2521.

Chen ZW, Chang CS, Leil TA, Olcese R, Olsen RW (2005) GABAA receptor-associated protein regulates GABAA receptor cell-surface number in Xenopus laevis oocytes. Mol Pharmacol 68:152-159.

Chi V, Chandy KG (2007) Immunohistochemistry: paraffin sections using the Vectastain ABC kit from vector labs. J Vis Exp:308.

Chu DC, Albin RL, Young AB, Penney JB (1990) Distribution and kinetics of GABAB binding sites in rat central nervous system: a quantitative autoradiographic study. Neuroscience 34:341-357.

Clague MJ (1998) Molecular aspects of the endocytic pathway. Biochem J 336 (Pt 2):271-282.

Clark S, Schwalbe J, Stasko MR, Yarowsky PJ, Costa AC (2006) Fluoxetine rescues deficient neurogenesis in hippocampus of the Ts65Dn mouse model for Down syndrome. Exp Neurol 200:256-261.

Clarke RS, Dundee JW, Carson IW (1973) Proceedings: A new steroid anaesthetic-althesin. Proc R Soc Med 66:1027-1030.

Colledge M, Scott JD (1999) AKAPs: from structure to function. Trends Cell Biol 9:216-221.

Connolly CN, Wafford KA (2004) The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. Biochem Soc Trans 32:529-534.

Davidson RJ, Jackson DC, Kalin NH (2000) Emotion, plasticity, context, and regulation: perspectives from affective neuroscience. Psychol Bull 126:890-909.

Dawson GR, Maubach KA, Collinson N, Cobain M, Everitt BJ, MacLeod AM, Choudhury HI, McDonald LM, Pillai G, Rycroft W, Smith AJ, Sternfeld F, Tattersall FD, Wafford KA, Reynolds DS, Seabrook GR, Atack JR (2006) An inverse agonist selective for alpha5 subunit-containing GABAA receptors enhances cognition. J Pharmacol Exp Ther 316:1335-1345.

de Haas SL, Franson KL, Schmitt JA, Cohen AF, Fau JB, Dubruc C, van Gerven JM (2009) The pharmacokinetic and pharmacodynamic effects of SL65.1498, a GABA-A alpha2,3 selective agonist, in comparison with lorazepam in healthy volunteers. J Psychopharmacol 23:625-632.

Deitrich RA, Dunwiddie TV, Harris RA, Erwin VG (1989) Mechanism of action of ethanol: initial central nervous system actions. Pharmacol Rev 41:489-537.

Delaney SM, Geiger JD (1996) Brain regional levels of adenosine and adenosine nucleotides in rats killed by high-energy focused microwave irradiation. J Neurosci Methods 64:151-156.

Depoortere H, Zivkovic B, Lloyd KG, Sanger DJ, Perrault G, Langer SZ, Bartholini G (1986) Zolpidem, a novel nonbenzodiazepine hypnotic. I. Neuropharmacological and behavioral effects. J Pharmacol Exp Ther 237:649-658.

Derry JM, Paulsen IM, Davies M, Dunn SM (2007) A single point mutation of the GABA(A) receptor alpha5-subunit confers fluoxetine sensitivity. Neuropharmacology 52:497-505.

DeSouza S, Fu J, States BA, Ziff EB (2002) Differential palmitoylation directs the AMPA receptor-binding protein ABP to spines or to intracellular clusters. J Neurosci 22:3493-3503.

Dias R et al. (2005) Evidence for a significant role of alpha 3-containing GABAA receptors in mediating the anxiolytic effects of benzodiazepines. J Neurosci 25:10682-10688.

Ernst M, Brauchart D, Boresch S, Sieghart W (2003) Comparative modeling of GABA(A) receptors: limits, insights, future developments. Neuroscience 119:933-943.

Ernst M, Bruckner S, Boresch S, Sieghart W (2005) Comparative models of GABAA receptor extracellular and transmembrane domains: important insights in pharmacology and function. Mol Pharmacol 68:1291-1300.

Essrich C, Lorez M, Benson JA, Fritschy JM, Luscher B (1998) Postsynaptic clustering of major GABAA receptor subtypes requires the gamma 2 subunit and gephyrin. Nat Neurosci 1:563-571.

Fairfax BP, Pitcher JA, Scott MG, Calver AR, Pangalos MN, Moss SJ, Couve A (2004) Phosphorylation and chronic agonist treatment atypically modulate GABAB receptor cell surface stability. J Biol Chem 279:12565-12573.

Fang C, Deng L, Keller CA, Fukata M, Fukata Y, Chen G, Luscher B (2006) GODZ-mediated palmitoylation of GABA(A) receptors is required for

normal assembly and function of GABAergic inhibitory synapses. J Neurosci 26:12758-12768.

Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat Rev Neurosci 6:215-229.

Farrar SJ, Whiting PJ, Bonnert TP, McKernan RM (1999) Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. J Biol Chem 274:10100-10104.

Feng J, Cai X, Zhao J, Yan Z (2001) Serotonin receptors modulate GABA(A) receptor channels through activation of anchored protein kinase C in prefrontal cortical neurons. J Neurosci 21:6502-6511.

Fernandez F, Morishita W, Zuniga E, Nguyen J, Blank M, Malenka RC, Garner CC (2007) Pharmacotherapy for cognitive impairment in a mouse model of Down syndrome. Nat Neurosci 10:411-413.

Florey E, Mc LH (1959) The effects of factor I and of gamma-aminobutyric acid on smooth muscle preparations. J Physiol 145:66-76.

Fritschy JM, Paysan J, Enna A, Mohler H (1994) Switch in the expression of rat GABAA-receptor subtypes during postnatal development: an immunohistochemical study. J Neurosci 14:5302-5324.

Fritschy JM, Benke D, Mertens S, Oertel WH, Bachi T, Mohler H (1992) Five subtypes of type A gamma-aminobutyric acid receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. Proc Natl Acad Sci U S A 89:6726-6730.

Fuhrmann JC, Kins S, Rostaing P, El Far O, Kirsch J, Sheng M, Triller A, Betz H, Kneussel M (2002) Gephyrin interacts with Dynein light chains 1 and 2, components of motor protein complexes. J Neurosci 22:5393-5402.

Gao B, Fritschy JM, Benke D, Mohler H (1993) Neuron-specific expression of GABAA-receptor subtypes: differential association of the alpha 1- and alpha 3-subunits with serotonergic and GABAergic neurons. Neuroscience 54:881-892.

Gingrich KJ, Roberts WA, Kass RS (1995) Dependence of the GABAA receptor gating kinetics on the alpha-subunit isoform: implications for

structure-function relations and synaptic transmission. J Physiol 489 (Pt 2):529-543.

Goren MZ, Kucukibrahimoglu E, Berkman K, Terzioglu B (2007) Fluoxetine partly exerts its actions through GABA: a neurochemical evidence. Neurochem Res 32:1559-1565.

Guattari B (1989) Simultaneous quantitative high-performance liquid chromatographic determination of cyclic adenosine 3',5'-monophosphate and adenosine in mouse brain after microwave irradiation: application to isoprenaline effect in vivo. J Chromatogr 489:394-398.

Haas KF, Macdonald RL (1999) GABAA receptor subunit gamma2 and delta subtypes confer unique kinetic properties on recombinant GABAA receptor currents in mouse fibroblasts. J Physiol 514 (Pt 1):27-45.

Harvey K, Duguid IC, Alldred MJ, Beatty SE, Ward H, Keep NH, Lingenfelter SE, Pearce BR, Lundgren J, Owen MJ, Smart TG, Luscher B, Rees MI, Harvey RJ (2004) The GDP-GTP exchange factor collybistin: an essential determinant of neuronal gephyrin clustering. J Neurosci 24:5816-5826.

Haucke V, Wenk MR, Chapman ER, Farsad K, De Camilli P (2000) Dual interaction of synaptotagmin with mu2- and alpha-adaptin facilitates clathrin-coated pit nucleation. Embo J 19:6011-6019.

Hayashi T, Rumbaugh G, Huganir RL (2005) Differential regulation of AMPA receptor subunit trafficking by palmitoylation of two distinct sites. Neuron 47:709-723.

Hebert MA, O'Callaghan JP (2000) Protein phosphorylation cascades associated with methamphetamine-induced glial activation. Ann N Y Acad Sci 914:238-262.

Hedblom E, Kirkness EF (1997) A novel class of GABAA receptor subunit in tissues of the reproductive system. J Biol Chem 272:15346-15350.

Herring D, Huang R, Singh M, Dillon GH, Leidenheimer NJ (2005) PKC modulation of GABAA receptor endocytosis and function is inhibited by mutation of a dileucine motif within the receptor beta 2 subunit. Neuropharmacology 48:181-194.

Herring D, Huang R, Singh M, Robinson LC, Dillon GH, Leidenheimer NJ (2003) Constitutive GABAA receptor endocytosis is dynamin-mediated and dependent on a dileucine AP2 adaptin-binding motif within the beta 2 subunit of the receptor. J Biol Chem 278:24046-24052.

Hill DR, Bowery NG (1981) 3H-baclofen and 3H-GABA bind to bicuculline-insensitive GABA B sites in rat brain. Nature 290:149-152.

Hinkle DJ, Macdonald RL (2003) Beta subunit phosphorylation selectively increases fast desensitization and prolongs deactivation of alpha1beta1gamma2L and alpha1beta3gamma2L GABA(A) receptor currents. J Neurosci 23:11698-11710.

Hirota K (2006) Special cases: ketamine, nitrous oxide and xenon. Best Pract Res Clin Anaesthesiol 20:69-79.

Hossain MZ, Murphy LJ, Hertzberg EL, Nagy JI (1994) Phosphorylated forms of connexin43 predominate in rat brain: demonstration by rapid inactivation of brain metabolism. J Neurochem 62:2394-2403.

Houston CM, Smart TG (2006) CaMK-II modulation of GABA(A) receptors expressed in HEK293, NG108-15 and rat cerebellar granule neurons. Eur J Neurosci 24:2504-2514.

Ishikawa K, Shibanoki S, Saito S, McGaugh JL (1982) Effect of microwave irradiation on monoamine metabolism in dissected rat brain. Brain Res 240:158-161.

Jacob TC, Moss SJ, Jurd R (2008) GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. Nat Rev Neurosci 9:331-343.

Jacob TC, Bogdanov YD, Magnus C, Saliba RS, Kittler JT, Haydon PG, Moss SJ (2005) Gephyrin regulates the cell surface dynamics of synaptic GABAA receptors. J Neurosci 25:10469-10478.

Jacob TC, Wan Q, Vithlani M, Saliba RS, Succol F, Pangalos MN, Moss SJ (2009) GABA(A) receptor membrane trafficking regulates spine maturity. Proc Natl Acad Sci U S A 106:12500-12505.

Johnston GA, Curtis DR, Beart PM, Game CJ, McCulloch RM, Twitchin B (1975) Cis- and trans-4-aminocrotonic acid as GABA analogues of restricted conformation. J Neurochem 24:157-160.

Jope RS, Johnson GV, Baird MS (1991) Seizure-induced protein tyrosine phosphorylation in rat brain regions. Epilepsia 32:755-760.

Jovanovic JN, Thomas P, Kittler JT, Smart TG, Moss SJ (2004) Brain-derived neurotrophic factor modulates fast synaptic inhibition by regulating GABA(A) receptor phosphorylation, activity, and cell-surface stability. J Neurosci 24:522-530.

Jursky F, Fuchs K, Buhr A, Tretter V, Sigel E, Sieghart W (2000) Identification of amino acid residues of GABA(A) receptor subunits contributing to the formation and affinity of the tert-butylbicyclophosphorothionate binding site. J Neurochem 74:1310-1316.

Kanematsu T, Jang IS, Yamaguchi T, Nagahama H, Yoshimura K, Hidaka K, Matsuda M, Takeuchi H, Misumi Y, Nakayama K, Yamamoto T, Akaike N, Hirata M (2002) Role of the PLC-related, catalytically inactive protein p130 in GABA(A) receptor function. EMBO J 21:1004-1011.

Katsura M, Iino T, Kuriyama K (1992) Changes in content of neuroactive amino acids and acetylcholine in the rat hippocampus following transient forebrain ischemia. Neurochem Int 21:243-249.

Kins S, Betz H, Kirsch J (2000) Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin. Nat Neurosci 3:22-29.

Kitamura A, Sato R, Marszalec W, Yeh JZ, Ogawa R, Narahashi T (2004) Halothane and propofol modulation of gamma-aminobutyric acidA receptor single-channel currents. Anesth Analg 99:409-415, table of contents.

Kittler JT, Moss SJ (2003) Modulation of GABAA receptor activity by phosphorylation and receptor trafficking: implications for the efficacy of synaptic inhibition. Curr Opin Neurobiol 13:341-347.

Kittler JT, McAinsh K, Moss SJ (2002) Mechanisms of GABAA receptor assembly and trafficking: implications for the modulation of inhibitory neurotransmission. Mol Neurobiol 26:251-268.

Kittler JT, Delmas P, Jovanovic JN, Brown DA, Smart TG, Moss SJ (2000) Constitutive endocytosis of GABAA receptors by an association with the adaptin AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. J Neurosci 20:7972-7977.

Kittler JT, Rostaing P, Schiavo G, Fritschy JM, Olsen R, Triller A, Moss SJ (2001) The subcellular distribution of GABARAP and its ability to interact with NSF suggest a role for this protein in the intracellular transport of GABA(A) receptors. Mol Cell Neurosci 18:13-25.

Kittler JT, Thomas P, Tretter V, Bogdanov YD, Haucke V, Smart TG, Moss SJ (2004) Huntingtin-associated protein 1 regulates inhibitory synaptic transmission by modulating gamma-aminobutyric acid type A receptor membrane trafficking. Proc Natl Acad Sci U S A 101:12736-12741.

Kittler JT, Chen G, Honing S, Bogdanov Y, McAinsh K, Arancibia-Carcamo IL, Jovanovic JN, Pangalos MN, Haucke V, Yan Z, Moss SJ (2005) Phospho-dependent binding of the clathrin AP2 adaptor complex to GABAA receptors regulates the efficacy of inhibitory synaptic transmission. Proc Natl Acad Sci U S A 102:14871-14876.

Kittler JT, Chen G, Kukhtina V, Vahedi-Faridi A, Gu Z, Tretter V, Smith KR, McAinsh K, Arancibia-Carcamo IL, Saenger W, Haucke V, Yan Z, Moss SJ (2008) Regulation of synaptic inhibition by phospho-dependent binding of the AP2 complex to a YECL motif in the GABAA receptor gamma2 subunit. Proc Natl Acad Sci U S A 105:3616-3621.

Klauck TM, Faux MC, Labudda K, Langeberg LK, Jaken S, Scott JD (1996) Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. Science 271:1589-1592.

Klausberger T, Roberts JD, Somogyi P (2002) Cell type- and input-specific differences in the number and subtypes of synaptic GABA(A) receptors in the hippocampus. J Neurosci 22:2513-2521.

Kleijnen MF, Shih AH, Zhou P, Kumar S, Soccio RE, Kedersha NL, Gill G, Howley PM (2000) The hPLIC proteins may provide a link between the ubiquitination machinery and the proteasome. Mol Cell 6:409-419.

Kneussel M, Brandstatter JH, Laube B, Stahl S, Muller U, Betz H (1999) Loss of postsynaptic GABA(A) receptor clustering in gephyrin-deficient mice. J Neurosci 19:9289-9297.

Kneussel M, Brandstatter JH, Gasnier B, Feng G, Sanes JR, Betz H (2001) Gephyrin-independent clustering of postsynaptic GABA(A) receptor subtypes. Mol Cell Neurosci 17:973-982.

Knight AR, Stephenson FA, Tallman JF, Ramabahdran TV (2000) Monospecific antibodies as probes for the stoichiometry of recombinant GABA(A) receptors. Receptors Channels 7:213-226.

Kofuji P, Wang JB, Moss SJ, Huganir RL, Burt DR (1991) Generation of two forms of the gamma-aminobutyric acidA receptor gamma 2-subunit in mice by alternative splicing. J Neurochem 56:713-715.

Kravitz EA, Kuffler SW, Potter DD (1963) Gamma-Aminobutyric Acid and Other Blocking Compounds in Crustacea. Iii. Their Relative Concentrations in Separated Motor and Inhibitory Axons. J Neurophysiol 26:739-751.

Krishek BJ, Amato A, Connolly CN, Moss SJ, Smart TG (1996) Proton sensitivity of the GABA(A) receptor is associated with the receptor subunit composition. J Physiol 492 (Pt 2):431-443.

Krishek BJ, Xie X, Blackstone C, Huganir RL, Moss SJ, Smart TG (1994) Regulation of GABAA receptor function by protein kinase C phosphorylation. Neuron 12:1081-1095.

Krnjevic K, Schwartz S (1967) The action of gamma-aminobutyric acid on cortical neurones. Exp Brain Res 3:320-336.

Kuffler SW, Edwards C (1958) Mechanism of gamma aminobutyric acid (GABA) action and its relation to synaptic inhibition. J Neurophysiol 21:589-610.

Laurie DJ, Wisden W, Seeburg PH (1992) The distribution of thirteen GABAA receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. J Neurosci 12:4151-4172.

Lavoie AM, Tingey JJ, Harrison NL, Pritchett DB, Twyman RE (1997) Activation and deactivation rates of recombinant GABA(A) receptor channels are dependent on alpha-subunit isoform. Biophys J 73:2518-2526.

Le Borgne R, Hoflack B (1998) Mechanisms of protein sorting and coat assembly: insights from the clathrin-coated vesicle pathway. Curr Opin Cell Biol 10:499-503.

Leil TA, Chen ZW, Chang CS, Olsen RW (2004) GABAA receptor-associated protein traffics GABAA receptors to the plasma membrane in neurons. J Neurosci 24:11429-11438.

Levi S, Logan SM, Tovar KR, Craig AM (2004) Gephyrin is critical for glycine receptor clustering but not for the formation of functional GABAergic synapses in hippocampal neurons. J Neurosci 24:207-217.

Li S, Wang C, Wang W, Dong H, Hou P, Tang Y (2008) Chronic mild stress impairs cognition in mice: from brain homeostasis to behavior. Life Sci 82:934-942.

Lin YF, Angelotti TP, Dudek EM, Browning MD, Macdonald RL (1996) Enhancement of recombinant alpha 1 beta 1 gamma 2L gamma-aminobutyric acidA receptor whole-cell currents by protein kinase C is mediated through phosphorylation of both beta 1 and gamma 2L subunits. Mol Pharmacol 50:185-195.

Liu F, Wan Q, Pristupa ZB, Yu XM, Wang YT, Niznik HB (2000) Direct protein-protein coupling enables cross-talk between dopamine D5 and gamma-aminobutyric acid A receptors. Nature 403:274-280.

Loebrich S, Bahring R, Katsuno T, Tsukita S, Kneussel M (2006) Activated radixin is essential for GABAA receptor alpha5 subunit anchoring at the actin cytoskeleton. EMBO J 25:987-999.

Majlessi N, Naghdi N (2002) Impaired spatial learning in the Morris water maze induced by serotonin reuptake inhibitors in rats. Behav Pharmacol 13:237-242.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104-9110.

Manchon M, Kopp N, Rouzioux JJ, Lecestre D, Deluermoz S, Miachon S (1987) Benzodiazepine receptor and neurotransmitter studies in the brain of suicides. Life Sci 41:2623-2630.

Manji HK, Drevets WC, Charney DS (2001) The cellular neurobiology of depression. Nat Med 7:541-547.

Margeta-Mitrovic M, Mitrovic I, Riley RC, Jan LY, Basbaum AI (1999) Immunohistochemical localization of GABA(B) receptors in the rat central nervous system. J Comp Neurol 405:299-321.

Marsden KC, Beattie JB, Friedenthal J, Carroll RC (2007) NMDA receptor activation potentiates inhibitory transmission through GABA receptor-associated protein-dependent exocytosis of GABA(A) receptors. J Neurosci 27:14326-14337.

Martin GR, Eglen RM, Hamblin MW, Hoyer D, Yocca F (1998) The structure and signalling properties of 5-HT receptors: an endless diversity? Trends Pharmacol Sci 19:2-4.

McClellan AM, Twyman RE (1999) Receptor system response kinetics reveal functional subtypes of native murine and recombinant human GABAA receptors. J Physiol 515 (Pt 3):711-727.

McDonald BJ, Moss SJ (1997) Conserved phosphorylation of the intracellular domains of GABA(A) receptor beta2 and beta3 subunits by cAMP-dependent protein kinase, cGMP-dependent protein kinase protein kinase C and Ca2+/calmodulin type II-dependent protein kinase. Neuropharmacology 36:1377-1385.

McDonald BJ, Amato A, Connolly CN, Benke D, Moss SJ, Smart TG (1998) Adjacent phosphorylation sites on GABAA receptor beta subunits

determine regulation by cAMP-dependent protein kinase. Nat Neurosci 1:23-28.

McKernan RM et al. (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha1 subtype. Nat Neurosci 3:587-592.

Meyer G, Kirsch J, Betz H, Langosch D (1995) Identification of a gephyrin binding motif on the glycine receptor beta subunit. Neuron 15:563-572.

Mobley PL, Gonzalez NE (1991) The use of microwave tissue fixation to demonstrate the in vivo phosphorylation of an acidic 80,000 molecular weight protein in the rat neocortex following treatment with soman. Life Sci 49:155-163.

Moragues N, Ciofi P, Tramu G, Garret M (2002) Localisation of GABA(A) receptor epsilon-subunit in cholinergic and aminergic neurones and evidence for co-distribution with the theta-subunit in rat brain. Neuroscience 111:657-669.

Moss SJ, Doherty CA, Huganir RL (1992) Identification of the cAMP-dependent protein kinase and protein kinase C phosphorylation sites within the major intracellular domains of the beta 1, gamma 2S, and gamma 2L subunits of the gamma-aminobutyric acid type A receptor. J Biol Chem 267:14470-14476.

Moss SJ, Gorrie GH, Amato A, Smart TG (1995) Modulation of GABAA receptors by tyrosine phosphorylation. Nature 377:344-348.

Navarro JF, Buron E, Martin-Lopez M (2002) Anxiogenic-like activity of L-655,708, a selective ligand for the benzodiazepine site of GABA(A) receptors which contain the alpha-5 subunit, in the elevated plus-maze test. Prog Neuropsychopharmacol Biol Psychiatry 26:1389-1392.

Newland CF, Cull-Candy SG (1992) On the mechanism of action of picrotoxin on GABA receptor channels in dissociated sympathetic neurones of the rat. J Physiol 447:191-213.

Nishikawa K, Jenkins A, Paraskevakis I, Harrison NL (2002) Volatile anesthetic actions on the GABAA receptors: contrasting effects of alpha

1(S270) and beta 2(N265) point mutations. Neuropharmacology 42:337-345.

Nohria V, Giller E (2007) Ganaxolone. Neurotherapeutics 4:102-105.

Nusser Z, Cull-Candy S, Farrant M (1997) Differences in synaptic GABA(A) receptor number underlie variation in GABA mini amplitude. Neuron 19:697-709.

Nusser Z, Sieghart W, Somogyi P (1998a) Segregation of different GABAA receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci 18:1693-1703.

Nusser Z, Hajos N, Somogyi P, Mody I (1998b) Increased number of synaptic GABA(A) receptors underlies potentiation at hippocampal inhibitory synapses. Nature 395:172-177.

O'Callaghan JP, Sriram K (2004) Focused microwave irradiation of the brain preserves in vivo protein phosphorylation: comparison with other methods of sacrifice and analysis of multiple phosphoproteins. J Neurosci Methods 135:159-168.

O'Callaghan JP, Martin PM, Mass MJ (1998) The MAP kinase cascade is activated prior to the induction of gliosis in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of dopaminergic neurotoxicity. Ann N Y Acad Sci 844:40-49.

O'Sullivan GA, Kneussel M, Elazar Z, Betz H (2005) GABARAP is not essential for GABA receptor targeting to the synapse. Eur J Neurosci 22:2644-2648.

Otsuka M, Iversen LL, Hall ZW, Kravitz EA (1966) Release of gamma-aminobutyric acid from inhibitory nerves of lobster. Proc Natl Acad Sci U S A 56:1110-1115.

Persohn E, Malherbe P, Richards JG (1992) Comparative molecular neuroanatomy of cloned GABAA receptor subunits in the rat CNS. J Comp Neurol 326:193-216.

Pirker S, Schwarzer C, Wieselthaler A, Sieghart W, Sperk G (2000) GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101:815-850.

Porter NM, Twyman RE, Uhler MD, Macdonald RL (1990) Cyclic AMP-dependent protein kinase decreases GABAA receptor current in mouse spinal neurons. Neuron 5:789-796.

Pritchett DB, Seeburg PH (1990) Gamma-aminobutyric acidA receptor alpha 5-subunit creates novel type II benzodiazepine receptor pharmacology. J Neurochem 54:1802-1804.

Pritchett DB, Sontheimer H, Shivers BD, Ymer S, Kettenmann H, Schofield PR, Seeburg PH (1989) Importance of a novel GABAA receptor subunit for benzodiazepine pharmacology. Nature 338:582-585.

Ragozzino D, Woodward RM, Murata Y, Eusebi F, Overman LE, Miledi R (1996) Design and in vitro pharmacology of a selective gamma-aminobutyric acidC receptor antagonist. Mol Pharmacol 50:1024-1030.

Ramming M, Kins S, Werner N, Hermann A, Betz H, Kirsch J (2000) Diversity and phylogeny of gephyrin: tissue-specific splice variants, gene structure, and sequence similarities to molybdenum cofactor-synthesizing and cytoskeleton-associated proteins. Proc Natl Acad Sci U S A 97:10266-10271.

Rathenberg J, Kittler JT, Moss SJ (2004) Palmitoylation regulates the clustering and cell surface stability of GABAA receptors. Mol Cell Neurosci 26:251-257.

Rijnsoever C, Tauber M, Choulli MK, Keist R, Rudolph U, Mohler H, Fritschy JM, Crestani F (2004) Requirement of alpha5-GABAA receptors for the development of tolerance to the sedative action of diazepam in mice. J Neurosci 24:6785-6790.

Rivera C, Voipio J, Payne JA, Ruusuvuori E, Lahtinen H, Lamsa K, Pirvola U, Saarma M, Kaila K (1999) The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. Nature 397:251-255.

Roberts E, Frankel S (1950) gamma-Aminobutyric acid in brain: its formation from glutamic acid. J Biol Chem 187:55-63.

Robinson RT, Drafts BC, Fisher JL (2003) Fluoxetine increases GABA(A) receptor activity through a novel modulatory site. J Pharmacol Exp Ther 304:978-984.

Rowlett JK, Platt DM, Lelas S, Atack JR, Dawson GR (2005) Different GABAA receptor subtypes mediate the anxiolytic, abuse-related, and motor effects of benzodiazepine-like drugs in primates. Proc Natl Acad Sci U S A 102:915-920.

Rudolph U, Mohler H (2004) Analysis of GABAA receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Annu Rev Pharmacol Toxicol 44:475-498.

Saliba RS, Pangalos M, Moss SJ (2008) The ubiquitin-like protein Plic-1 enhances the membrane insertion of GABAA receptors by increasing their stability within the endoplasmic reticulum. J Biol Chem 283:18538-18544.

Saliba RS, Michels G, Jacob TC, Pangalos MN, Moss SJ (2007) Activity-dependent ubiquitination of GABA(A) receptors regulates their accumulation at synaptic sites. J Neurosci 27:13341-13351.

Sanna E, Mostallino MC, Busonero F, Talani G, Tranquilli S, Mameli M, Spiga S, Follesa P, Biggio G (2003) Changes in GABA(A) receptor gene expression associated with selective alterations in receptor function and pharmacology after ethanol withdrawal. J Neurosci 23:11711-11724.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805-809.

Saxena NC, Macdonald RL (1996) Properties of putative cerebellar gamma-aminobutyric acid A receptor isoforms. Mol Pharmacol 49:567-579.

Schaerer MT, Kannenberg K, Hunziker P, Baumann SW, Sigel E (2001) Interaction between GABA(A) receptor beta subunits and the multifunctional protein gC1q-R. J Biol Chem 276:26597-26604.

Schofield PR, Darlison MG, Fujita N, Burt DR, Stephenson FA, Rodriguez H, Rhee LM, Ramachandran J, Reale V, Glencorse TA, et al. (1987) Sequence and functional expression of the GABA A receptor shows a ligand-gated receptor super-family. Nature 328:221-227.

Sellers EM, Kaplan HL, Busto UE (1992) Abuse liability of bretazenil and other partial agonists. Clin Neuropharmacol 15 Suppl 1 Pt A:409A.

Serwanski DR, Miralles CP, Christie SB, Mehta AK, Li X, De Blas AL (2006) Synaptic and nonsynaptic localization of GABAA receptors containing the alpha5 subunit in the rat brain. J Comp Neurol 499:458-470.

Shen X, Xu KF, Fan Q, Pacheco-Rodriguez G, Moss J, Vaughan M (2006) Association of brefeldin A-inhibited guanine nucleotide-exchange protein 2 (BIG2) with recycling endosomes during transferrin uptake. Proc Natl Acad Sci U S A 103:2635-2640.

Sheng G, Chang GQ, Lin JY, Yu ZX, Fang ZH, Rong J, Lipton SA, Li SH, Tong G, Leibowitz SF, Li XJ (2006) Hypothalamic huntingtin-associated protein 1 as a mediator of feeding behavior. Nat Med 12:526-533.

Shin HW, Morinaga N, Noda M, Nakayama K (2004) BIG2, a guanine nucleotide exchange factor for ADP-ribosylation factors: its localization to recycling endosomes and implication in the endosome integrity. Mol Biol Cell 15:5283-5294.

Shin RM, Masuda M, Miura M, Sano H, Shirasawa T, Song WJ, Kobayashi K, Aosaki T (2003) Dopamine D4 receptor-induced postsynaptic inhibition of GABAergic currents in mouse globus pallidus neurons. J Neurosci 23:11662-11672.

Sieghart W (2006) Structure, pharmacology, and function of GABAA receptor subtypes. Adv Pharmacol 54:231-263.

Sieghart W, Sperk G (2002) Subunit composition, distribution and function of GABA(A) receptor subtypes. Curr Top Med Chem 2:795-816.

Sieghart W, Fuchs K, Tretter V, Ebert V, Jechlinger M, Hoger H, Adamiker D (1999) Structure and subunit composition of GABA(A) receptors. Neurochem Int 34:379-385.

Sigel E, Buhr A (1997) The benzodiazepine binding site of GABAA receptors. Trends Pharmacol Sci 18:425-429.

Sigel E, Mamalaki C, Barnard EA (1982) Isolation of a GABA receptor from bovine brain using a benzodiazepine affinity column. FEBS Lett 147:45-48.

Slepnev VI, De Camilli P (2000) Accessory factors in clathrin-dependent synaptic vesicle endocytosis. Nat Rev Neurosci 1:161-172.

Smart TG (1992) A novel modulatory binding site for zinc on the GABAA receptor complex in cultured rat neurones. J Physiol 447:587-625.

Smart TG (1997) Regulation of excitatory and inhibitory neurotransmitter-gated ion channels by protein phosphorylation. Curr Opin Neurobiol 7:358-367.

Smart TG, Hosie AM, Miller PS (2004) Zn2+ ions: modulators of excitatory and inhibitory synaptic activity. Neuroscientist 10:432-442.

Smotrys JE, Linder ME (2004) Palmitoylation of intracellular signaling proteins: regulation and function. Annu Rev Biochem 73:559-587.

Snyder GL, Allen PB, Fienberg AA, Valle CG, Huganir RL, Nairn AC, Greengard P (2000) Regulation of phosphorylation of the GluR1 AMPA receptor in the neostriatum by dopamine and psychostimulants in vivo. J Neurosci 20:4480-4488.

Song M, Messing RO (2005) Protein kinase C regulation of GABAA receptors. Cell Mol Life Sci 62:119-127.

Sperk G, Schwarzer C, Tsunashima K, Fuchs K, Sieghart W (1997) GABA(A) receptor subunits in the rat hippocampus I: immunocytochemical distribution of 13 subunits. Neuroscience 80:987-1000.

Stupien G, Florian C, Roullet P (2003) Involvement of the hippocampal CA3-region in acquisition and in memory consolidation of spatial but not in object information in mice. Neurobiol Learn Mem 80:32-41.

Svenningsson P, Tzavara ET, Liu F, Fienberg AA, Nomikos GG, Greengard P (2002) DARPP-32 mediates serotonergic neurotransmission in the forebrain. Proc Natl Acad Sci U S A 99:3188-3193.

Svenningsson P, Tzavara ET, Qi H, Carruthers R, Witkin JM, Nomikos GG, Greengard P (2007) Biochemical and behavioral evidence for antidepressant-like effects of 5-HT6 receptor stimulation. J Neurosci 27:4201-4209.

Taylor PM, Thomas P, Gorrie GH, Connolly CN, Smart TG, Moss SJ (1999) Identification of amino acid residues within GABA(A) receptor beta subunits that mediate both homomeric and heteromeric receptor expression. J Neurosci 19:6360-6371.

Terunuma M, Jang IS, Ha SH, Kittler JT, Kanematsu T, Jovanovic JN, Nakayama KI, Akaike N, Ryu SH, Moss SJ, Hirata M (2004) GABAA receptor phospho-dependent modulation is regulated by phospholipase C-related inactive protein type 1, a novel protein phosphatase 1 anchoring protein. J Neurosci 24:7074-7084.

Thomas P, Mortensen M, Hosie AM, Smart TG (2005) Dynamic mobility of functional GABAA receptors at inhibitory synapses. Nat Neurosci 8:889-897.

Thomson AM, Bannister AP, Hughes DI, Pawelzik H (2000) Differential sensitivity to Zolpidem of IPSPs activated by morphologically identified CA1 interneurons in slices of rat hippocampus. Eur J Neurosci 12:425-436.

Tia S, Wang JF, Kotchabhakdi N, Vicini S (1996) Distinct deactivation and desensitization kinetics of recombinant GABAA receptors. Neuropharmacology 35:1375-1382.

Tretter V, Moss SJ (2008) GABA(A) Receptor Dynamics and Constructing GABAergic Synapses. Front Mol Neurosci 1:7.

Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant GABAA receptor subtype. J Neurosci 17:2728-2737.

Tretter V, Jacob TC, Mukherjee J, Fritschy JM, Pangalos MN, Moss SJ (2008) The clustering of GABA(A) receptor subtypes at inhibitory synapses is facilitated via the direct binding of receptor alpha 2 subunits to gephyrin. J Neurosci 28:1356-1365.

Tunnicliff G, Schindler NL, Crites GJ, Goldenberg R, Yochum A, Malatynska E (1999) The GABA(A) receptor complex as a target for fluoxetine action. Neurochem Res 24:1271-1276.

Twyman RE, Rogers CJ, Macdonald RL (1989) Differential regulation of gamma-aminobutyric acid receptor channels by diazepam and phenobarbital. Ann Neurol 25:213-220.

Uji A, Matsuda M, Kukita T, Maeda K, Kanematsu T, Hirata M (2002) Molecules interacting with PRIP-2, a novel Ins(1,4,5)P3 binding protein type 2: Comparison with PRIP-1. Life Sci 72:443-453.

Unwin N (1993) Nicotinic acetylcholine receptor at 9 A resolution. J Mol Biol 229:1101-1124.

Valluzzi JA, Chan K (2007) Effects of fluoxetine on hippocampal-dependent and hippocampal-independent learning tasks. Behav Pharmacol 18:507-513.

Wang H, Bedford FK, Brandon NJ, Moss SJ, Olsen RW (1999) GABA(A)-receptor-associated protein links GABA(A) receptors and the cytoskeleton. Nature 397:69-72.

Wang J, Liu S, Haditsch U, Tu W, Cochrane K, Ahmadian G, Tran L, Paw J, Wang Y, Mansuy I, Salter MM, Lu YM (2003) Interaction of calcineurin and type-A GABA receptor gamma 2 subunits produces long-term depression at CA1 inhibitory synapses. J Neurosci 23:826-836.

Wang JW, David DJ, Monckton JE, Battaglia F, Hen R (2008) Chronic fluoxetine stimulates maturation and synaptic plasticity of adult-born hippocampal granule cells. J Neurosci 28:1374-1384.

Whiting P, McKernan RM, Iversen LL (1990) Another mechanism for creating diversity in gamma-aminobutyrate type A receptors: RNA splicing directs expression of two forms of gamma 2 phosphorylation site. Proc Natl Acad Sci U S A 87:9966-9970.

Whiting PJ (2006) GABA-A receptors: a viable target for novel anxiolytics? Curr Opin Pharmacol 6:24-29.

Whiting PJ, Bonnert TP, McKernan RM, Farrar S, Le Bourdelles B, Heavens RP, Smith DW, Hewson L, Rigby MR, Sirinathsinghji DJ, Thompson SA, Wafford KA (1999) Molecular and functional diversity of the expanding GABA-A receptor gene family. Ann N Y Acad Sci 868:645-653.

Wieland HA, Luddens H, Seeburg PH (1992) A single histidine in GABAA receptors is essential for benzodiazepine agonist binding. J Biol Chem 267:1426-1429.

Wisden W, Laurie DJ, Monyer H, Seeburg PH (1992) The distribution of 13 GABAA receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci 12:1040-1062.

Wooltorton JR, Moss SJ, Smart TG (1997) Pharmacological and physiological characterization of murine homomeric beta3 GABA(A) receptors. Eur J Neurosci 9:2225-2235.

Zhang HG, ffrench-Constant RH, Jackson MB (1994) A unique amino acid of the Drosophila GABA receptor with influence on drug sensitivity by two mechanisms. J Physiol 479 (Pt 1):65-75.

Zhang L, Spigelman I, Carlen PL (1991) Development of GABA-mediated, chloride-dependent inhibition in CA1 pyramidal neurones of immature rat hippocampal slices. J Physiol 444:25-49.

॥ राम ॥