ABSTRACT

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$GABA_A$ receptor is a ligand-gated ion channel that conducts negatively charged chloride ions. Influx of this ion leads to hyperpolarization of neurons; thus, suppression of the neuronal excitability. Alterations in GABAergic neurotransmission may contribute to depression and anxiety. While neurosteroids can regulate the responsiveness of the $GABA_A$ receptor in allosteric manner, certain intracellular signaling pathways can also regulate the function of the $GABA_A$ receptors through phosphorylation of its subunits. One pathway that is regulated by both neurotrophic factors and steroid hormones is the ERK/MAPK pathway. This pathway is involved in cell proliferation, maturation, and even, cell death. The role of this pathway in the regulation of the $GABA_A$ receptor, however, is not well studied and is the subject of my dissertation.

Initial studies conducted by the Singh and Dillon laboratories showed that pharmacological inhibition of the ERK/MAPK pathway potentiated the $\alpha1\beta2\gamma2$ configuration of the $GABA_A$ receptor, expressed in HEK-t cells. This suggested that the ERK/MAPK pathway was involved in the negative regulation of the $GABA_A$ receptor function. In silico analysis revealed that the Thr 375 residue within the $\alpha1$ subunit was a plausible target of the ERK/MAPK pathway. As a result, I hypothesized that the activation of the ERK/MAPK pathway inhibited

$GABA_A$ receptor function through the direct phosphorylation of the Thr 375 residue, resulting in receptor internalization.

Supporting this hypothesis was data showing that mutation of the Thr 375 residue to Alanine prevented the enhancement of GABA-gated currents elicited by inhibiting the ERK/MAPK pathway. However, using the HEK-t cell line transfected with the $\alpha1\beta2\gamma2$ configuration of the $GABA_A$ receptor, I determined that the activation of the ERK/MAPK pathway by HGF did not influence the peak amplitude of the GABA-gated currents. Further, the potentiation of the GABA-gated currents was apparently not due to internalization of the receptor. Collectively, while we believe that the Thr 375 within the $\alpha1$ subunit is relevant to the effect of ERK/MAPK pathway inhibition, it was not a direct target of the ERK/MAPK pathway.

ERK/MAPK PATHWAY REGULATION OF $GABA_A$ RECEPTOR FUNCTION

DISSERTATION

Presented to the Graduate Council of the

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For the Degree of

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Akiko Dohi, B.Sc.

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LIST OF ABBREVIATIONS

4D4 cells	HEK-t cells stably expressing α1β2γ2-GABAA receptor
Akt	protein kinase B
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumin
Cl-	chloride ion
CNS	central nervous system
DMSO	dimethyl sulfoxide
DNA	deoxynucleic acid
EC30	effective concenration 30
ERK	extracellular signal regulated kinase
FITC	fluorecein isothiocyanate
FLAG	8 amino acid tag (DYKDDDDK)
GABA	gamma-amino butyric acid
GAD	glutamate decarboxylase
HEK-t cells	human embryonic kidney cells
HGF	hepatocyte growth factor
HRP	horse radish peroxidase
MAPK	mitogen activated protein kinase

MEK	MAPKK
mIPSC	miniature postsynaptic current
p-	phospho-
PI3K	phosphatidyl inositol triphosphate kinase
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
PMDD	premenstrual dysphoric disorder
PMS	premenstrual syndrome
Pro or P	proline
SDS-PAGE	sodium dodecyl sulfate polyacrylamine gel electrophoresis
SEM	standard error of the mean
Ser or S	serine
Thr or T	threonine
TM	transmembrane
Tyr or Y	tyrosine

CHAPTER 1

INTRODUCTION

γ-amino butyric acid (GABA)

GABA is the principal inhibitory small amino acid neurotransmitter produced by GABAergic neurons in the mammalian central nervous system (CNS), and is a ligand for its GABA receptors (Bormann, 1988). GABAergic neuronal activities in CNS regulate the function of the excitatory neurocircuitry, and this regulation depends on the diversity and numbers of the GABAergic local circuit neurons (Letinic et al., 2002).

GABA is synthesized from its precursor, glutamate. Glutamate is the most abundant excitatory amino acid neurotransmitter in the brain. GABAergic neurons take up glutamate through glutamate transporter. Glutamate is converted to GABA by glutamic acid decarboxylase (GAD). Two isoforms of the GAD are recognized, GAD67 and GAD65, both of which are expressed in the brain (Erlander et al., 1991). These two isoforms are derived from the transcription of different genes. GAD 67 is transcribed from the Gad1 gene while GAD 65 is transcribed from Gad2. The respective molecular weights of the translated proteins are 67 and 65 kDa. GAD65 is closely associated with synaptic vesicles while GAD67 is more evenly distributed throughout the cytoplasm of GABAergic neurons in the brain. Therefore, GAD65 is more likely responsible for producing GABA-filled synaptic vesicles that get released upon sustained neuronal stimulation (Martin and Rimvall, 1993).

High affinity GABA transporters located at the presynaptic membranes of GABAergic neurons as well as surrounding astrocytes take up the released GABA from the synaptic cleft and inactivate the neurotransmission process. After GABA is transported back into presynaptic neuron, most GABA molecules are taken up by the vesicular GABA transporter into synaptic vesicles and recycled as a neurotransmitter (Chaudhry et al., 1998; Sarup et al., 2003; Madsen et al., 2008). GABA that is taken up by astrocytes goes through the astrocytic glutamate-glutamine cycle. Here, GABA is converted to glutamate and then to glutamine. Glutamine is released from the astrocytes and taken up by the glutamine transporter on the GABAergic neurons. Glutamine is then converted back to glutamate and to GABA to complete the cycle (Liang et al., 2006). Interestingly, GABA can be utilized within the neurons for something else other than being recycled as a neurotransmitter. In the GABA shunt, GABA-transaminase converts GABA into succinate semialdehyde (SSA), and SSA dehydrogenase catalyses the oxidation of SSA to succinic acid (succinate) (Ramos et al., 1985). Succinate, in turn, fuels the TCA cycle leading to the production of adenosine-5'-triphosphate (ATP), the cell's principle energy source (Bach et al., 2009). GABA-transaminase inhibitors, such as vigabatrin, are used as a treatment for epilepsy so that GABA is used more towards neurotransmission (Wang et al., 2008).

GABAergic neurons are located throughout the CNS, including caudate nucleus, substantia nigra, dentate gyrus, hippocampus, and cerebellum. Dysfunction of the GAD is linked to a wide range of diseases, such as major depression, anxiety disorders (Hettema et al., 2006), schizophrenia (Zink et al., 2004), bipolar disorder (Woo et al., 2004), Stiff Person Syndrome (Murinson, 2004), and diabetes mellitus (Bingley et al., 1994; Knip and Siljander, 2008; Tsirogianni et al., 2009).

The $GABA_A$ receptor

The $GABA_A$ receptor is a member of a ligand-gated ion channel super family (Barnard, 1992) and specifically, is a GABA-gated chloride channel. It belongs to the Cys-loop superfamily of the ligand gated ion channels along with nicotinic, acetylcholine, glycine, 5-hydroxytryptamine type 3, and zinc activated cation receptors (Davies et al., 2003) where a cysteine residue within the extracellular domain makes covalent disulfide bond with another cysteine to make a small loop.

The $GABA_A$ receptor is composed of five proteins (pentamer) that are embedded in the cell membranes of postsynaptic neurons (Schofield et al., 1987). The five subunits of the receptor form a channel at the center of the assembly for the conduction of negatively charged chloride ion. When GABA binds to the $GABA_A$ receptor, the channel opens and allows influx of chloride ions, which subsequently decreases the excitability of the post-synaptic neurons by hyperpolarization. GABA inhibits virtually every neuron in the mature brain.

The $GABA_A$ receptors are found throughout the brain, and activation of the receptors are involved in sedation, relief of anxiety, and motor incoordination. Compounds that enhance the activity of GABA at the $GABA_A$ receptor, such as benzodiazepines, barbiturates, alcohol, and a number of general anesthetic reagents, such as propofol, etomidate, and thiopental (Olsen et al., 1986; Sieghart, 1995; Belelli et al., 1999; Rudolph and Mohler, 2006), results in neuronal inhibition leading to antiepileptic, anxiolytic, muscle relaxing, sedative, and hypnotic effect. These anesthetic compounds all act as positive allosteric modulators to potentiate inhibition of the CNS mediated by $GABA_A$ receptors (Burt and Kamatchi, 1991).

$GABA_A$ receptor subunits

Characteristics of the $GABA_A$ receptors depend on its subunit composition. Each subunit has a large extracellular N-terminal domain located on the outside of the cell membrane, four transmembrane (TM1-4) regions with a large intracellular domain between TM3 and TM4. TM2 of the five subunits line up at the center of the assembly to make the pore for the negatively charged chloride ion conduction. The extracellular domain is responsible for GABA binding, and the intracellular domain is often a target of phosphorylation, resulting in modification of the receptor function.

Seventeen $GABA_A$ receptor subunits have been listed to date: α (1-6), β (1-3), γ (1-3), δ, ε, θ, π, and ρ (Schofield et al., 1987; Levitan et al., 1988; Khrestchatisky et al., 1989; Lolait et al., 1989; Pritchett et al., 1989; Schofield et al., 1989; Shivers et al., 1989; Ymer et al., 1989a; Ymer et al., 1989b; Kato, 1990; Luddens et al., 1990; Malherbe et al., 1990a; Malherbe et al., 1990b; Pritchett and Seeburg, 1990; Sommer et al., 1990; Ymer et al., 1990; Bateson et al., 1991; Cutting et al., 1991; Keir et al., 1991; Kofuji et al., 1991; Wilson-Shaw et al., 1991; Olsen and Sieghart, 2008). The ρ subunit makes a homo pentamer and is highly expressed in the retina. This GABA receptor used to be called $GABA_C$ receptor. Recently it was re-classified and became part of the subclass of the $GABA_A$ receptor (Barnard et al., 1998).

The majority of the $GABA_A$ receptors that are expressed in the brain are composed of two αs, two βs, and one γ, δ, ε, θ, or π, and α1β2γ2-configuration of the $GABA_A$ receptor is the most common form in the brain. The $GABA_A$ receptor subunits show unique regional distribution. The expression depends on the neuronal type and subcellular localization of the receptor (McKernan and Whiting, 1996; Rudolph and Mohler, 2004). For example, α1 subunit is expressed throughout the brain while α6 subunit is expressed extensively in cerebellar granule

cells (Kato, 1990). About half of α subunits expressed in cerebellum is α6 (Nusser et al., 1999). The mRNAs for α1, β2, and γ2 subunits are co-localized in many brain regions such as olfactory bulb mitral cells, hippocampal pyramidal cells, and cerebellar Purkinje cells (Shivers et al., 1989) while the δ subunit mRNA is found at cerebellar granule cells.

Binding sites for various ligands that modulate the $GABA_A$ receptor are located between the subunits. GABA binding site is located between α and β subunit while the modulator of the $GABA_A$ receptor, benzodiazepine, binding site is between α and γ subunit. The composition of the subunits changes its pharmacological properties (Allan et al., 1988). Benzodiazepines are often used to characterize the different $GABA_A$ receptor subunit properties. $GABA_A$ receptors composed of α1, α2, α3, or α5 subunits together with β and γ subunits are benzodiazepine-sensitive (Rudolph and Mohler, 2004). They are located at the synaptic cleft where the presynaptic GABAergic neurons release the GABA and mediate most of the phasic inhibition in the brain, with exception of α5 (Brunig et al., 2002). Not only is phasic inhibition through synaptic $GABA_A$ receptors important in the brain but also, the tonic form of inhibition that occurs through extrasynaptic $GABA_A$ receptors activated by low concentrations of ambient GABA (Farrant and Nusser, 2005). The $GABA_A$ receptors containing α4 or α6 subunits together with β and δ subunits are located predominantly at the extrasynaptic site, but there are some at the presynaptic site (Draguhn et al., 2008). These forms of the $GABA_A$ receptor mediate tonic inhibition and are insensitive to benzodiazepine modulation (Brunig et al., 2002). Consistent with this role, there is growing evidence that the decrease in δ subunit containing $GABA_A$ receptors during pregnancy is a compensatory mechanism to overcome high level of neurosteroids (Maguire et al., 2009). Therefore, specific subunit composition of the $GABA_A$ receptor determines the receptor's characteristics (Olsen et al., 1990; McKernan and Whiting,

1996). A single subunit can form a functional Cl^- channel (homomer); however, those channels are much less functional and assembled inefficiently (Blair et al., 1988). It is unlikely that any functional receptor subtypes *in vivo* are formed this way (Deng et al., 1991) with the exception of the homo pentamer composed of ρ subunits in retina.

$GABA_A$ receptors are assembled from their component subunits in the endoplasmic reticulum (ER) and expressed on the neuronal cell surface. The expression of some subunits, quite interestingly, can be influenced by others. For example, disruption of α6 subunit expression leads to disappearance of the δ subunit in the cerebellum. In addition, γ2, β2, β3 subunit expressions are all decreased by the lack of α6 subunit expression (Nusser et al., 1999). Deleting the expression of the δ subunit increases the expression of the γ2 subunit while decreasing the expression of the α4 subunit (Korpi et al., 2002). This indicates that modification of the expression of the δ subunit can dictate whether α4- or α6-containing $GABA_A$ receptors are formed. The α1 subunit also plays an important role in the surface expression of the $GABA_A$ receptor (Maljevic et al., 2006). For example, when the α1 subunit was mutated, the assembled $GABA_A$ receptor was not expressed at the cell surface.

The Functional Regulation of The $GABA_A$ receptor

Neurosteroid Regulation of $GABA_A$ receptor

Several steroidal hormones, termed neurosteroids, allosterically modulate the activity of $GABA_A$ receptor. Endogenous steroid hormones are synthesized in the periphery as well as in the CNS locally (Belelli and Lambert, 2005; Mukai et al., 2006; Herd et al., 2007). Because of the lipophilic nature of the steroidal hormones, those produced in the periphery can cross the blood-brain barrier and act on the CNS. Traditionally the actions of the steroid hormones are genomic, which implies that the consequences of these hormones are exerted through the

regulation of gene transcription. Briefly, the dogmatic view of steroid hormone action is as follows: The steroid hormones bind to the cytosolic receptors. This results in a conformational change and dissociation from their chaperone proteins. The ligand-bound receptors then translocate to the nucleus, dimerize and subsequently bind to a sequence of DNA within the promoter region of the target gene, called the steroid response element. This genomic action of steroidal hormones requires hours to days (Tsai et al., 1994).

However, steroidal hormones can also induce intracellular signaling. This "non-genomic" action is thought to be initiated at the plasma membrane, either through classical hormone receptors that can associate with the plasma membrane, or a relatively recently defined class of membrane steroid hormone receptors that are integral membrane proteins. For example, with respect to progesterone, a membrane progesterone receptor was recently cloned that is predicted to have a 7-transmembrane spanning domain (Zhu et al., 2003). Although the membrane steroid hormone receptor would predict a faster action, at least when compared to the classical actions, non-genomic actions still requires a few minutes at least. Alternatively, certain steroidal hormones can produce immediate changes in neuronal excitability. This time scale is much shorter than the genomic actions and/or non-genomic cell signaling involving events. The action of the steroidal general anesthetic agent, 5α-pregnane-3α-ol-11,20-dione (alphaxalone), is $GABA_A$ receptor mediated (Harrison and Simmonds, 1984).

The endogenous steroid hormones that modulate $GABA_A$ receptor function include the progesterone metabolite, 5α-pregnan-3α-ol-20-one (allopregnanolone), , 5β-pregnan-3α-ol-20-one, and the deoxycorticosterone metabolite, 5α-pregnan-3α,21-diol-20-one (Crawley et al., 1986; Majewska et al., 1986; Belelli et al., 1990; Lundgren et al., 2003). In addition, submicromolar to micromolar concentrations of allopregnanolone can activate the $GABA_A$

receptor by itself (Majewska et al., 1986; Callachan et al., 1987; Rodriguez Gil et al., 2002). Allopregnanolone exhibits its allosteric modulation of $GABA_A$ receptor at low nanomolar concentrations (Fodor et al., 2005). It promotes the open state of the GABA-gated chloride channel (Callachan et al., 1987). The sensitivity of the $GABA_A$ receptor to the neuroactive steroids is determined by the stoichiometry of the subunit composition. α2-, α4-, and α5-containing $GABA_A$ receptors are less sensitive to allopregnanolone when compared to α1-, α3-, or α6-containing receptors with β1γ2 subunits. α6-containing receptor showed the maximal effect of the progesterone metabolite. β subunits do not have much of the influence in the sensitivity. γ2-containing receptor shows the most effect while γ1-containing receptor gives the least effect of the steroid. The $GABA_A$ receptor that contains the α4β3δ subunits shows much stronger modulatory effect of the allopregnanolone than the one where the δ subunit is replaced by the γ subunit (Belelli et al., 2002). The subcellular distribution of different subunits may be another degree of $GABA_A$ receptor regulation for the influence of neurosteroids on neuroexcitability.

Neuroactive steroid levels are modulated under various physiological and pathological conditions, such as stress, the menstrual cycle, pregnancy, and aging (Purdy et al., 1991; Paul and Purdy, 1992; Concas et al., 1998; Schumacher et al., 2003; Maguire et al., 2009). Other factors also influence the endogenous level of allopregnanolone. These include, for example, ethanol, whose intake increases the levels of allopregnanolone in mice and rats (Finn et al., 2004; Sanna et al., 2004). Some antidepressants, such as fluoxetine, also increase the level of allopregnanolone without influencing other steroidal hormone levels (Uzunova et al., 1998).

Due to the potentiating effect on the $GABA_A$ receptor, the level of the progesterone metabolites are thought to be involved in the cause of hormonal associated neuropsychiatric

disorders, such as postpartum depression, premenstrual dysphoric disorder, and a neurological disorder called catamenial epilepsy (Nappi et al., 2001; Klatzkin et al., 2006; Reddy and Rogawski, 2009). Accordingly, restoration of deficits in these disorders has been considered as a therapy. For example, treatment of catamenial epilepsy with progesterone during the luteal phase appears to be effective (Herzog, 2009). Although the anti epileptic effect may be through the metabolite of progesterone, allopregnanolone, since the seizure frequency increases when finasteride, 5α-reductase inhibitor, is administered, which inhibits the metabolism of progesterone to allopregnanolone (Herzog and Frye, 2003).

$GABA_A$ receptors and their phospho-regulation

$GABA_A$ receptors are heteropentamers and contain a large N-terminal extracellular domain followed by four TM domains with a large cytoplasmic loop between TM3 and TM4. This intracellular loop is a major interaction site for scaffold proteins and various protein kinases (both serine/threonine kinases and tyrosine kinases) (Ortells and Lunt, 1995). As implied by the interaction of various signaling proteins with the $GABA_A$ receptor, the functions of the $GABA_A$ receptors are indeed regulated by phosphorylation of the intracellular domain. The serine (Ser), threonine (Thr), and tyrosine (Tyr) residues of intracellular loop domains of the β and γ subunits are especially known to be targeted by a number of protein kinases. Those protein kinases include protein kinase C (PKC), protein kinase A (PKA), cGMP-dependent protein kinase (PKG), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), Akt, and Src (Browning et al., 1990; McDonald and Moss, 1994; Brandon et al., 2001; Wang et al., 2003).

β subunits are phosphorylated on conserved serine residues either at Ser409 or Ser410 by PKC, PKA, Ca^{2+}/calmodulin-dependent protein kinase (Moss et al., 1992a; Moss et al., 1992b; Krishek et al., 1994; McDonald and Moss, 1997). Ser408 of β3 subunit is also PKA substrate

(McDonald and Moss, 1997). γ subunits are also known to be phosphorylated by PKA, PKC and tyrosine kinase SRC (Kirkness et al., 1989; Browning et al., 1990; Krishek et al., 1994). In addition, both the γ_2S and γ_2L are phosphorylated by PKC at Ser327, and γ_2L is also phosphorylated at Ser343 by PKC (Moss et al., 1992a). Src phosphorylates Tyr365 and Tyr367 of the γ2 subunit (Moss et al., 1995; Valenzuela et al., 1995). Interestingly while PKA reduces the amplitude of miniature inhibitory postsynaptic current (mIPSC) in CA1neurons (Poisbeau et al., 1999), chronic activation of the PKA leads to the increase in surface expression of the $GABA_A$ receptors in cerebellar granule cells (Ives et al., 2002). In addition, while PKA increases the α1 subunit surface expression, α6 subunit is not affected. However, transiently transfected cells with the α1β1γ2s configuration of the $GABA_A$ receptor shows PKA-induced inhibition of GABA-gated current (Moss et al., 1992a; Moss et al., 1992b; Angelotti et al., 1993). The results of the PKA-induced phosphorylation appear to be cell system dependent. PKC phosphorylates the same β1 serine residue as PKA, and it shows the inhibitory effect on GABA-gated current. Both γ2S and γ2L subunits are phosphorylated by PKC at position 307 of their intracellular loop. The γ2L subunit contains additional phosphorylation site at 343.

Among the consequences of $GABA_A$ receptor phosphorylation is its influence on the sensitivity to ligand. For example, the sensitivity of ethanol is dependent on the phosphorylation of γ subunit by PKC (Wafford and Whiting, 1992). This PKC induced phosphorylation of the $GABA_A$ receptor leads to the internalization of the receptor. Subsequently the decreased receptor density results in the inhibition of the GABA-gated current (Herring et al., 2005). This PKC-induced inhibition of the GABA-gated current appears to be temperature dependent, which gives the additional evidence that the PKC induced inhibition due to the internalization of the receptor (Machu et al., 2006). Interestingly, in dentate granule cells, PKC shows seemingly an

opposite effect. In these cells, PKC increased the amplitude of mIPSCs (Poisbeau et al., 1999). Therefore, as seen for PKA, the effects of PKC on the regulation of the $GABA_A$ receptors might also be cell system dependent.

In contrast to the PKC-induced enhancement of GABAA receptor function, activation of the Akt/PI3K pathway has been reported to have the opposite effect. For example, activation of the Akt/PI3K pathway by insulin results in the phosphorylation of the β subunit of the $GABA_A$ receptor, leading to increased GABA-gated currents through rapid translocation of $GABA_A$ receptors from intracellular compartments to the plasma membrane (Wan et al., 1997; Wang et al., 2003; Vetiska et al., 2007). Interestingly, when the Akt/PI3K and the ERK/MAPK pathways are activated simultaneously by brain derived neurotrophic factor (BDNF) (Bhave et al., 1999), the inhibitory effect on $GABA_A$ receptor function prevails instead of strengthening the inhibitory synaptic activity. This effect of BDNF appears to be mediated by the reduction in surface expression of $GABA_A$ receptors, mIPSCs, and GABA-gated currents (Brunig et al., 2001; Mizoguchi et al., 2003). So far, all known phosphorylation-dependent regulation of $GABA_A$ receptor function involve β and γ subunits; but little to none is known about the phosphorylation of α subunits and its consequent effects on $GABA_A$ receptor function.

ERK/MAPK pathways on $GABA_A$ receptors

Estrogen, progesterone, BDNF, and insulin have something in common; they all induce the activation of the ERK/MAPK intracellular signaling pathways (Dudek et al., 1997; Gunn-Moore et al., 1997; Bhave et al., 1999; Herbert et al., 2000; Singh, 2001; Zhu et al., 2002; Kotzka et al., 2004; Mannella and Brinton, 2006). Our previous studies suggested that the ERK/MAPK pathway was involved in inhibitory regulation of GABA-gated currents in HEK-t cell system, and identified the Thr 375 residue of the $GABA_A$ receptor α1 subunit to be involved

in this regulation (Bell-Horner et al., 2006). Whether the activation of the ERK/MAPK pathway translates to decreased GABA-gated currents has not been studied. Recently it was found that the ERK/MAPK pathway directly targets a different class of ion channel, the Kv4.2 potassium channel (Schrader et al., 2006). With this precedent, I suggested that $GABA_A$ receptor is phospho-regulated by the same way, with the Thr 375 as the relevant target within the $\alpha 1$ subunit.

The ERK/MAPK pathway

MAPK cascades are a family of protein kinases containing at least three protein kinases in series: MAPK kinase kinases (MAPKKKs), MAPK kinases (MAPKKs or MEKs), and MAPKs, such as extracellular-signal regulated kinase (ERK) 1 and 2. MAPKs are involved in the regulation of numerous cellular processes, including proliferation, differentiation, and cell death (Pearson et al., 2001). This cascade normally starts from a molecule (such as a growth factor) binding to its receptor, such as a receptor tyrosine kinase (RTK), at the plasma membrane. Stimulation of these receptors results in autophosphorylation on tyrosine residues within intracellular domains of the receptors, enabling the binding of scaffolding proteins such as the Grb2:Sos complex via their SH2 domains. Sos is a guanine nucleotide exchange factor (GEF), which facilitates the shift from GDP-bound Ras to GTP-bound Ras. Ras-GTP recruits Raf to the plasma membrane, and thereby promotes its activation. Activated Raf phosphorylates and activates MEK, which in turn, phosphorylates ERK, and ERK gets activated. The activated ERK is involved in the regulation of proliferation, survival, and differentiation (Baccarini, 2005).

ERK1 and 2 are 90% identical and ubiquitously expressed serine/threonine kinases that have molecular weight of 44 kDa and 42 kDa respectively (Boulton et al., 1990). They contain a threonine-glutamate-tyrosine (TEY) dual phosphorylation motif in the activation loop and both T

and Y have to be phosphorylated by MEK1 and 2 in order to become fully active. ERK1/2 often interact with other proteins through specific protein-binding motifs. One such sequence is (R/K)X(R/K)X_{2-4}(L/I)X(L/I), named the docking site for ERK and JNK with LXL motif (Docking site for ERK/JNK with dileucine (DEJL) motif or D domain). Substrates of ERK1/2 also contain a consensus phosphorylation sequence, P-x-S/T-P or minimal phosphorylation sequence, S/T-P (Pearson et al., 2001). Another member of the MAPK family, ERK5, is a 98 kDa molecule that shares some sequence homology with ERK 1 and 2 including the TEY motif (Lee et al., 1995). The catalytic domain of ERK5 is reported to be highly homologous to ERK 2 (Kasler et al., 2000). ERK5 is involved in embryonic development, but some level of ERK5 is also detected in adult brain of (Regan et al., 2002; Yan et al., 2003). ERK5 is phosphorylated and activated by MEK5, and the MEK5-ERK5 interaction is highly specific (Zhou et al., 1995). It was found that the specific MEK1/2 inhibitor, U0126, also inhibits ERK5 (Mody et al., 2001) presumably due to the homology shared in MEK1 and MEK5 (Zhou et al., 1995; Mody et al., 2001). Therefore, the data generated in this dissertation, using U0126, will need to be re-evaluated for the involvement of ERK5.

Goal of this study

The study here was built on, and is a continuation of, previous findings resulting from the collaboration of the Singh and Dillon labs. These prior studies suggested that the ERK/MAPK pathway was involved in the negative regulation of the $GABA_A$ receptor function through either trafficking or gating of the receptor, and that the Thr 375 amino acid residue of α1 subunit might be the direct target of the ERK/MAPK pathway. By focusing on the $\alpha1\beta2\gamma2$ subunit configuration of the $GABA_A$ receptor, being the predominant form expressed in the mature vertebrate brain, this body of work was expected to further our understanding of the

mechanism(s) by which this configuration of the $GABA_A$ receptor function is regulated by the ERK/MAPK pathway. This in turn, was expected to increase our knowledge of how the primary mode of inhibitory neurotransmission in our brain is regulated. The information learned from this study may then be applied to the disorders involving $GABA_A$ receptor, including depression, anxiety, drug abuse, epilepsy, and more.

Clinical significance

As stated previously, regulation (or dysregulation) of the $GABA_A$ receptor, may underlie, at least in part, certain mood disorders. Among these is the Premenstrual Syndrome (PMS). PMS is experienced by 80% of women. Among these women, 5-7% exhibit more severe symptoms, and based on DMS-IV criteria, are diagnosed with premenstrual dysphoric disorder (PMDD) (Campbell et al., 1997; Hylan et al., 1999). Behavioral hallmarks of these syndromes include anxiety and depression (Halbreich, 2003). Since the symptoms of PMS and PMDD are generally noted during only specific periods of the menstrual cycle (the luteal phase), fluctuations in gonadal hormones, particularly estrogen and progesterone, have been implicated in the etiology of these affective disorders. However, the mechanism by which these hormones influence anxiety and depression is less clear. Among the possible mechanistic targets that may be involved is the $GABA_A$ receptor (Nutt and Malizia, 2001). Given that the $GABA_A$ receptor is linked to the development of anxiety and depression, it is possible that the fluctuations in estrogen and progesterone may influence the behavioral symptoms of PMS and PMDD through its actions on the $GABA_A$ receptor (Kalueff, 2007).

Another potentially relevant connection between estrogen, progesterone and the $GABA_A$ receptor is based on the following observations. Both estrogen and progesterone are known to increase the level of brain-derived neurotrophic factor (BDNF) and insulin (Costrini and

Kalkhoff, 1971; Gibbs, 1999; Karege et al., 2002; Shirayama et al., 2002; Karege et al., 2005; Lommatzsch et al., 2006; Gonzalez Deniselle et al., 2007). Both BDNF and insulin are known to be involved in affective disorders, and recently, it has been reported that BDNF is a negative modulator of $GABA_A$ receptor function, while insulin is a positive modulator (Wan et al., 1997; Cheng and Yeh, 2003; Wang et al., 2003; Vetiska et al., 2007). Thus, the effects of estrogen or progesterone on the $GABA_A$ receptor (and thus, on anxiety and/or depression) may be mediated by these factors. Alternatively, as discussed below, the cellular mechanisms triggered by BDNF and insulin may be the key mediators of the effects of estrogen and progesterone on mood, and will be explored through the research proposed.

Understanding how the $GABA_A$ receptor functions and is regulated by each signaling pathway will substantially increase our understanding of the complexities by which $GABA_A$ receptors function. Importantly, it may reveal new insight into how hormones, such as estrogen and progesterone, regulate $GABA_A$ receptors, since estrogen and progesterone can elicit the ERK/MAPK pathways. Thus, we expect to provide a link between hormone-induced signaling and signaling-induced regulation of $GABA_A$ receptor function, so as to reveal possible mechanisms to explain such affective disorders as PMS and PMDD, and hopefully provide the opportunity for improving the quality of life for those women who suffer from these disorders.

CHAPTER 2

MATERIALS AND METHODS

Cell culture

Stably and transiently transfected cell lines were used in this study. Human embryonic kidney cells (HEK-t) and 4D4 cells (HEK-t cells stably expressing recombinant rat α1, β2, γ2s subunit configuration of $GABA_A$ receptors) were studied in this investigation. Both HEK-t cells and 4D4 cells were generously provided by Dr. Dillon at UNTHSC. They were both propagated in minimum essential medium (MEM) (Invitrogen) supplemented with 10% fetal bovine serum (SAFC biosciences), L-glutamine (Mediatech, Inc.) and penicillin-streptomycin solution (Mediatech, Inc.), and maintained at 37°C in a humidified environment containing 5% CO_2. In addition to the media above, 4D4 cell media also contained G-418 sulfate (EMD Chemicals Inc.). After various treatments, the cells were harvested, homogenized, and centrifuged, and the supernatant was subsequently collected and analyzed for total protein concentration using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Inc.). Alternatively, they were processed for flow cytometry.

Transient transfection

Rat wild type $GABA_A$ receptor subunit β2, γ2, and FLAG-tagged rat α1 subunit were generously provided by Cynthia Czajkowski (University of Wisconcin, Madison), and were subcloned into pcDNA3.1. The FLAG-tag located at the N-terminal of the α1 subunit. The HEK-t cells were plated on day 1, transfected on day 3, and experiments were conducted on those cells on day 4. For immunoprecipitation and Western blot, 1 million HEK-t cells were plated in 100

mm plate, and 2 μg of FLAG-α1, 2 μg of β2, and 10 μg of γ2 subunits are used. For flow cytometry, 50,000 HEK-t cells were placed in 60 mm plate, and half of the amount of subunits DNA was used for transient transfection. Transient expression of $GABA_A$ receptors was accomplished using TransIT (Mirus Bio LLC) following the manufacture's instruction.

Chemicals

U0126 (Cell Signaling Technology, Inc.), U0124 (Cell Signaling Technology, Inc.), phorbol-12-myristate-13-acetate (PMA; Calbiochem), okadaic acid (Alexis Biochemicals), calyculin A (Cell Signaling Technology, Inc.), gramicidin (Sigma-Aldrich), diazepam (Sigma-Aldorich) were dissolved in sterile dimethyl sulfoxide (DMSO; Sigma-Aldrich). Hepatocyte growth factor (HGF; Chemicon) and GABA (Sigma-Aldrich) were prepared in sterilized water.

Treatment of cultures

HEK-t cells and 4D4 cells were treated with various pharmacological reagents and a growth factor: 10 μM U0126 – inhibition of the ERK/MAPK pathway, 25 and 100 nM PMA – PKC pathway activation, 100 nM okadaic acid – phosphatase inhibitor, 10 nM calyculin A – phosphatase inhibitor, 10 pg/mL – 50 ng/mL HGF – ERK/MAPK pathway activator, 5 μM GABA – $GABA_A$ receptor ligand. Final concentration of 0.1% DMSO was used.

Harvesting cells

Cells were washed with ice-cold PBS and immersed into a lysis buffer containing a final concentration of 50 mM pH 7.4 Tris base, 150 mM sodium chloride, 1 mM pH 8.0 ethylene glycol tetraacetic acid (EGTA), 1 mM pH 10.0 sodium orthovanadate, 100 mM sodium fluoride, 5 μM zinc chloride, 10% glycerol, 1% Triton X-100, 1 mM phenylmethanesulfonylfluoride (PMSF) and protease inhibitor cocktail containing ABESF, aprotinin, leupeptin, bestatin, pepstatin A, and E-64. For immunoprecipitation, Triton X-100 was replaced by NP-40. For 100

mm petri dishes, the cells were washed with ice cold phosphate buffered saline (PBS) and lysed with 500 μL lysis buffer above. The force of volume expulsion through the pipette was used to lift the cells off of the plates. The cell – lysis buffer mixture was transferred to a microcentrifuge tube. The mixture was homogenized through triturating them using a sterile 22 gauge needle and the force from the syringe. The mixture was centrifuged at 100,000 × *g* for 15 min at 4°C, and the resulting supernatants (lysates) were normalized for protein content using Lowry Protein Assay (Bio-Rad Laboratories, Inc.) according the manufacture's protocol.

SDS-PAGE and Western blot analysis

After the protein concentration was determined by the protein assay, 80 μg of protein was transferred to a new microcentrifuge tube. After the 4× Laemill buffer containing Tris HCl, glycerol, sodium dodecyl sulfate, ethylene diamine tetraacetic acid (EDTA), EGTA, and bromophenol blue was added to the cell lysate and denatured by boiling them at 100°C, the lysate was loaded onto a sodium dodecyl sulfate 10% polyacrylamide gel (SDS-PAGE), subjected to electrophoresis, and subsequently transferred onto a 0.22 μm pore size polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Inc.) overnight. The membrane was blocked with Tris-buffered saline solution containing 0.2% Tween (TBS-t) and 3% bovine serum albumin (BSA) before application of the primary antibody. The blots were probed with various antibodies (Rb = rabbit, Gt = goat, Ms = mouse, HRP = horse radish peroxidase conjugated).

Primary Ab	Brand	Secondary Ab	Brand
p-ERK 1/2	Cell Signaling	Gt anti-Rb-HRP	Pierce
ERK1(C-16)-G	Santa Cruz	Rb anti-Gt-HRP	Pierce
ERK2(C-14)-G	Santa Cruz	Rb anti-Gt-HRP	Pierce
p-Akt	Cell Signaling	Gt anti-Rb-HRP	Pierce
Akt	Cell Signaling	Gt anti-Rb-HRP	Pierce
p-ERK5	Cell Signaling	Gt anti-Rb-HRP	Pierce
actin (I-19)	Cell Signaling	Rb anti-Gt-HRP	Pierce
pan actin Ab-5	Neomarkers	Gt anti-Ms-HRP	Pierce
alpha1-6	Santa Cruz	donkey anti-Rb	Pierce
FLAG-tag	Sigma	Rb True Blot anti-Rb IgG	eBioscience
p-Thr-Pro	Cell Signaling	Gt anti-Ms IgM	Jackson Immunoresearch
p-Thr	Cell Signaling	Rb True Blot anti-Rb IgG	eBioscience

Antibody binding to its target on the membrane was detected using a secondary antibody conjugated to horseradish peroxidase (HRP; Pierce Chemical Co.) and visualized using Westpico chemiluminescence (Pierce Chemical Co.) with the UVP imaging system (UVP, LLC) and Alpha Innotech imaging system (Alpha Innotech Corporation). The membrane was then stripped with Re-blot mild or strong solution (Chemicon) at room temperature for 30 min and washed with TBS-t extensively. After stripping, the blot was blocked with either 3% BSA or 5% skim milk, the blot was re-probed with appropriate controls to confirm equal loading of protein across lanes. p-Akt, p-ERK, p-Thr-Pro, and p-Thr blots were re-probed with anti-ERK1/2, anti-actin, or anti-FLAG antibodies to ensure equal loading the samples across lanes.

Immunoprecipitation

The HEK-t cells that were transfected and treated with various reagents were harvested with lysis buffer that contained Nonidet P-40 instead of triton X-100 in order to reduce the stringent nature of triton X-100. After centrifugation and protein assay, resulting lysates (at a concentration between 1-5 mg/mL) were incubated with the anti-FLAG antibody that was conjugated to agarose gel (EZview Red Anti-FLAG M affinity gel; Sigma-Aldrich). The mixture was kept at 4°C overnight on a nutator for the maximal binding. After the mixture was

centrifuged, they were analyzed through SDS-PAGE and Western blot to detect p-Thr-Pro, p-Thr, ERK1/2, or p-ERK1/2 immunoreactivity. An Anti-FLAG antibody was used to detect the FLAG-tagged α1 subunit at the molecular weight of 51 kDa to confirm the expression of the $GABA_A$ receptor in HEK-t cells and to ensure the equal loading the samples.

Flow cytometric analysis

Everything after the treatment of cells was conducted either on ice or at 4°C, and all buffers were kept on ice. 4D4 or HEK-t cells were treated with HGF, DMSO, U0126, or Phorbol-12-myristate-13-acetate (PMA; EMD Chemicals Inc.) for the appropriate period of time. To collect cells for flow cytometry analysis, monolayer cultures of HEK-t cells were dissociated using 0.05 % trypsin EDTA (Cellgro) for 30 seconds at room temperature. The cells were collected, centrifuged, and re-suspended in 1 mL staining buffer (BD Biosciences), which contains 0.2% bovine serum albumin. The use of trypsin as a suitable cell detaching agent that preserves the FLAG-tag that is located at the extracellular N-terminal domain was confirmed in other studies (Lo et al., 2008). Following washes with the staining buffer, cells were counted with automatic cell counter, Cellometer Auto T4 Plus (Nexelom Bioscience LLC) 300,000 cells were then blocked with blocking buffer (staining buffer containing 2 % goat serum) at 4°C on a nutator for an hour to reduce the non-specific binding sites for the antibodies. After a wash, the cells were incubated with antibody. The cells were incubated with anti-FLAG IgG directly conjugated to FITC (Sigma-Aldrich) in incubation buffer (staining buffer containing 0.8% goat serum) for 1hr at 4°C on a nutator. From each sample, 50,000 labeled cells were counted with Accuri C6 flow cytometer (Accuri cytometers Inc.) and analyzed. Nonviable cells were excluded from analysis based on forward and side scatter profiles. Data were analyzed off line using FlowJo 7.1 (TreeStar, Inc.), and the mean fluorescent intensity was calculated. The

fluorescence index of each experimental condition was then normalized to each control condition for comparison. One-way analysis of variance with Dunnett's post-test was used to determine whether there were any significant differences in surface expression, as indexed by the mean fluorescent intensity, as a function of pharmacological treatments. Statistical analysis was conducted using Prism 4.0 (Graphpad Software Inc.). The data were expressed as mean ± S.E. For labeling $GABA_A$ receptor β subunit, an additional step had to be taken for the incubation of secondary antibody. After the primary antibody incubation with 1 μg of mouse anti-$GABA_A$ receptor β2/β3 subunit monoclonal antibody (Millipore Corporate) for 1 hour on a nutator, the cells were washed and the re-suspended in 100 μL incubation buffer. The secondary antibody, rabbit anti-mouse IgG-FITC (Sigma-Aldrich), was added to the cells, and the cells were incubated another 1 hour.

Electrophysiology

Prior to plating the 4D4 cells, coverslips were treated and coated with poly-L-lysine (Sigma-Aldrich) dissolved in serum free media and placed in 35 mm culture dishes. 20,000 4D4 cells (α1β2γ2-stably transfected HEK293 cells) were split and plated on the culture dishes with coated coverslips. Cells were analyzed electrophysiologically 2 days after the plating. Whole cell and perforated patch recordings were conducted at room temperature (22-25°C) to assess the GABA-gated Cl^- currents with the membrane potential clamped at -60 mV. Patch pipettes of borosilicate glass (1B150F; Word Precision Instruments, Inc.) were pulled to a desired diameter using pipette puller (Flaming/Brown, P-87/PC; Sutter Instrument Co.) to a diameter of 1-2 μm and a tip resistance of 1-1.25 MΩ. The pipette solution for conventional whole-cell recording contained 140 mM CsCl, 10 mM EGTA-Na^+, 10 mM HEPES-Na^+, 4 mM Mg^{2+}-ATP, pH 7.2. For perforated-patch recording, a stock solution of 4 mg/mL gramicidin (Sigma-Aldrich) in

DMSO was prepared and diluted into the pipette solution above to a final concentration of 2.5, 5, or 10 μg/mL. The pipette solution was kept on ice. Coverslips containing cultured cells were placed in the small recording chamber on the stage of an inverted light microscope (Olympus IMT-2; Olympus) and superfused continuously with external solution containing 125 mM NaCl, 5.5. mM KCl, 0.8 mM $MgCl_2$, 3.0 mM $CaCl_2$, 20 mM HEPES, 10 mM D-glucose, pH 7.3 at a rate of approximately 30 mL/min. GABA-induced Cl^- currents were obtained using Axo-clamp 200A amplifier (Axon Instruments) equipped with a CV-4 headstage. Currents were low-pass filtered at 5 kHz, monitored simultaneously on an oscilloscope and a chart recorder (Gould TA240; Gould Instrument Systems Inc.), and stored on a computer using an on-line data acquisition system (pClamp 6.0; Axon Instruments) for subsequent off-line analysis.

Perforated whole cell patch recording

Perforated patch was established using Gramicidin (Sigma-Aldrich). It was added to the pipette solution in order to make small pores within the patched area. Gramicidin was dissolved into sterile DMSO to make 2mg/mL stock solution, and this stock solution was added into pipette solution. The final concentration of the gramicidin in the pipette was 2.5, 5.0, or 10.0 μg/mL depending on the experiments. Two consecutive stable control currents within 5% difference to ensure the patch is stable. HGF was bath-applied for the duration of 10-20 min during which a 5 sec pulse of 5μM GABA (at the EC_{30}) was applied at 2 min intervals. The EC_{30} of GABA was used in order to minimize desensitization of the receptors. The cells were then washed out with N-saline and the currents were recorded every 5 min after discontinuing the treatments.

Statistical analysis

For electrophysiological analysis for perforated patch clamp and conventional whole cell patch clamp), concentration-response profiles for GABA were analyzed using the following logistic equation from Origin 5.0 (Origin Lab).

$$I/I_{max} = 1/(1+EC_{50}/[ligand])^{n}_{H}$$

Where I_{max} = maximum current amplitude, EC_{50} = the half-maximal effective concentration, [ligand] = concentration of GABA in μM, and H = the Hill coefficient. Means ± s.e.m. were calculated for all recorded variables. Analysis of group differences was conducted using an ANOVA. Differences were accepted as significant if the p value was less than 0.05. Statistical analysis was performed using GraphPad Prism 4.

CHAPTER 3

RESULTS

The $GABA_A$ receptors are a heteropentameric ligand gated ion channel. Most of these receptors in mammalian brain are composed of two αs, two βs, and one γ subunit. Each subunit has a large extracellular N-terminal where the GABA binding site is located, four transmembrane domains, and a large intracellular domain between TM3 and TM4. This intracellular domain is often a target for numerous protein kinases. The resulting phosphorylation of the $GABA_A$ receptor results, in turn, in the regulation of the receptor function. For example, sIPSC and the receptor density at cell surface are affected by the phospho-regulations. While most studies that have addressed the role of phosphorylation on $GABA_A$ receptor function has focused on β and γ subunits, consideration of the α subunit as a relevant target in the regulation has not been well studied.

Sequence analysis of the α subunits shows that all but one (the α5 subunit) of the rat α subunits contain either full (P-x-[S/T]-P) or minimal ([S/T]-P) consensus phosphorylation site for ERK1/2 and ERK5. In addition, all of the human α subunits contain the sites. These consensus sequences were not observed in the β and γ subunits.

The ERK/MAPK pathway is one of many cell signal transduction pathways. This pathway is involved in regulations of gene transcription, cell survival, and even cell death depending on the duration of the pathway activation. Recently a report showed that the ERK

modulated potassium channels by direct phosphorylation (Schrader et al., 2006), supporting the potential importance of the ERK/MAPK pathway in regulating ion channel function. As it relates to my studies, it is worth noting that the effect of the ERK/MAPK pathway activity on $GABA_A$ receptor function has not been studied thoroughly.

Steroidal hormones and growth factors such as estrogen, progesterone, testosterone, insulin, and BDNF are known to activate the ERK/MAPK pathway. In this pathway, extracellular signal received at receptors on a cell surface is transduced to activate Ras, a small guanine nucleotide exchange protein. A cascade of the kinase activations follows the activation of Ras, which then leads to the activation of Raf, a serine/threonine kinase, followed by the activation of MEK, a mitogen-activated ERK-activating kinase. MEK activation leads to the activation and phosphorylation of the major effector of this pathway, extracellular-signal regulated kinase (ERK).

Brief Summary of the Previous Study

My dissertation is greatly based on work conducted as a collaboration between the laboratories of Dr. Dillon and Dr. Singh, in which I participated. This section is devoted to summarize the findings of the previous work (Bell-Horner et al., 2006). The relationship between the ERK/MAPK pathway and $GABA_A$ receptor function was studied. $\alpha1\beta2\gamma2$ configuration of the $GABA_A$ receptor was studied since this composition was the most abundantly (~60%) and ubiquitously expressed form of $GABA_A$ receptor in mature mammalian brain. Electrophysiological studies were conducted to evaluate the effect of ERK/MAPK pathway inhibition, using pharmacological MEK1/2 and MEK5 inhibitor, U0126, and another inhibitor, PD98059, on the peak amplitude of the GABA-gated current. The perforated patch clamp technique, rather than whole cell patch clamp, was used since the perforated patch clamp

methodology preserves the intracellular signaling molecules whereas conventional whole cell patch dialyses out the intracellular milieu. Ten minutes of 10 μM U0126 or 50 μM PD98059 incubation increased the peak amplitude of the GABA-gated current when perforated patch technique was used (Figure 1 A & B). Co-application of U0126 with GABA did not show any effect, which illustrated that the effect was not due to the direct binding of the U0126 to the $GABA_A$ receptor. In order to see if this potentiation effect of ERK/MAPK pathway inhibitor on GABA-gated current was specific to inhibition of the kinase activity of MEK and ERK, an inactive analog of U0124 was used. U0124 binds to the MEK but does not inhibit its kinase activity. U0124 showed no effect on the GABA-gated current, which strongly supported the involvement of the ERK/MAPK pathway in the regulation of the $GABA_A$ receptor function (Figure 2). In addition, the potentiation effect of U0126 disappeared when the conventional whole cell patch technique was applied (Figure 3 A & B). This indicated that the U0126 effect required the intact intracellular environment, presumably since the signaling molecules were dialyzed into the patched pipette. The MEK inhibitory effect of U0126 was confirmed through Western blot analysis (Figure 4).

In order to test the importance of the putative ERK phosphorylation site within α1 subunit, Thr 375 was mutated to alanine. Consistent with the implied importance of this residue as a target of the ERK/MAPK pathway, application of U0126 onto cells expressing the $GABA_A$ receptor containing the mutated receptor did not show the potentiation effect on GABA-gated currents (Figure 5). Interestingly, not only did U0126 fail to potentiate GABA-gated currents, but now, resulted in an inhibition. This illustrated the Thr 375 of the α1 subunit is part of the mechanism to the regulation of the $GABA_A$ receptor function by the ERK/MAPK pathway. My

experiments explored the role of the ERK/MAPK pathway in the regulation of the $GABA_A$ receptor function further.

Based on the data cited above, my hypothesis was activation of the ERK/MAPK pathway would directly target the Thr 375 within the α1 subunit. The phosphorylation of the Thr 375 would lead to the internalization of the $GABA_A$ receptor. Subsequently it would decrease the $GABA_A$ receptor density at cell surface and inhibit the GABA-gated current.

The experimental model

To be consistent, the α1β2γ2-configuration of $GABA_A$ receptors was used. HEK-t cells stably transfected with rat α1β2γ2-configuration of $GABA_A$ receptor (4D4 cells) were used in the investigation as it was used previously. These stably-transfected HEK-t cells represent a useful model to study the inter-relationship between the ERK/MAPK pathway and the $GABA_A$ receptor. HEK-t cells transiently transfected with the eight amino acid FLAG-tagged (DYKDDDDK) rat α1 along with β2 and γ2-configuration of $GABA_A$ receptor were also used for immunoprecipitation and flow cytometry experiments, with the latter used specifically to investigate the receptor density on the cell surface. Even though HEK-t cells are not neuronal in origin, they provide a valuable model for which to study the regulation of the $GABA_A$ receptor.

The importance of the putative ERK phosphorylation site (Thr 375) in α1 subunits on ERK/MAPK pathway-regulated $GABA_A$ receptor function

The α subunits of $GABA_A$ receptor contain a consensus ERK phosphorylation sequence within its large intracellular domain. The α1 subunit, the most ubiquitously expressed α subunit in the brain, contains a minimal consensus ERK phosphorylation sequence, Thr 375, followed by proline (Pro). The electrophysiological data in the previous study showed that the α1(T375A) mutant resulted in the opposite effect of U0126 in GABA-gated currents, suggesting the

involvement of the Thr 375 in the regulation of the $GABA_A$ receptor function through the ERK/MAPK pathway (Bell-Horner et al., 2006). The importance of the putative ERK1/2 and/or ERK5 phosphorylation site Thr 375 in the intracellular loop of the α1 subunit was evaluated to see if this site was a direct target of the ERK/MAPK pathway. Using an immunoprecipitation-based strategy and using an anti phospho-threonine-proline (p-Thr-Pro) antibody (one that recognizes only phospho-threonine residues that are next to a proline), the involvement of the Thr 375 residue of α1 subunit in the ERK/MAPK pathway regulation of the $GABA_A$ receptor function. To assess the possibility of Thr 375 being the direct target of the ERK1/2 and/or ERK5, the phosphorylation level of the α1 subunit was evaluated while activating the ERK/MAPK pathway with HGF. Within the intracellular loop of the α1 subunit, there was only 1 site found where Thr was followed by proline (the putative "minimal consensus phosphorylation site" for ERK1/2). Therefore, if there is any change in phosphorylation status based on p-Thr-Pro immunoreactivity, it should be due to the Thr 375 amino acid residue. It is worth pointing out that HEK-t cells transiently transfected with $GABA_A$ receptor rat FLAG-α1, β2, and γ2 subunits were used for this investigation since commercially available anti-α subunit antibody did not show a strong binding to be able to detect the α1 subunit in 4D4 cells (Figure 6).

Determining optimal duration and concentration of MAPK pathway activator

Pharmacological inhibition of the ERK/MAPK pathway by U0126 resulted in the enhancement of GABA-gated currents in α1β2γ2-expressing HEK-t cells (Bell-Horner et al., 2006). This suggested that activity of the ERK/MAPK pathway at basal level was involved in its inhibition of the $GABA_A$ receptor function. Also BDNF, a growth factor that activates the ERK/MAPK pathway (Obara et al., 2007), attenuates GABA-gated currents in granule cells

(Cheng and Yeh, 2003). However, in view of data that suggests that the Akt/PI3K pathway works in opposition to the ERK/MAPK pathway, we sought to identify an factor that would activate the ERK/MAPK pathway without eliciting an increase in the activity of the Akt/PI3K pathway. To this end, we identified HGF as a suitable candidate. HGF elicited the phosphorylation of ERK1/2 but not of Akt (see details below). This ligand would be used extensively throughout my experiments to evaluate the role of the ERK/MAPK pathway on GABA-gated currents.

4D4 cells were treated with HGF, and the ERK/MAPK pathway was activated through the endogenous HGF receptor, Met. Met is a receptor tyrosine kinase (Higuchi et al., 2004) and elicits phosphorylation of ERK1/2 through activation of the ERK/MAPK pathway cascade. Depending on the cell model, HGF has also been reported to activate other pathways. They include the Akt/PI3K pathway (Kakazu et al., 2004), p38 MAPK (Ogihara et al., 2003), and phospholipase C (Okano et al., 1993). In order to determine if the phosphorylation status of the two main MAPK pathway effector kinases, ERK1/2, was regulated by HGF, we treated 4D4 cells with HGF and assessed ERK phosphorylation using Western blot analysis. We also used this method to decide the duration and concentration of the treatment for the subsequent electrophysiology experiments. Initial concentration of HGF tested was based on the literature (Duan et al., 2004). Time course analysis confirmed that 50 ng/mL HGF elicited the phosphorylation of ERK1/2 where maximal phosphorylation of ERK1/2 was noted following 5 minutes of treatment (Figure 7). The phosphorylation level finally declined to nearly basal levels after 2 hours.

The phosphorylation status of Akt was also evaluated since the Akt/PI3K pathway was involved in potentiation of the GABA-gated current by increasing the receptor density of the

$GABA_A$ receptor (Wang et al., 2003). The results showed that the treatment on 4D4 cells with HGF appeared to have no effect on the PI3K pathway effector, Akt (Figure 7). Therefore, HGF was deemed an appropriate ligand for our experiments since HGF treatment led to the activation of the ERK/MAPK pathway without seeing the effect of the Akt/MAPK pathway activation in our experimental model.

In order to determine the optimal concentration of HGF for the activation of the ERK/MAPK pathway, the 4D4 cells were treated with different concentration of HGF, ranging from 10 pg/mL to 10 ng/mL (Figure 8). The duration of the treatment was 5 min. The results showed that 10 ng/mL of HGF induced robust phosphorylation of ERK1/2.

p-Thr-Pro site of the α1 subunit

Transient expression of the rat $GABA_A$ receptor FLAG-α1β2γ2 subunit in HEK-t cells was confirmed through immunoprecipitation (Figure 9). The transiently transfected HEK-t cells were treated with 10 ng/mL HGF for the activation of the ERK/MAPK pathway for 5, 15, or 30 minutes, and the phosphorylation status of the α1 subunit Thr 375 residue was evaluated after immunoprecipitation with FLAG-tag that was put on the α1 subunit. The Western blot analysis showed that there was no phosphorylated Thr-Pro residue within α1 subunit in untreated control (Figure 10 A). The HGF-induced activation of the ERK/MAPK pathway also did not affect the p-Thr-Pro level of the α1 subunit. The increased use of the primary antibody from 1:2,000 to 1:500 showed the same results (Figure 10 B). The same experiment was repeated using increased amount of total protein (from 1 mg to 5 mg) for the immunoprecipitation (Figure 10 C). Although the result showed a band close to the 50 kDa area, it was higher than the predicted size, which suggested that it was not the α1 subunit.

After GABA binds to the $GABA_A$ receptor, the receptor changes its confirmation so that chloride ion influx occurs. The potentiation effect that resulted from the U0126 on GABA-gated current occurred while GABA was applied to the receptor. Therefore, we tested the hypothesis that the direct phosphorylation of the $GABA_A$ receptor by ERK1/2 required a prior conformational change resulting from the binding of GABA to its receptor. GABA was therefore co-applied with HGF for 15 minutes and the Thr 375 residue within $\alpha 1$ subunit was evaluated with the same method. The results showed that any conformational change resulting from the application of GABA did not affect the phosphorylation status of the $GABA_A$ receptor (Figure 10 D). More commonly used anti p-Thr antibody was used to probe any p-Thr within the $\alpha 1$ subunit (Figure 10 E); however, it too did not reveal any signs of phosphorylation of the FLAG-immunoprecipitated $\alpha 1$ subunit.

Phosphorylation status is a balance between kinase and phosphatase activities. As such, we also addressed the possibility that the phosphorylation state of the alpha subunit was very labile, and tested the hypothesis that inhibition of phosphatase activity would allow us to visualize phosphorylation of the alpha subunit more readily. Two different kinds of phosphatase inhibitors were used in attempt to slow down the overall dephosphorylation process by the phosphatases and shift the balance more towards the kinase activity so that the phosphorylated sites would be preserved. First the HEK-t cells transiently expressing $GABA_A$ receptor FLAG-$\alpha 1\beta 2\gamma 2$ subunits were pre-treated with commonly used phosphatase inhibitor, okadaic acid for 20 minutes and the ERK/MAPK pathway was activated with the HGF for 15 minutes. The concentration of the okadaic acid (100 nM) was determined based on the previous reports using HEK-t cells (Zhang et al., 2009). The p-Thr-Pro level was analyzed with Western blot after immunoprecipitation with anti FLAG antibody (Figure 11 A). The blot did not show any p-Thr-

Pro band around 50 kDa. Increasing the starting total protein for the immunoprecipitaion from 2 mg to 5 mg did not show any sign of p-The-Pro band around 50 kDa area, either (Figure 11 B).

Control experiments to assess the inhibitory effects of okadaic acid on phosphatase were assessed through Western blot to ensure it was inhibiting the phosphatase (Figure 11 C). 100 nM okadaic acid effectively increased the HGF-induced phosphorylation of the ERK1/2. Okadaic acid is known to inhibit phosphatase PP2A over PP1. In order to inhibit both PP1 and PP2A, calyculin A was used. The same experiment was repeated replacing okadaic acid with calyculin A. The inhibitory activity of calyculin A on phosphatase was evaluated by Western blot (Figure 12 A). The p-Thr bands in calculi treated cells intensified, which proved the phosphatase-kinase balance shifted towards kinase. Calyculin A was used to inhibit the phosphatases in transiently transfected HEK-t cells, and the procedure was repeated to see if pretreatment with the dual PP2A/PP1 phosphatase inhibitor would allow the visualization of the phosphorylation status of the $GABA_A$ receptor $\alpha 1$ subunit at Thr-Pro residue (Figure 12 B). The results showed that there was no increase in p-Thr-Pro within $\alpha 1$ subunit. In attempt to capture any p-Thr site within the $\alpha 1$ subunit, anti p-Thr-Pro antibody was replaced with the less residue-specific anti p-Thr antibody (Figure 12 C). This too revealed no consequence of HGF on the phosphorylation status of the alpha1 subunit of the $GABA_A$ receptor. Therefore, it is now concluded that the activation of the ERK/MAPK pathway by HGF does not result in the phosphorylation of the $\alpha 1$ subunit at Thr 375.

Association of the $GABA_A$ receptor with the ERK1/2

Although the Thr 375 may not be the direct target for the ERK/MAPK pathway, it might still be an important site for the $GABA_A$ receptor and ERK1/2 protein-protein association. Using immunoprecipitation technique and Western blot analysis, the physical association of the

$GABA_A$ receptor and the ERK1/2 was evaluated. After immunoprecipitation with FLAG-tag, the blot was probed for p-ERK1/2 (Figure 13 A) and ERK1/2 (Figure 13 B). p-ERK1/2 blot showed no bands; therefore, $GABA_A$ receptor did not have any protein-protein association with phosphorylated ERK1/2. On the other hand, ERK1/2 blot showed that the FLAG-tag immunoprecipitated proteins including α1 subunit appeared to show the physical association with non-phosphorylated form of ERK1/2. Therefore, it suggested that there was some association between $GABA_A$ receptor and ERK2. However, the mock transfected HEK-t cell sample also showed the ERK2 band (Figure 13 C). We preliminarily ruled out the possibility that the anti-Flag antibody was recognizing a sequence within ERK1/2 since neither ERK1 nor ERK2 contained an amino acid sequence resembling the FLAG-tag; therefore, it is possible that some other FLAG-domain containing protein happened to be associated with ERK2. To summarize, there was no physical association between $GABA_A$ receptor and phosphorylated or non-phosphorylated form of ERK1/2.

The effects of the MAPK pathway activation on GABA-gated currents

Effect of the HGF-induced MAPK pathway activation on GABA-gated currents

Using the perforated cell patch technique, 10 ng/mL of HGF was bath-applied to the 4D4 cells for at least 10 minutes and EC_{30} of GABA (5 μM) was applied for 5 seconds. EC_{30} was chosen in order to avoid desensitization of the receptor. The current amplitudes were recorded, and the relative mean peak current amplitude of the GABA-gated current during the HGF treatment was compared to the control to assess the effect of the MAPK pathway activation on the GABA-gated current. Final concentration of 5 μg/mL gramicidin was used for the perforation. After 2 stable control currents were established in physiological normal saline (N-saline) bath, and 10 ng/mL HGF was bath-applied for 10 minutes. It was switched back to N-

saline for the washout after the treatment. GABA-gated currents were recorded with 5 minutes intervals during the establishment of the stable control currents, 2 minutes intervals during the HGF treatment, then 5 minutes intervals during washout. The results showed that although the 10 ng/mL HGF treatment showed inhibitory effects on relative mean GABA-gated current in some 4D4 cells, it was not consistent and was not statistically significant (Figure 14). The same experiment was repeated using conventional whole patch technique (Figure 15). The data showed that the HGF activation of the ERK/MAPK pathway did not show any effect on the GABA-gated current in this configuration.

Physiological saline does not influence the phosphorylation status of kinases

Electrophysiology is often conducted in normal (N)-saline solution instead of the media that the cells are gown in. To make sure that N-saline did not affect the outcome of the electrophysiology and HGF effectively induced phosphorylation of ERK1/2 in N-saline, Western blot analysis was conducted in cells incubated with N-saline instead of media (Figure 16). The media was replaced with filter-sterilized N-saline 30 minutes before the beginning of 10 ng/mL HGF treatment at various durations. The Western blot analysis confirmed that the N-saline did not affect the ability of the HGF to induce the activation of the ERK/MAPK pathway in 4D4 cells. It also had no effect on the basal phosphorylation level of the ERK1/2. N-saline did not affect the Akt/PI3K pathway, either.

Since U0126, at the concentration used in the previous study (10 μM), is known to not only inhibit the ERK1/2 phosphorylation, but also ERK5 phosphorylation (Mody et al., 2001), we decided to measure the phosphorylation status of ERK5 as well (Figure 16). The molecular weight of ERK5 is about 100 kDa. Although the intensity of the ERK5 molecular weight area showed small increase in its intensity in response to HGF, it was substantially less than the

robust phosphorylation of the ERK1/2 at time points studied (5, 15, and 30 min). N-saline did not affect the basal level of p-ERK5. Therefore, the focus was put of ERK1/2 in this investigation using HGF as the ERK/MAPK pathway activator.

The influence of temperature on the phosphorylation status of ERK1/2

Temperature often affects the trafficking process of the receptors in various cell types, and lower temperatures often slow down or halt the process (Herring et al., 2005; Veyrat-Durebex et al., 2005; Machu et al., 2006). Both perforated and conventional whole cell patch electrophysiological recordings of GABA-gated current for the assessment of the ERK/MAPK pathway using HGF were conducted at room temperature. Therefore, HGF-induced activation of the ERK/MAPK pathway at room temperature was evaluated using Western blot analysis to ensure that the HGF effectively activated the ERK/MAPK pathway at room temperature during the electrophysiological recordings (Figure 17). The media of the 4D4 cells were replaced with room temperature N-saline 1 hour before the HGF treatment to mimic the time required to establish the perforation process. The cells were kept at room temperature. The final concentration of 10 ng/mL HGF was added to the N-saline and incubated at room temperature. The time course results showed that the HGF-induced activation of the MAPK pathway was slower compared to when the 4D4 cells were treated at 37°C. At 5 minutes of HGF treatment at room temperature, the phosphorylation of the ERK1/2 was higher than the basal level. However, the robust phosphorylation was observed at 15 minutes of the treatment. Based on these data, we inferred that the duration of the HGF application that was used in the electrophysiological recordings (10 minutes) was most likely sufficient for the HGF to activate the ERK/MAPK pathway.

The effects of the ERK/MAPK pathways on the trafficking of $GABA_A$ receptors

Insulin-induced activation of PI-3K/Akt pathway results in increased GABA-gated currents through rapid translocation of $GABA_A$ receptors from intracellular compartments to the plasma membrane (Wan et al., 1997; Wang et al., 2003; Vetiska et al., 2007). Meanwhile BDNF, a growth factor that activates both the Akt/PI3K and the ERK/MAPK pathways (Zhu et al., 2002; Obara et al., 2007), leads to the internalization of the $GABA_A$ receptor thus results in inhibition of the GABA-gated currents in granule cells (Cheng and Yeh, 2003). However, nothing is known about how the ERK/MAPK pathway influences $GABA_A$ receptor trafficking. The previous electrophysiological study using the ERK/MAPK pathway inhibitor, U0126, suggested that the ERK/MAPK pathway was involved in the inhibitory regulation of GABA-gated currents (Bell-Horner et al., 2006). Therefore, we hypothesized that the potentiation of the GABA-gated currents that was observed through the use of U0126 may be due to the greater insertion of the $GABA_A$ receptor into the plasma membrane or inhibition of $GABA_A$ receptor internalization. Flow cytometric analysis was used to determine if the potentiation effect of U0126 was due to the increase of the $GABA_A$ receptor density at the cell surface.

First, the 4D4 cells that were stably expressing $GABA_A$ receptor $\alpha1\beta2\gamma2$ subunits were probed with anti $GABA_A$ receptor β subunit antibody under non-permeabilized conditions, and relative mean fluorescent intensity was compared to non-transfected HEK-t cells (Figure 18). The results showed that the non-transfected HEK-t cells showed no significant difference from 4D4 cells. In addition, the secondary antibody itself showed the similar intensity to the 4D4 cells that was probed with primary and secondary antibodies. Therefore, non-specific binding was too high so that the detection of the $GABA_A$ receptor was not possible. Therefore, HEK-t cells were transfected with FLAG-tagged $\alpha1$ along with $\beta2$ and $\gamma2$ subunits to avoid the use of the

secondary antibody. The FLAG-tag is an eight amino acid residue located the N-terminal of the extracellular domain. Using this HEK-t cells transiently expressing $GABA_A$ receptor FLAG-$\alpha1\beta2\gamma2$ subunits, the ERK/MAPK pathway was inhibited with 10 μM U0126 for 15 or 30 minutes at 37°C and the surface expression of $GABA_A$ receptor level was detected using anti FLAG-FITC antibody using flow cytometer. The results were compared to vehicle treated control (Figure 19 and 20). Either 15 or 30 minutes of U0126 treatment did not show any statistically significant change in relative mean fluorescent intensity, which indicated that the receptor density at the surface of the HEK-t cells did not change after U0126 treatment. The vehicle treatment itself did not show any statistically significant difference compared to no treatment control either.

The HGF activation of the ERK/MAPK pathway did not show any significant effect on GABA-gated current in electrophysiological recordings (Figure 14 & 15). To confirm the activation of the ERK/MAPK pathway does not affect the trafficking of the receptor in this cell type, the flow cytometric experiment was repeated with the activation of the pathway with HGF. The HEK-t cells transiently expressing $GABA_A$ receptor FLAG-$\alpha1\beta2\gamma2$ subunits were treated with 10 ng/mL HGF at 37°C, and the change in $GABA_A$ receptor density was monitored using anti-FLAG antibody though the flow cytometer (Figure 21). The HGF treatment groups were compared to no treatment control. The data showed that HGF induced activation of ERK/MAPK pathway had no significant effect on the $GABA_A$ receptor cell surface density.

Activation of PKC is known to inhibit the GABA-gated current (Machu et al., 2006) by internalizing the $GABA_A$ receptor (Herring et al., 2005). In order to confirm the reliability of the flow cytometry method in this study and validate the findings so far, the activator of PKC, PMA was used to confirm the decrease in $GABA_A$ receptor density by internalization. Transiently

transfected HEK-t cells were treated with final concentration of 25 and 100 nM PMA. Those concentrations were based on the previous studies on the neuronal cells and HEK-t cells (Herring et al., 2005; Machu et al., 2006). The results showed that 15 minutes treatment with either 25 or 100 nM PMA did not affect the cell surface expression while 30 minutes incubation with100 nM PMA showed statistically significant decrease in surface expression of the receptor (Figure 22 and 23). Therefore, the flow cytometric evaluation of the $GABA_A$ receptor density on the cell surface was valid, and this confirmed the activation of the ERK/MAPK pathway by HGF did not have any effect on the trafficking of the $GABA_A$ receptor in HEK-t cells.

Summary

To summarize the findings of this study, the activation of the ERK/MAPK pathway using HGF in HEK-t cells did not influence the GABA-gated current. The potentiation effect that was seen through the application of the ERK/MAPK pathway inhibitor, U0126, was not due to the increase in receptor density at the cell surface. Rather it might be the change in the regulation of $GABA_A$ receptor gating. Also the activation of the ERK/MAPK pathway using HGF did not change the density of the $GABA_A$ receptor. The Thr 375 that is located within $\alpha 1$ subunit was not the direct target of the ERK1/2 or ERK5. And finally there was no association between $GABA_A$ receptor $\alpha 1$ subunit and ERK1/2.

CHAPTER 4

ILLUSTRATIONS

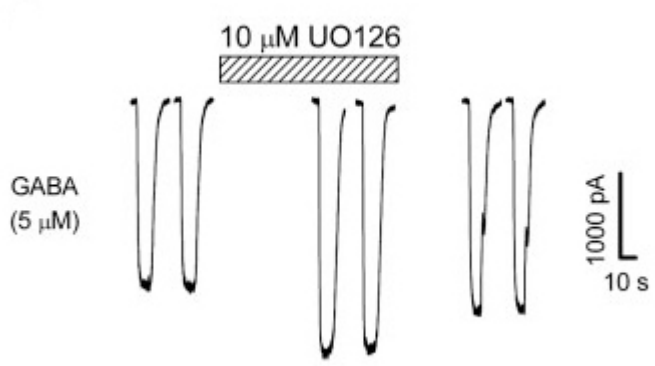
Figure 1. MEK inhibition enhances GABA-gated currents in $\alpha 1\beta 2\gamma 2$ $GABA_A$ receptors when the intracellular environment is maintained (Adopted from Bell-Horner et al. 2006)

(A) MEK1/2 inhibition by 10 μM U0126 led to an enhancement of the GABA-gated current.

(B) Relative mean current ampltudes were compared between the groups ($p = 0.01$, n = 6).

Figure 1. MEK inhibition enhances GABA-gated currents in $\alpha1\beta2\gamma2$ $GABA_A$ receptors when the intracellular environment is maintained (Adopted from Bell-Horner et al. 2006)

(A)



(B)

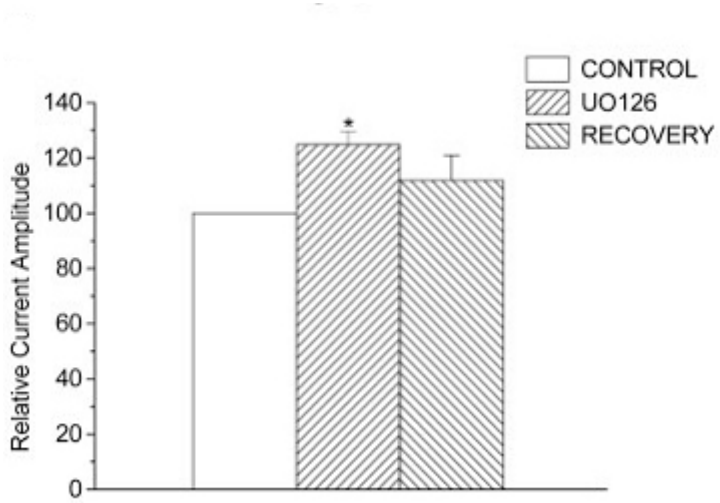


Figure 2. U0124 showed no effect on GABA-gated currents (Adopted from Bell-Horner et al. 2006)

Inactive analog of U0126, U0124, failed to alter GABA-gated currents.

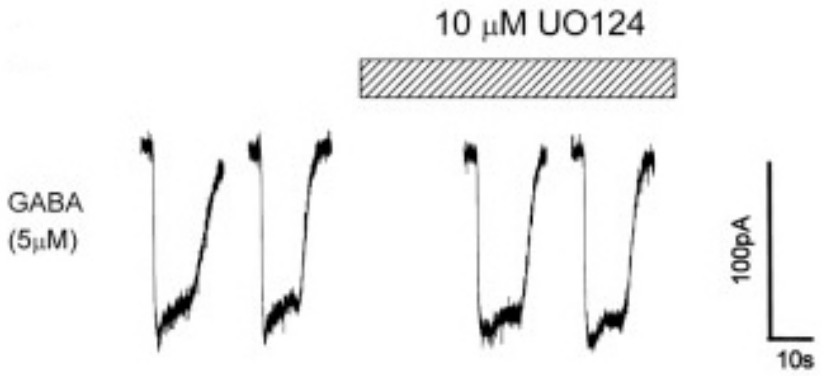
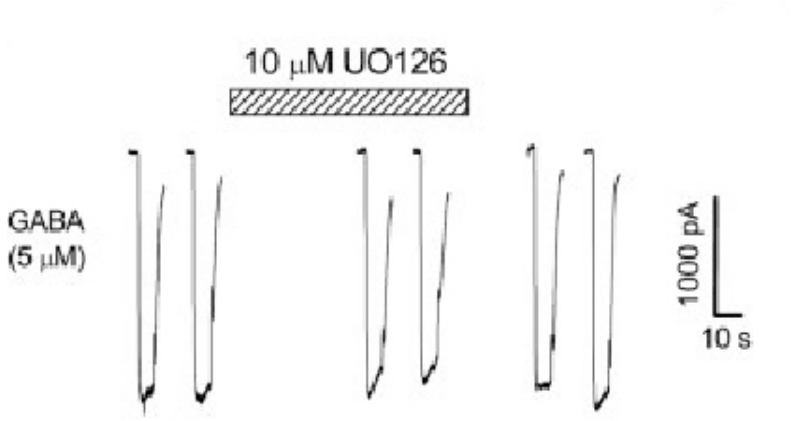


Figure 3. U0126 effect disappears when conventional whole cell patch is used (Adopted from Bell-Horner et al. 2006)

(A) U0126 failed to show the potentiation effect on GABA-gated current when conventional whole cell patch was used.



(B) The ability of U0126 to enhance $GABA_A$ receptor function is dependent on an intact intracellular environment (n = 4).

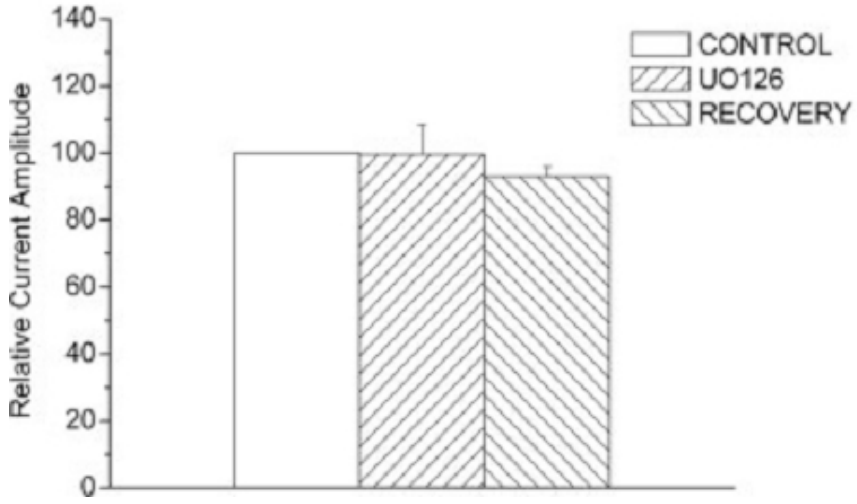


Figure 4. U0126 inhibits basal ERK phosphorylation in α1β2γ2-transfected HEK-t cells (Adopted from Bell-Horner et al. 2006)

The MEK inhibitory effect of U0126 (10 μM, 10 minutes) was confirmed through Western blot analysis.

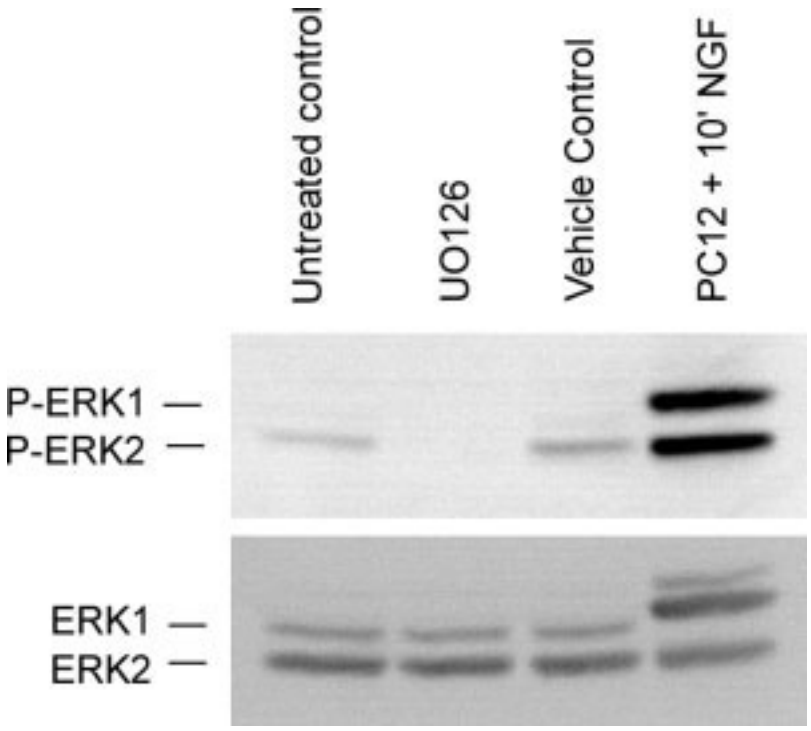


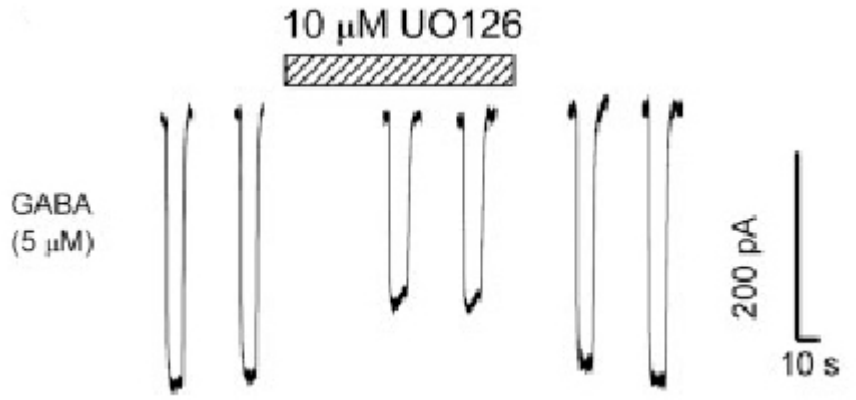
Figure 5. Mutation of the putative ERK phosphorylation site within the α1 subunit abolishes the U0126-induced enhancement of GABA-gated currents (Adopted from Bell-Horner et al. 2006)

(A) Thr 375 was mutated to alanine. The mutation abolished the ability of U0126 to potentiate GABA-gated currents. In addition, it showed completely opposite effect (inhibition).

(B) Relative mean current amplitudes were compared between the groups. It showed that the relative mean current amplitude was significantly less when compared to the control ($p = 0.05$, $n = 4$).

Figure 5. Mutation of the putative ERK phosphorylation site within the α1 subunit abolishes the U0126-induced enhancement of GABA-gated currents (Adopted from Bell-Horner et al. 2006)

(A)



(B)

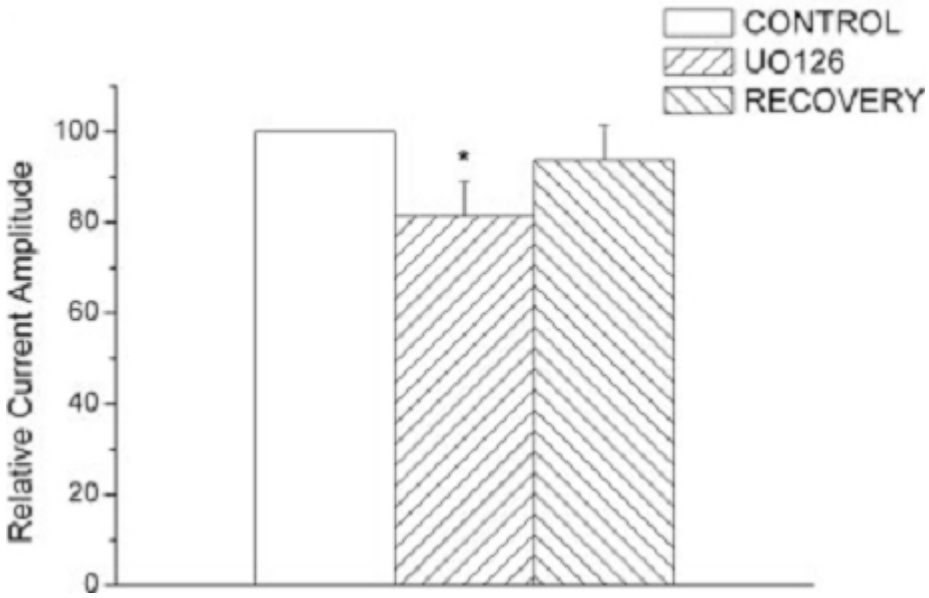


Figure 6. Commercially available anti-α1 subunit antibody is not effective

Indicated amount of total 4D4 cell lysate was analyzed with Western blot and probed with commercially available anti α subunit antibody. Despite the high amount of protein used for the analysis, the antibody showed weak binding, and the binding was non-specific.

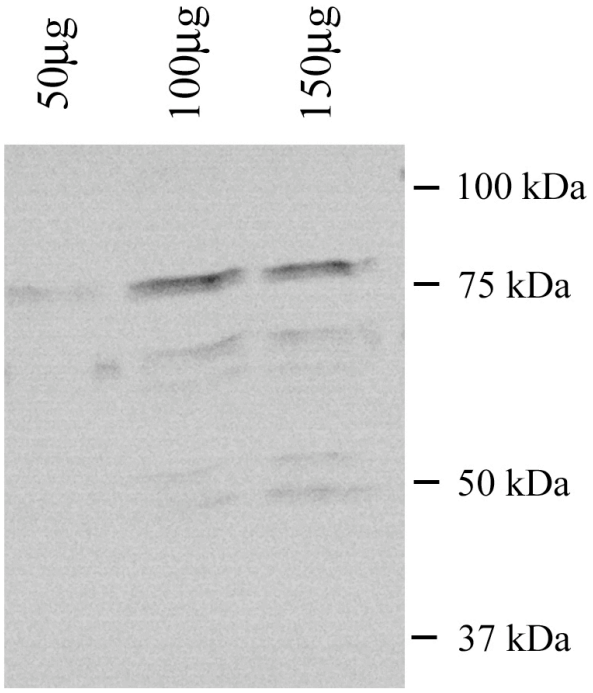


Figure 7. HGF activates the ERK/MAPK pathway in time dependent manner while it does not affect the Akt/PI3K pathway in 4D4 cells

HEK-t cells stably expressing rat $GABA_A$ receptor $\alpha1\beta2\gamma2$ subunits (4D4 cells) were treated 50 ng/mL of hepatocyte growth factor (HGF) at 37°C and phosphorylation level of Akt and ERK1 and ERK2 were evaluated through Western blot analysis. The maximal phosphorylation of ERK1 (44 kDa) and ERK 2 (42 kDa) was observed at 5 min and decreased thereafter. The phosphorylation level of these kinases finally went down close to its basal level after 2 hours of its treatment. Due to the phosphorylation, there was upward phosphorylation super shift in the molecular weight especially in ERK2. Therefore, actin was used as a control for the equal loading of the each well. Neuro growth factor (NGF) treated PC12 cell lysate was used for the positive control of p-ERK1/2. Phosphorylation status of Akt was also assessed to see if HGF would also activate the Akt/PI3K pathway. The phosphorylation of the Akt in 4D4 cells did not get any influence from HGF treatment.

Figure 7. HGF activates the ERK/MAPK pathway in time dependent manner while it does not affect the Akt/PI3K pathway in 4D4 cells

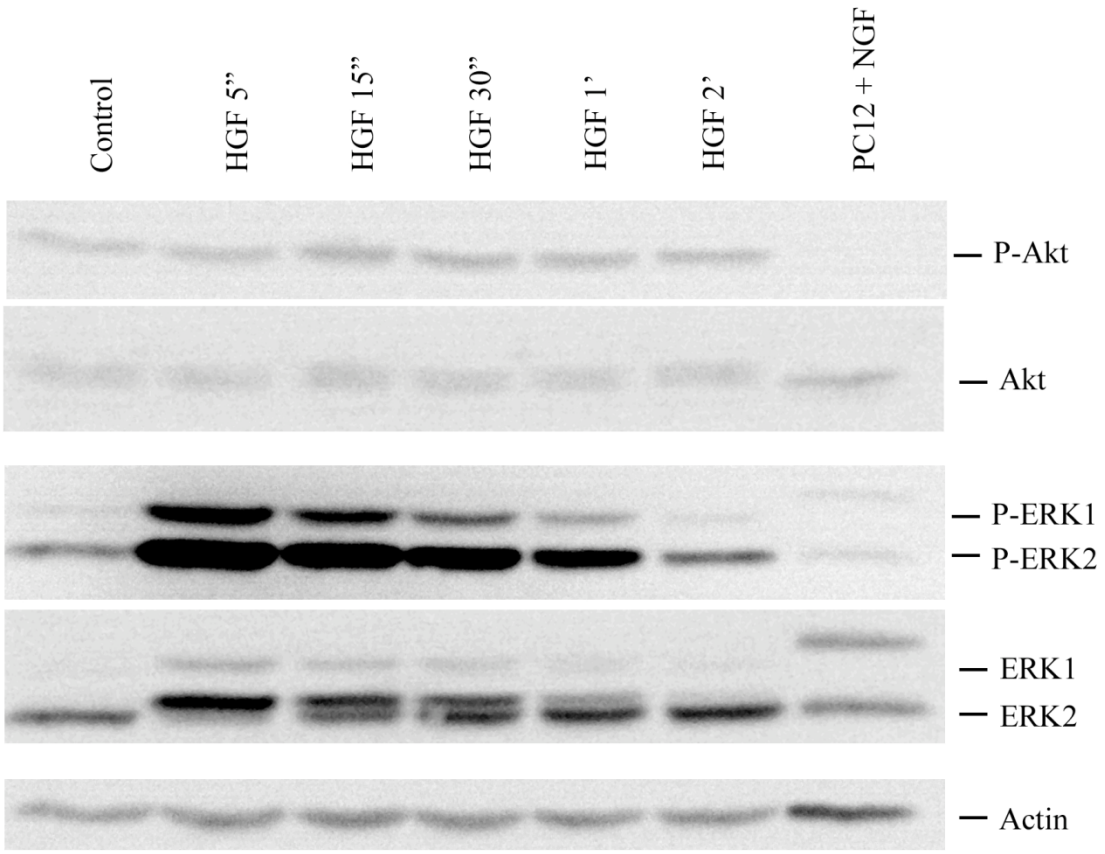


Figure 8. HGF induced activation of the ERK/MAPK pathway is concentration dependent in 4D4 cells

4D4 cells were treated with various concentrations of HGF for 5 minutes at 37°C. Western blot analysis showed that the HGF induced the activation of the ERK1/2 in concentration dependent manner while it did not affect the phosphorylation level of Akt.

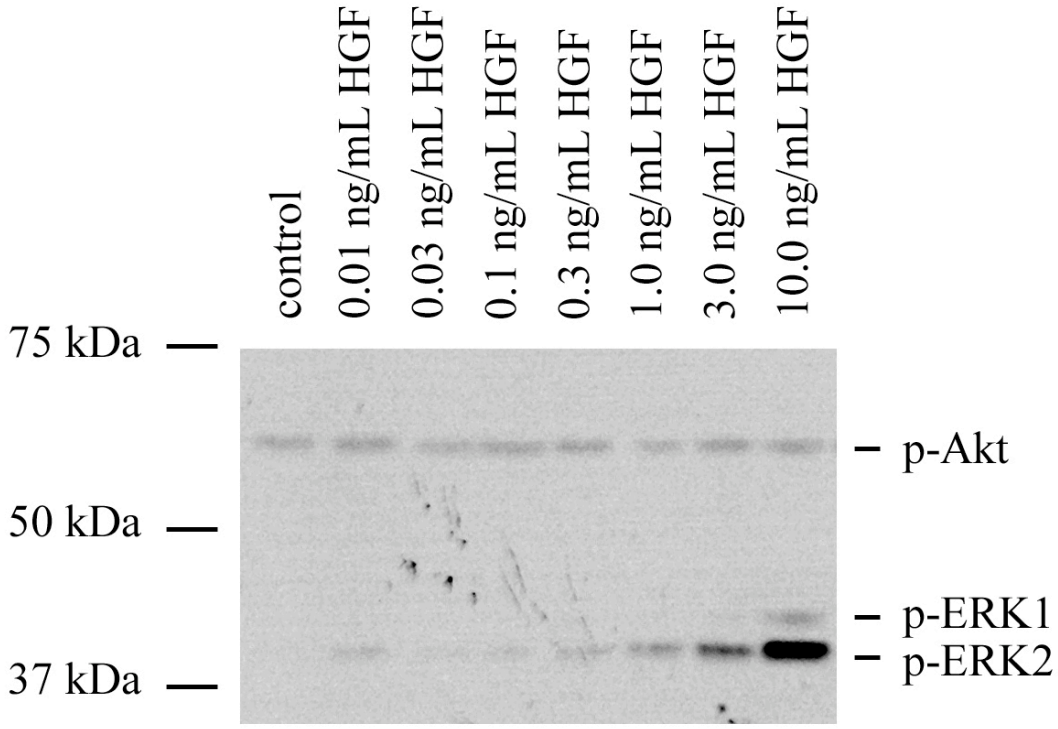


Figure 9. FLAG-tagged α1 with β2 and γ2 subunit of the $GABA_A$ receptor was transiently expressed in HEK-t cells

$GABA_A$ receptor FLAG-α1β2γ2 subunit was transiently expressed in HEK-t cells. To confirm the expression of receptors, α1 subunit was immunoprecipitated with anti-FLAG antibody and probed with anti FLAG antibody in Western blot analysis. The FLAG-tagged α1 subunit was expressed in this cell line, and its molecular weight was right around at 50 kDa (α1 subunit: 51 kDa). In addition the anti FLAG antibody was found to be highly specific.

Figure 9. FLAG-tagged α1 with β2 and γ2 subunit of the $GABA_A$ receptor was transiently expressed in HEK-t cells

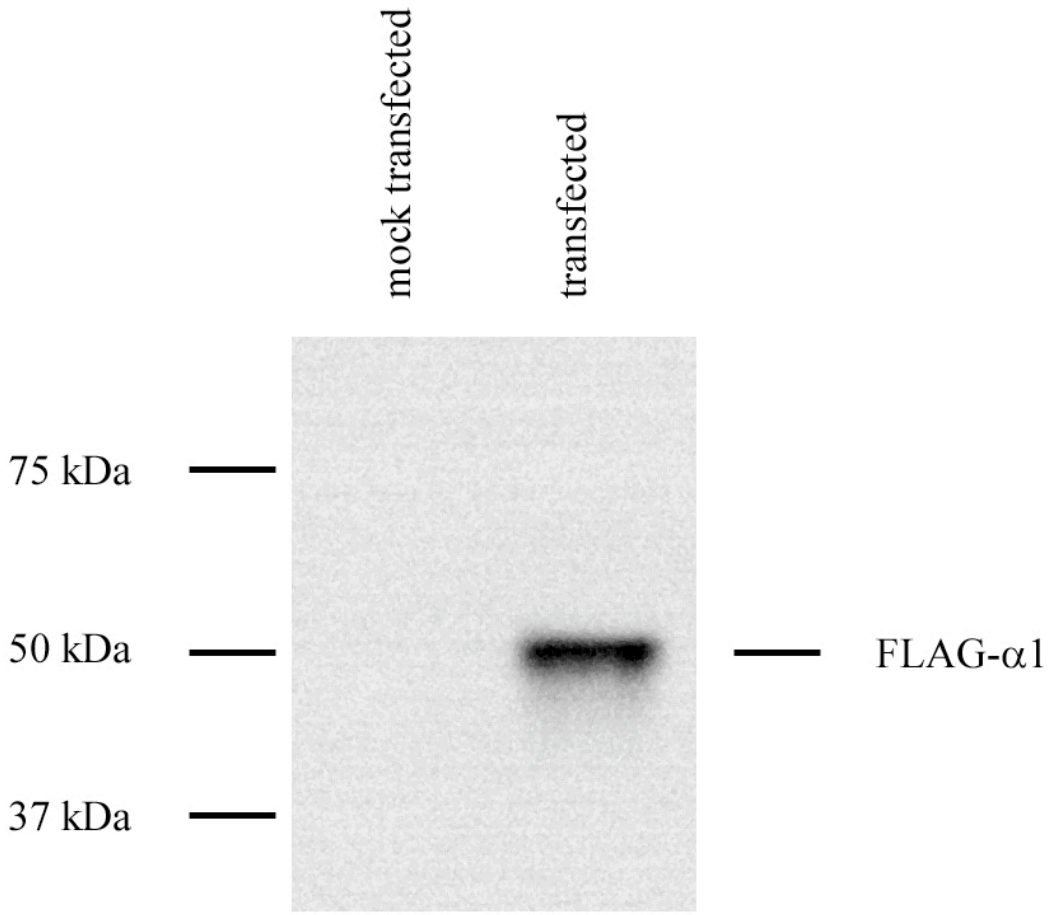


Figure 10. Activation of the ERK/MAPK pathway does not result in phosphorylation of Thr 375 residue of $GABA_A$ receptor α1 subunit

HEK-t cells were transfected with $GABA_A$ receptor FLAG-α1β2γ2 subunit, and the ERK/MAPK pathway was activated with 10 ng/mL HGF for various period of time at 37°C. FLAG-tagged α1 subunit was immunoprecipitated and Western blot analysis was conducted with anti-p-Thr-Pro antibody.

(A) 1 mg total protein was used to for the immunoprecipitation. p-Thr-Pro was not detected at 51 kDa where α1 subunit would migrate.

(B) The use of anti p-The-Pro antibody was increased from 1:2,000 to 1:500. There was no possible FLAG-α1 subunit band detected.

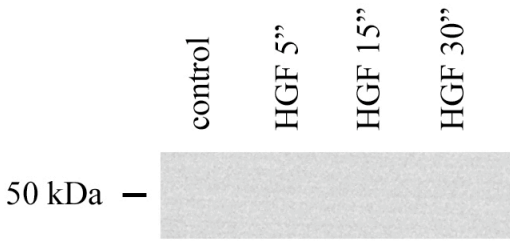
(C) The starting total protein for the immunoprecipitation was increased from 1 mg to 5 mg. Although there was a detectable band around 50 kDa, it was slightly higher than where FLAG-α1 subunit should be seen.

(D) 50 μM GABA was co-applied with HGF for the conformational change of the $GABA_A$ receptor can take place. 2 mg total protein was used for the immunoprecipitation. However, there was no p-The-Pro band around 50 kDa was detected.

(E) Anti p-Thr antibody was used to see if any phosphorylated Thr residue is detected. However, no bands were detected.

Figure 10. Activation of the ERK/MAPK pathway does not result in phosphorylation of Thr 375 residue of $GABA_A$ receptor α1 subunit.

(A)



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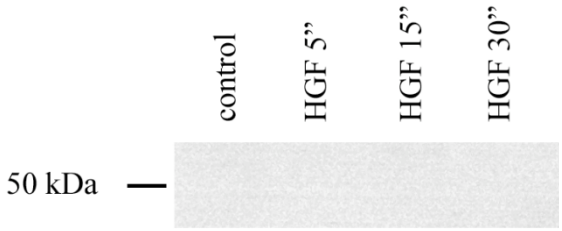


(C)

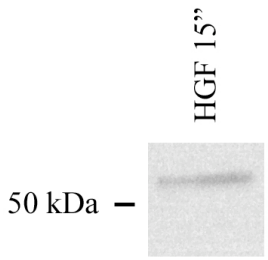
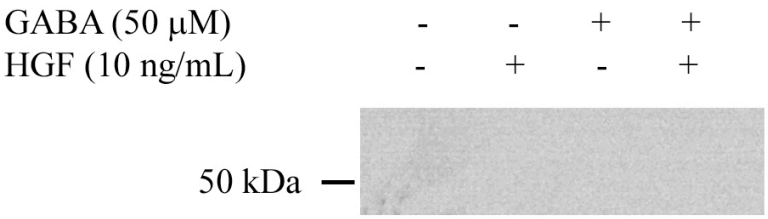


Figure 10. Activation of the ERK/MAPK pathway does not result in phosphorylation of Thr 375 residue of $GABA_A$ receptor α1 subunit.

(D)



(E)

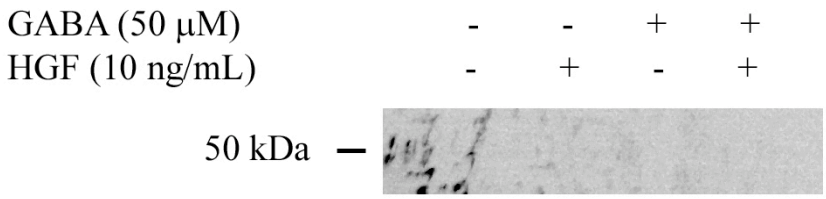


Figure 11. Okadaic acid pre-treatment did not affect the phosphorylation status of Thr375 residue within FLAG-tagged α1 subunit

Transfected HEK-t cells were pre-treated with 100 nM okadaic acid for 20 minutes in attempt to slow down the phosphatase activity and preserve the phosphorylated sites. However, it did not have any effect on the phosphorylation status of the Thr 375 in FLAG-tagged α1 subunit.

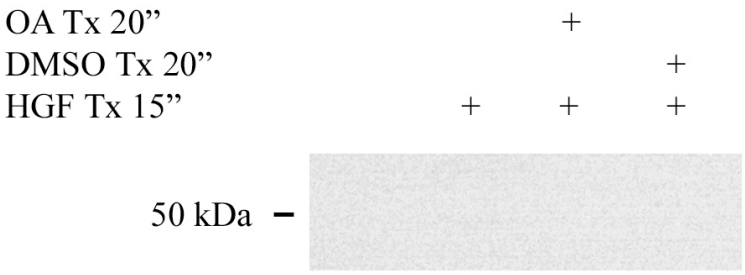
(A) 2 mg total protein was used for the immunoprecipitation. No bands were observed around 50 kDa area

(B) The amount of protein used for the immunoprecipitation was increased to 5 mg. The Western blot analysis showed the same results.

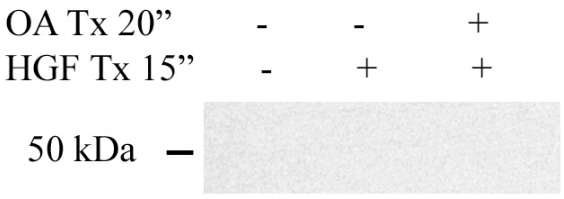
(C) To ensure the inhibitory effect of okadaic acid on phosphatase activity at the concentration of 100 nM, phosphorylation status of the ERK1 and ERK2 were evaluated through Western blot. It showed that the intensified p-ERK1/2 band due to the okadaic acid pre-treatment.

Figure 11. Okadaic acid pre-treatment did not affect the phosphorylation status of Thr 375 residue within FLAG-tagged α1 subunit

(A)



(B)



(C)

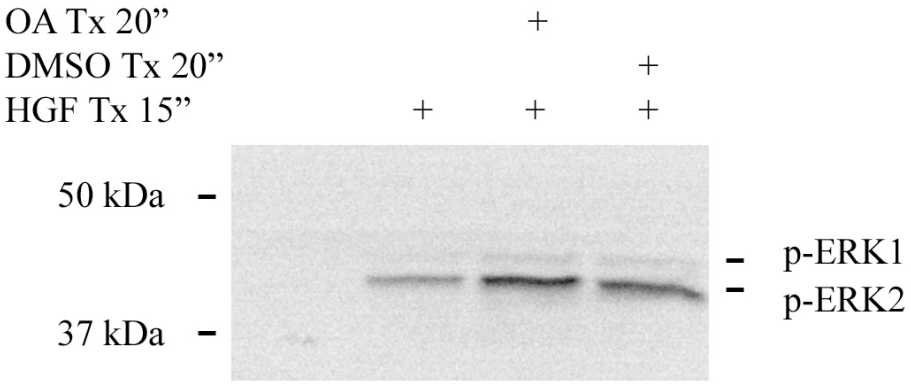


Figure 12. Inhibition of phosphatases by Calyculin A does not affect the phosphorylation status of the Thr 375 of FLAG-α1 subunit

HEK- cells transiently expressing $GABA_A$ receptor FLAG-α1β2γ2 subunits were treated with a phosphatase inhibitor, calyculin A. GABA was also co-applied to see if the conformational change is required for the direct phosphorylation of Thr 375 by ERK1/2. FLAG-tagged α1 subunit was immunoprecipitated and analyzed with Western blot. The results showed that the Thr 375 did not get any phosphorylation effect at the p-Thr-Pro site. Calyculin A was validated through Western blot analysis of the transfected HEK-t cells.

(A) In order to validate the use of calyculin A, transiently transfected HEK-t cells were treated with calyculin A without any other treatment. Western blot analysis showed that calyculin A increased the phosphorylated Thr level throughout the blot.

(B) Calyculin A did not affect the phosphorylation status of the Thr 375. Anti p-Thr-Pro antibody was used to detect the Thr 375 got phosphorylated.

(C) Same blot was probed with more generic anti p-Thr antibody. Despite the inhibition of the phosphatases, the phosphorylation status of the α1 subunit did not change around 50 kDa.

Figure 12. Inhibition of phosphatases by Calyculin A does not affect the phosphorylation status of the Thr 375 of FLAG-α1 subunit

(A)

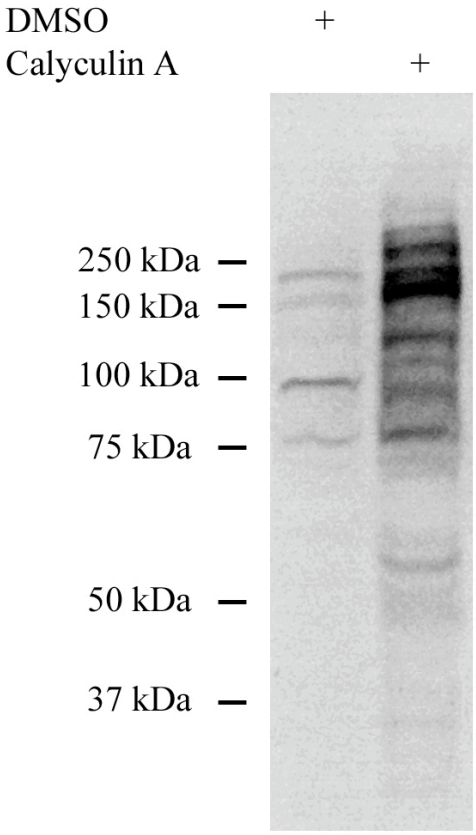
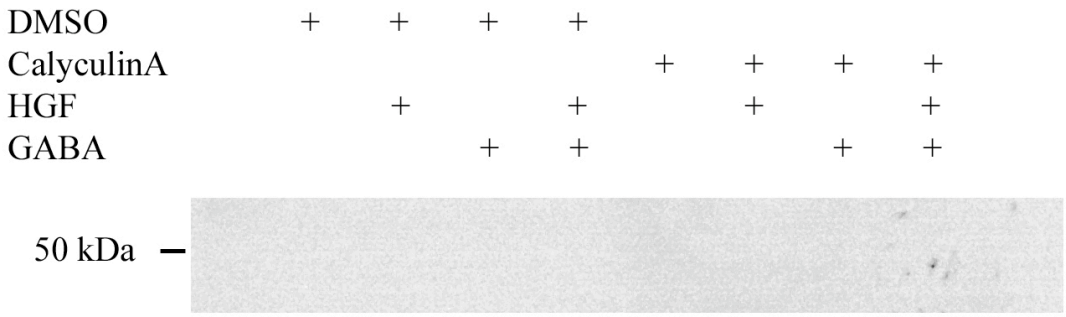


Figure 12. Inhibition of phosphatases by Calyculin A does not affect the phosphorylation status of the Thr 375 of FLAG-α1 subunit

(B)



(C)

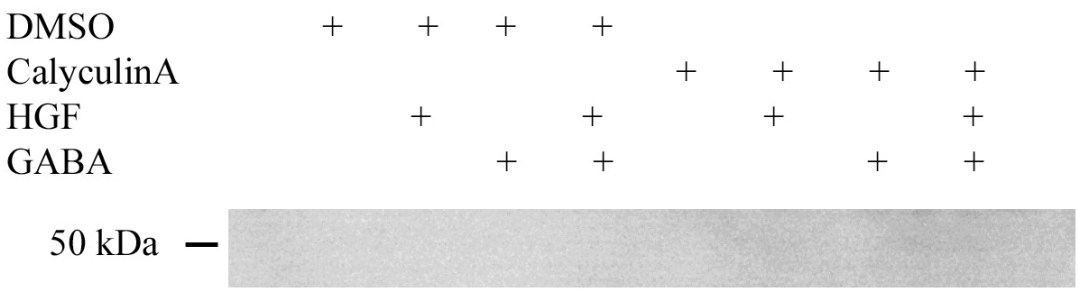


Figure 13. $GABA_A$ receptor does not associate with ERK1/2

The immunoprecipitated $GABA_A$ receptor α1 subunit was analyzed with Western blot to determine if there is any protein-protein association. The blot showed that immunoprecipitation using FLAG-tag on the α1 subunit also precipitated ERK2 while phosphorylated ERK1 nor ERK2 were detected; however, when the HEK-t cells were mock transfected, it also showed the precipitation of ERK2 using anti FLAG-tag antibody. Therefore, although ERK2 does not have the FLAG-tag sequence (DYKDDDDK), there must be a site the native conformation of the ERK2 show some similarity to the FLAG-tag.

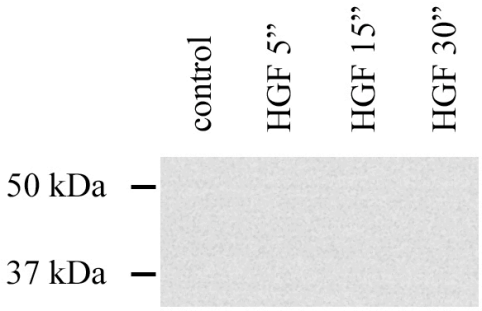
(A) After immunoprecipitation with anti FLAG antibody, the Western blot was probed with anti p-ERK1/2 antibody. The results showed there was no association between $GABA_A$ receptor α1 subunit.

(B) The same blot was probed with total ERK1 and total ERK2 antibodies. It showed some association between the non-phosphorylated ERK2 and the $GABA_A$ receptor FLAG-α1 subunit.

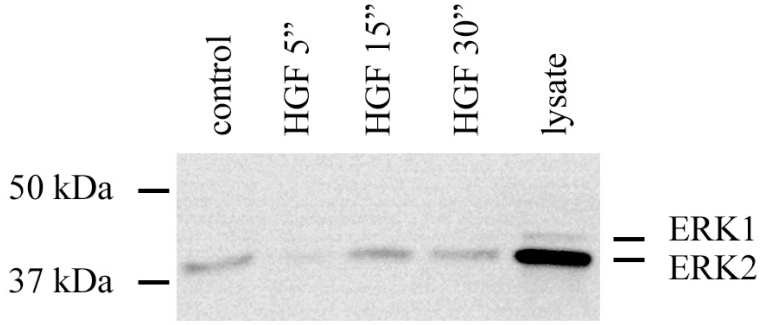
(C) Mock transfected HEK-t cells showed that immunoprecipitation with FLAG-tag precipitated the ERK2.

Figure 13. $GABA_A$ receptor does not associate with ERK1/2.

(A) Immunoprecipitated with FLAG-tag and probed with anti p-ERK1/2



(B) Immunoprecipitated with FLAG-tag and probed with anti total ERK1 and ERK2



(C) ERK2 was detected in mock transfected HEK-t cell; therefore, the results in Figure 13 B appeared to be false positive

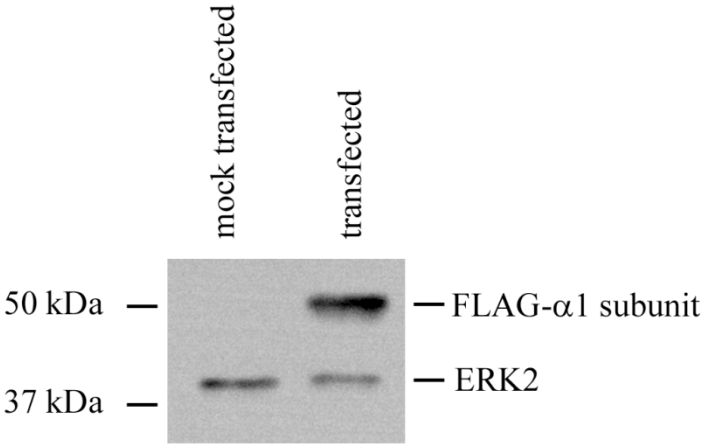


Figure 14. The ERK/MAPK activation by HGF does not affect the GABA-gated current in perforated patch recording

Electrophysiological recording was conducted on 4D4 cells at room temperature. 10 ng/mL of HGF was bath applied on 4D4 cells for 10 minutes. 5 μM (EC_{30}) was applied for 5 seconds, and the mean peak amplitude was normalized and compared to the control current that was established before the application of HGF. Perforation was established with final concentration of 5 μg/mL gramicidin that was added to the pipette solution. This summary graph showed that the ERK/MAPK pathway activation by 10 ng/mL HGF application did not affect the GABA-gated current at room temperature ($P > 0.05$). Washout did not recover to the control level, and the amplitude was inconsistent. The graph was created and statistically analyzed using Prism 4.0 ($n = 6$).

Figure 14. The ERK/MAPK activation by HGF does not affect the GABA-gated current in perforated patch recording

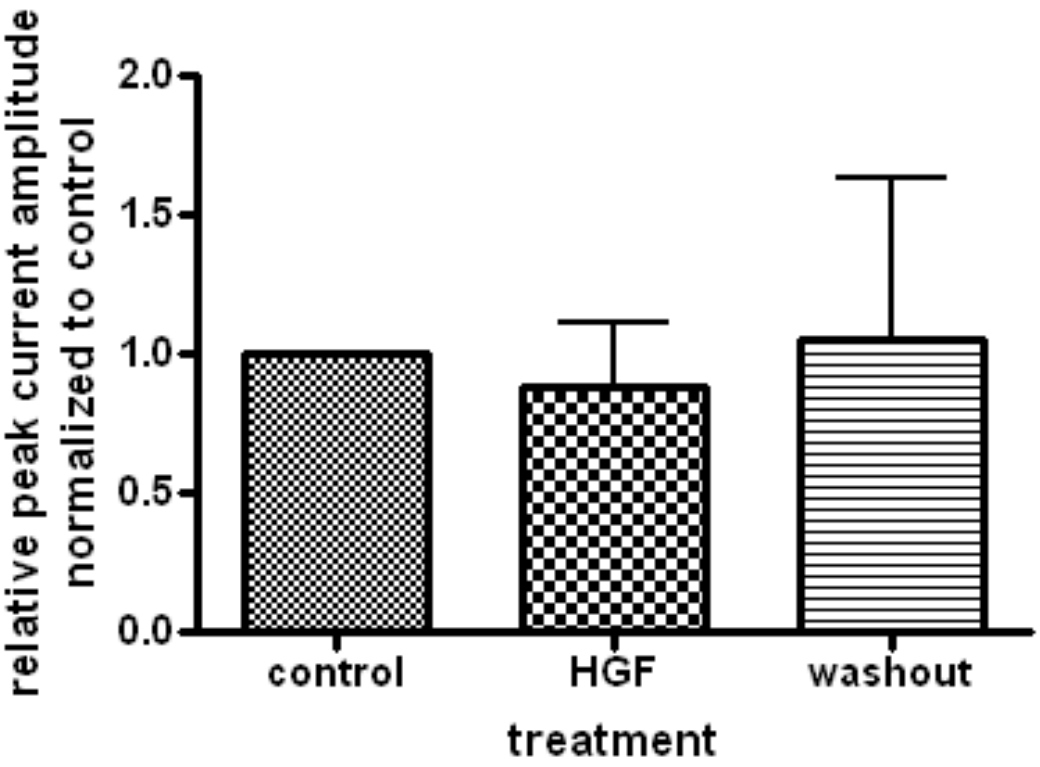


Figure 15. The ERK/MAPK activation by HGF does not affect the GABA-gated current in conventional whole cell patch recording

The electrophysiological recording was repeated with more conventional whole cell patch on 4D4 cells. Activation of the ERK/MAPK pathway by 10 ng/mL HGF did not affect the GABA-gated current ($P > 0.05$, $n = 3$).

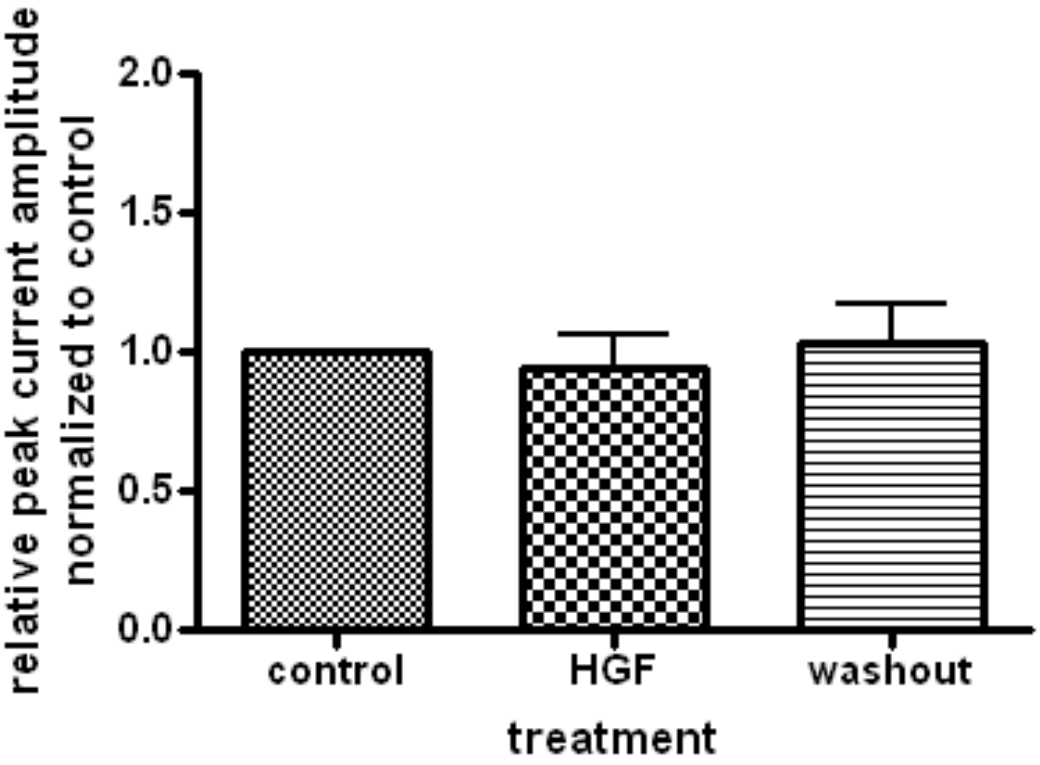


Figure 16. N-saline does not affect the HGF-induced activation of the ERK/MAPK pathway

The 4D4 media was replaced with 37°C N-saline 30 minutes before the activation of the ERK/MAPK pathway with 10 ng/mL HGF in 4D4 cells. Western blot analysis showed that the N-saline did not affect the phosphorylation level of ERK1/2, ERK5, or Akt. It did not affect the basal level of their phosphorylation, either. Actin was used as a control for the equal loading of the each well.

Figure 16. N-saline does not affect the HGF-induced activation of the ERK/MAPK pathway

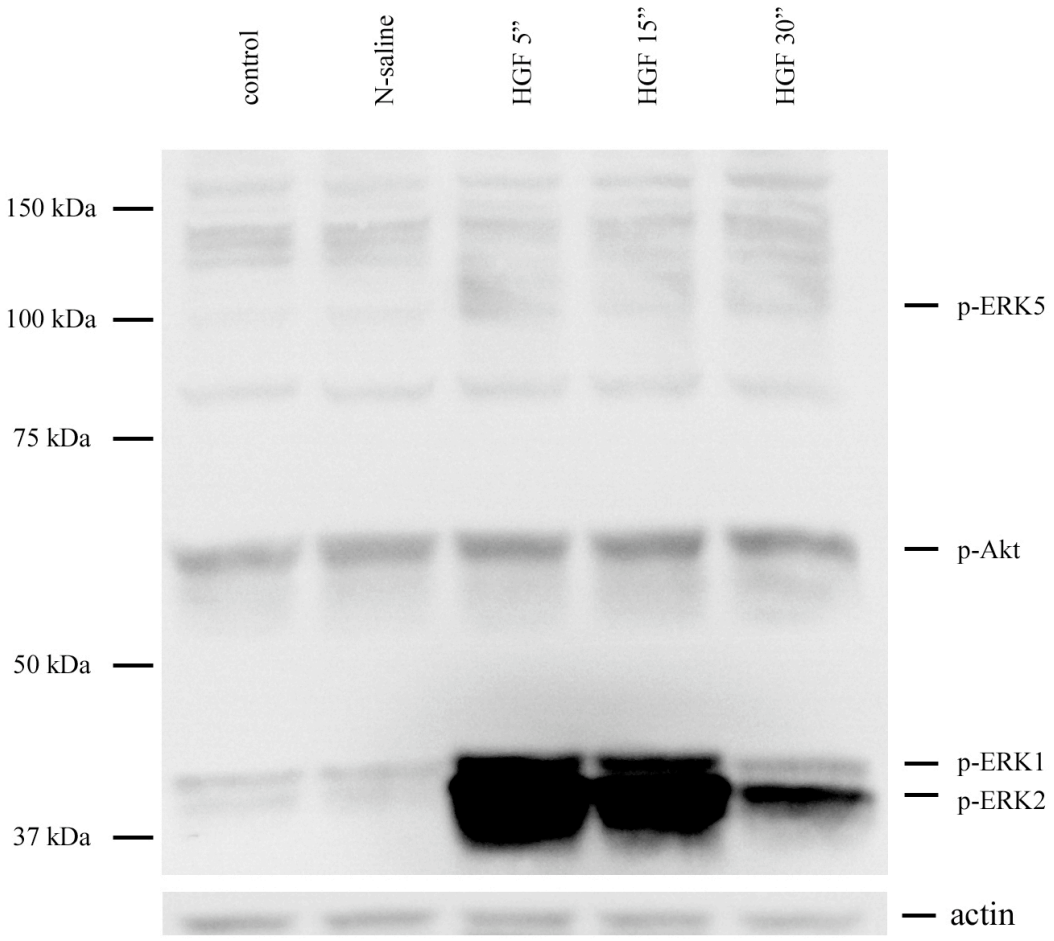


Figure 17. The activation of the ERK/MAPK pathway by HGF in 4D4 cells slows down at room temperature

The ERK/MAPK pathway was activated by 10 ng/mL HGF in HEK-t cells at room temperature. Western blot analysis showed that the 5 minutes incubation of the HEK-t cells with 10 ng/mL HGF was not enough to see robust phosphorylation of the ERK1 and ERK2 while it was when incubated at 37°C. Although those kinases showed higher intensity of the HGF-induced phosphorylation compared to the control at room temperature, their intensity level appeared less than when compared to the 37°C incubation (Figure 16).

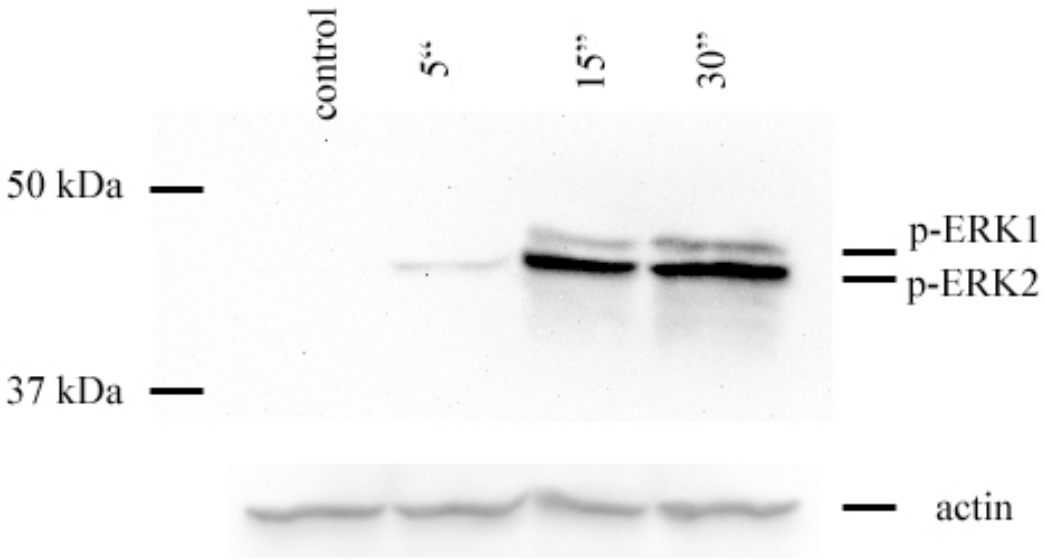


Figure 18. Commercially available monoclonal anti $GABA_A$ receptor β subunit antibody showed high non-specific binding

Monoclonal anti $GABA_A$ receptor β subunit antibody was used as a primary antibody to detect the cell surface density of the $GABA_A$ receptor. Using flow cytometric technique 4D4 cells were probed with this antibody, and the mean fluorescent intensity of non-transfected HEK-t cells were normalized and compared to the 4D4 cells. Although the HEK-t cells were not transfected, the surface receptor density of those cell lines showed no significant difference. In addition, the secondary antibody that is conjugated to FITC showed high non-specific binding. Therefore, it was necessary to avoid the use of the secondary antibody.

4D4+12: 4D4 cells + anti FLAG primary antibody + secondary antibody

4D4+2: 4D4 cells + secondary antibody only

HEKt+12: HEK-t cells with anti FLAG primary antibody + secondary antibody

HEKt+2: HEK-t cells + secondary antibody only

Figure 18. Commercially available monoclonal anti $GABA_A$ receptor β subunit antibody showed high non-specific binding

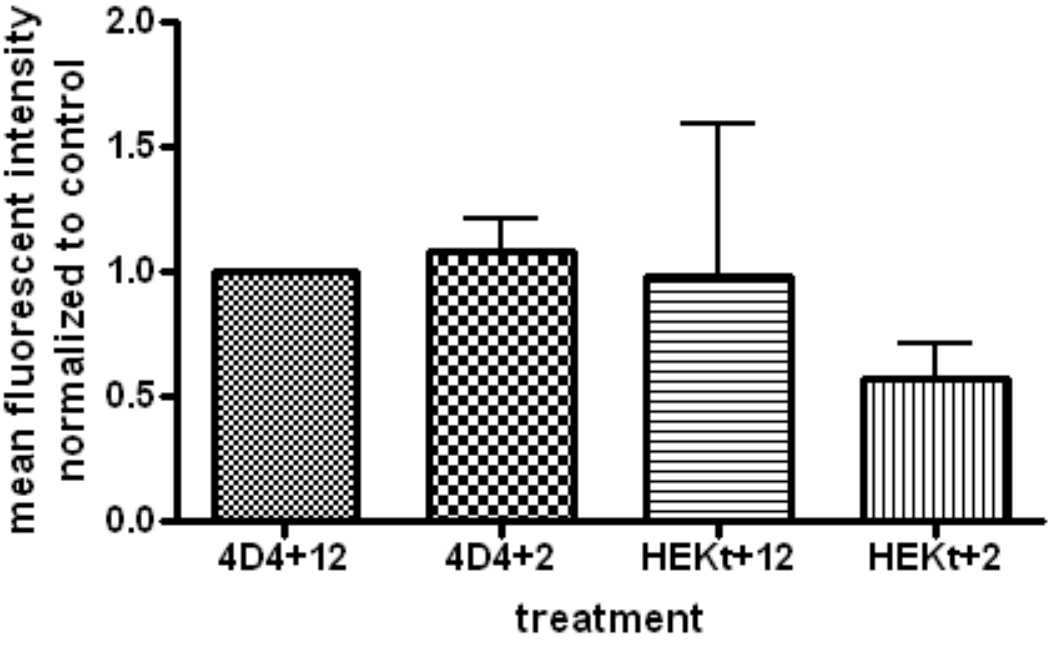


Figure 19. The inhibition of the ERK/MAPK pathway with U0126 for 15 minutes did not change the surface expression of the $GABA_A$ receptor

U0126-induced inhibition of the ERK/MAPK pathway showed potentiation of the GABA-gated current in 4D4 cells in the previous study (Bell-Horner et al., 2006). The flow cytometric analysis at non-permeabilized condition using HEK-t cells transiently expressing $GABA_A$ receptor FLAG-$\alpha 1\beta 2\gamma 2$ subunit showed that 15 minutes 10 μM U0126 treatment at 37°C did not affect the trafficking of the receptor ($P > 0.05$, $n = 3$). Although 10 μM U0126 appeared to increase the surface expression of the $GABA_A$ receptor, it was statistically insignificant. Vehicle itself (DMSO) did not have any statistically significant effect.

Mock: mock transfected HEK-t cells + anti FLAG-FITC antibody

No Tx: transfected HEK-t cells + anti FLAG-FITC antibody

V15: transfected HEK-t cells treated with DMSO for 15 minutes + anti FLAG-FITC antibody

U15: transfected HEK-t cells treated with 10 μM U0126 for 15 minutes + anti FLAG-FITC antibody

Figure 19. The inhibition of the ERK/MAPK pathway with U0126 for 15 minutes did not change the surface expression of the $GABA_A$ receptor

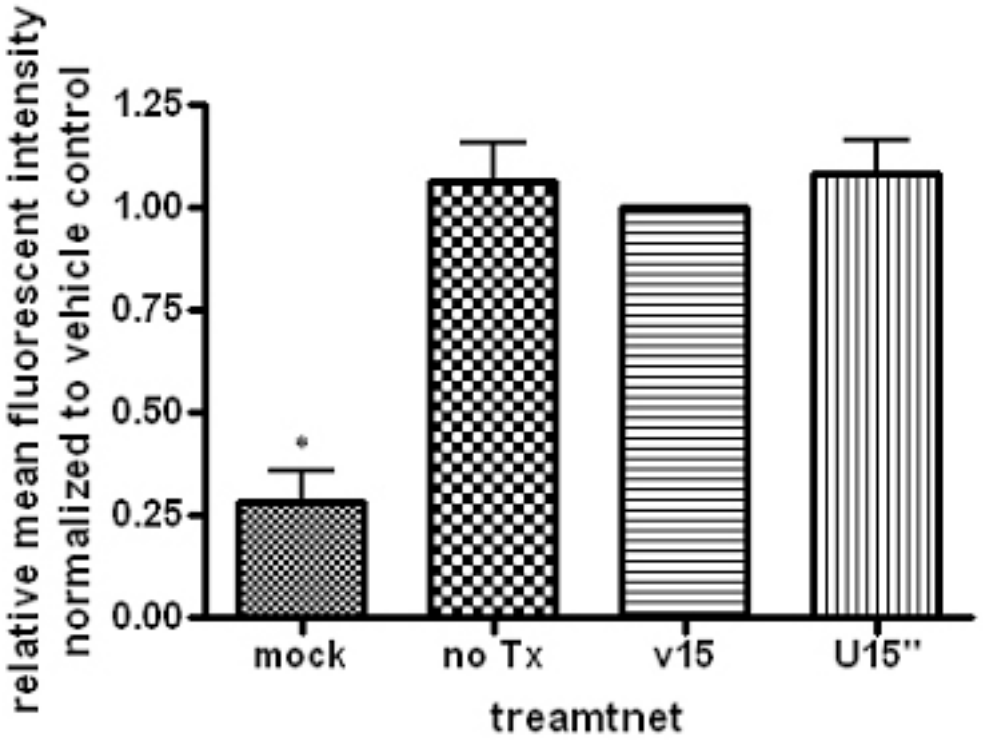


Figure 20. The 30 minutes inhibition of the ERK/MAPK pathway with U0126 did not change the surface expression of the $GABA_A$ receptor

U0126-induced inhibition of the ERK/MAPK pathway was extended to 30 minutes. The surface expression of the $GABA_A$ receptor did not have any statistically significant difference compared to 30 minutes vehicle control ($P > 0.05$, $n = 3$).

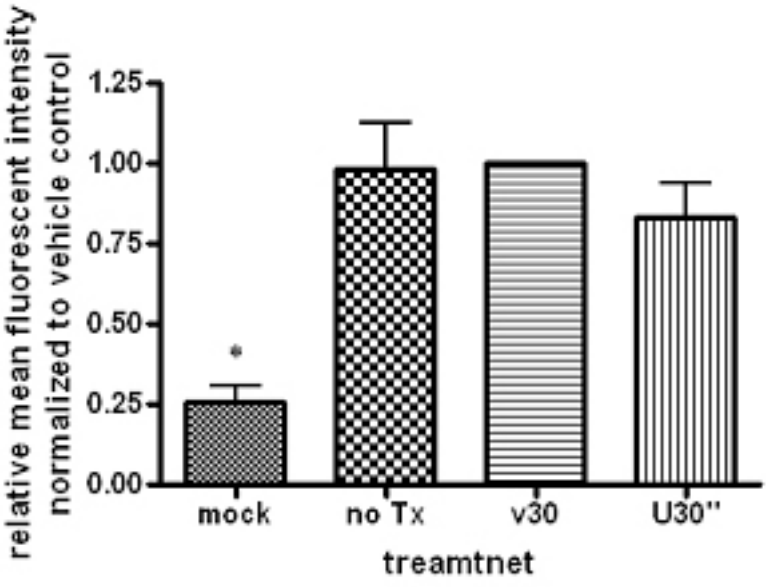


Figure 21. Activation of the ERK/MAPK pathway by HGF does not affect the trafficking of the $GABA_A$ receptor

The ERK/MAPK pathway was activated with 10 ng/mL HGF for 5, 15, or 30 minutes in transiently expressing $GABA_A$ receptor FLAG-$\alpha 1\beta 2\gamma 2$ subunit. Flow cytometric analysis showed that the activation of the ERK/MAPK pathway did not affect the cell surface expression of the receptor ($P > 0.5$, $n = 4$). Also it showed that the anti FLAG-FITC antibody used was highly specific ($P < 0.01$).

(-): untransfected HEK-t cells only

Ab: untransfected HEK-t cells with anti FLAG-FITC antibody

mock: HEK-t cells were mock transfected and anti FLAG-FITC was added

no Tx: transfected HEK-t cells with anti FLAG-FITC, used as a control

HGF5, 15, 30: transfected HEK-t cells treated with 10 ng/mL HGF for indicated duration (minutes)

Figure 21. Activation of the ERK/MAPK pathway by HGF does not affect the trafficking of the $GABA_A$ receptor

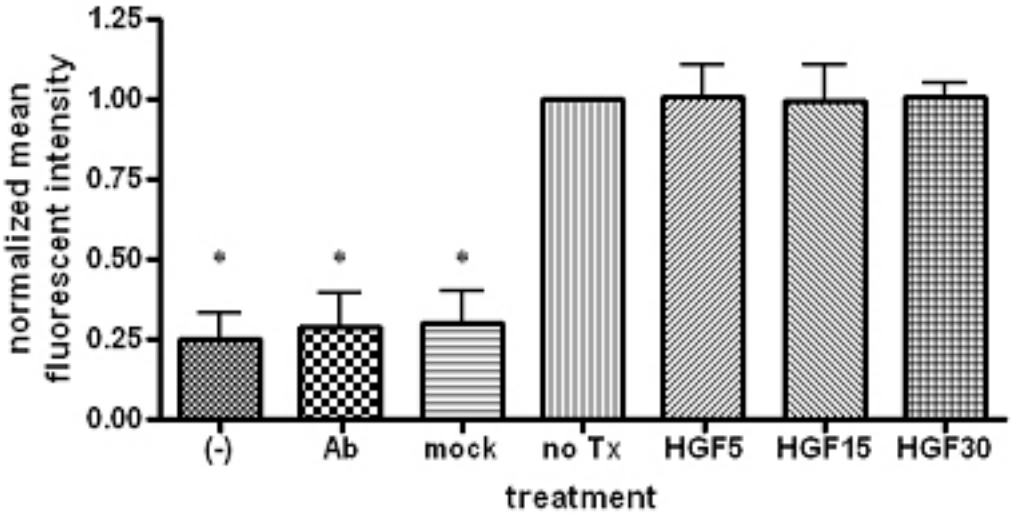


Figure 22. Activation of the PKC showed no effect on the cell surface expression of the $GABA_A$ receptor at 15 minutes

PKC was activated with 25 and 100 nM PMA in HEK-t cells transiently expressing $GABA_A$ receptor FLAG-α1β2γ2 subunits and incubated for 15 minutes at 37°C. The flow cytometric analysis using anti-FLAG-FITC antibody showed that there was no statistically significant effect on the cell surface expression of the $GABA_A$ receptor ($P > 0.05$, $n = 4$).

Mock: mock transfected HEK-t cells + anti-FLAG-FITC antibody

No Tx: transfected HEK-t cells + anti-FLAG-FITC antibody

V15: transfected HEK-t cells treated with DMSO for 15 minutes + anti-FLAG-FITC antibody

25PMA15: transfected HEK-t cells treated with 25 μM PMA for 15 minutes + anti-FLAG-FITC antibody

100PMA15: transfected HEK-t cells treated with 100 μM PMA for 15 minutes + anti-FLAG-FITC antibody

Figure 22. Activation of the PKC showed no effect on the cell surface expression of the $GABA_A$ receptor at 15 minutes

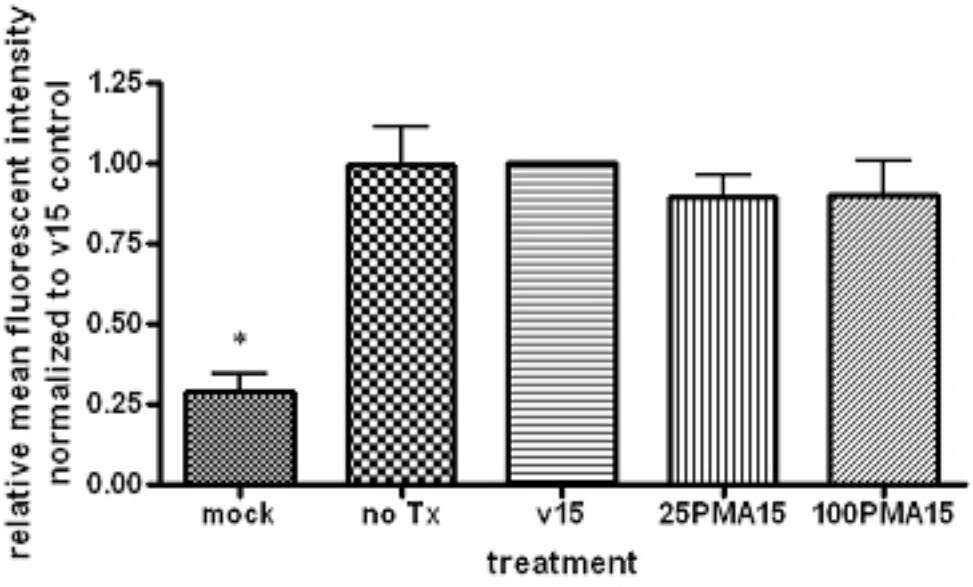
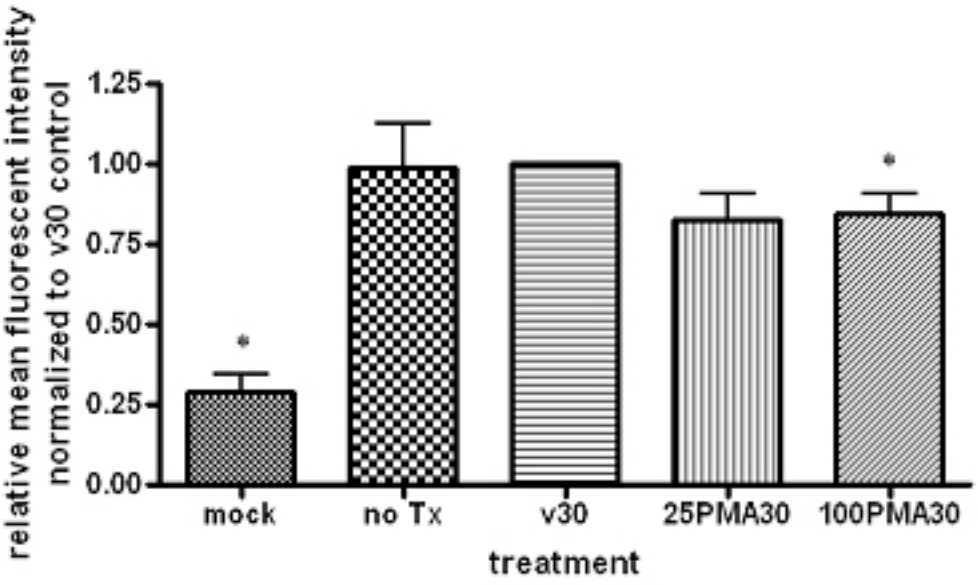


Figure 23. Activation of the PKC showed internalization of the $GABA_A$ receptor at 30 minutes

PKC was activated with 25 and 100 nM PMA for 30 minutes at 37°C. It showed that 100 nM PMA effectively internalized the $GABA_A$ receptor as reported using a confocal microscope (Herring et al., 2005). These results served as a positive control to other flow cytometric analysis used in this study. This technique was reliable, and the data were valid. ($P < 0.05$, $n = 4$).



CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

Summary

The relationship between the ERK/MAPK pathway and $GABA_A$ receptor function, trafficking, and biochemical modulation in HEK-t cells was investigated in this dissertation (Supplemental figure 1). The results showed that while the ERK/MAPK pathway inhibition by U0126 potentiated the GABA-gated peak amplitude of the current (Bell-Horner et al., 2006), activation of this pathway by HGF, which activated the ERK/MAPK pathway but not the Akt/PI3K pathway, did not have any effect on the amplitude. In addition, neither inhibition of the ERK/MAPK pathway with U0126 nor the activation of this pathway with HGF changed the $GABA_A$ receptor cell surface density in this cell model. Therefore, it appeared that the ERK/MAPK pathway inhibition-induced potentiation of the GABA-gated current observed was due to the regulation of gating of the receptor instead of influencing receptor trafficking. Interestingly, mutating the presumptive target of the ERK/MAPK pathway, the Thr 375 residue of the $GABA_A$ receptor $\alpha 1$ subunit to alanine, abolished the U0126-induced potentiation of GABA-gated current (Bell-Horner et al., 2006). However, this amino acid residue was found not to be a direct target of the ERK/MAPK pathway based on a series of biochemical analyses. Further, the $GABA_A$ receptor $\alpha 1$ subunit did not have protein-protein association with ERK1/2 under control or ERK/MAPK pathway-activated conditions in this cell model.

Issues with the perforated patch technique

The perforated patch technique was used in the electrophysiological recordings of the GABA-gated current in order to preserve the intracellular milieu that contains the various signaling components of interest. This method prevents the signaling molecules from dialyzing into the recording pipette tip, which would happen when conventional whole cell patch is used. The recording protocol used in this study was adapted from prior studies conducted in the Dillon laboratory. After the pipette tip containing gramicidin for the perforation is attached to the recording cell, 5 μM of GABA (EC_{30}) is applied to see the progress of the perforation. When two consecutive recordings show a peak current amplitude within 10%, the perforated patch is deemed to have reached its maximum opening and is stable. This value is set as the control currents. After these steps, the HGF bath application starts. Based on this procedure, it is worth noting that the starting time for the application of the HGF was not set at any pre-defined time point, but rather based on achieving a stable baseline recording. This brought up an issue of the stability of the perforation. Since the voltage is clamped, the amplitude can fluctuate depending on the resistance. In other words, the perforation could produce two consecutive peak current amplitudes that were stable, but potentially, the perforation could continue later. If so, perceived increases in current amplitude following drug treatment at later time points could, potentially, occur by chance. To investigate this issue, gramicidin-perforation control experiments were conducted, in which three different concentrations of gramicidin were used to assess the duration and stability of the perforation. GABA was applied using the same 5 minutes interval paradigm as used previously and the peak current amplitude was monitored. The results showed that the final concentration of 2.5 μg/mL gramicidin caused the patch to continue to perforate after two

hours of recording (Supplemental figure 2). The standard error in this setting was relatively small. When 5.0 μg/mL gramicidin was used, it began to produce stable peak current amplitude around one hour into the perforation and reached a plateau (Supplemental figure 3). When the data sets that did not follow a pattern to form a stable perforation were eliminated, it produced a reasonable stable pattern of perforation, reaching a plateau. We hoped that increasing the concentration of the gramicidin would enable reaching its plateau faster than that observed with 5.0 μg/mL; however, when 10.0 μg/mL gramicidin was used, the perforation still continued and did not show any sign of stabilizing to a plateau (Supplemental figure 4). Since the use of 2.5 μg/mL gramicidin showed the smallest standard error (least fluctuation), it was used experimentally to see the effect of the ERK/MAPK pathway activation (by HGF) on GABA-gated currents. HGF application was initiated 55 minutes following the establishment of perforation. HGF application was prolonged to 20 minutes instead of 10 minutes (Supplemental figure 5). However, as seen in the summary graph, the perforation continued while the HGF was applied. Therefore, 5.0 μg/mL gramicidin may have been the optimal concentration. Therefore, perforated patch recording of the GABA-gated current using 5.0 μg/mL gramicidin within this recording protocol would be helpful to confirm if the ERK/MAPK pathway has any effect on GABA-gated currents.

Another issue within the electrophysiological experiments was that these experiments were conducted at room temperature, instead of at physiological (35 – 37°C) temperature. Western blot analysis showed that experiments conducted at room temperature resulted in a delayed maximal induction of the ERK/MAPK pathway activation following HGF treatment (Figure 17). Therefore, the downstream effect of the ERK/MAPK pathway would require potentially more time than the 10 minutes of HGF application used in the electrophysiological

recordings at room temperature. Temperature controlled experiments would be needed in future studies to better define the effect of activation of the ERK/MAPK pathway on GABA-gated current.

Temperature issue and endogenous β subunit expression

In the investigation of $GABA_A$ receptor trafficking using flow cytometric analysis, the inhibition of the ERK/MAPK pathway by U0126 showed no effect. This experiment was conducted using the same concentration of U0126 as that used in the electrophysiology experiments, but at physiological temperature (37°C). The electrophysiological data in the previous study, showing the potentiation of the GABA-gated current, was seen in 10 minutes of U0126 application at room temperature. It is possible that any effect of the ERK/MAPK pathway inhibition might have occurred before 15 minutes of its application due to the physiological temperature.

Originally, an anti-β subunit antibody was used to detect the change in cell surface receptor density. It was chosen due to the weak specificity of the commercially available anti α subunit antibody. However, the use of this anti β subunit antibody showed high non-specific binding when 4D4 and untransfected HEK-t cells were compared. HEK-t cells expressing endogenous β3 subunit may have been the cause of the apparent high non-specific binding (Davies et al., 2000) since the antibody does not discriminate β2 from β3 subunit. Choosing other secondary antibody-FITC conjugates could also help resolve the high non-specific binding issue of the secondary antibody.

Cross-talk between Akt/PI3K, the ERK/MAPK, and PKC pathway in trafficking of the $GABA_A$ receptor

The Akt/PI3K pathway has been implicated in the trafficking of the $GABA_A$ receptor and increases the receptor density at cell surface (Wan et al., 1997; Wang et al., 2003). HGF has been shown by others to induce the activation of Akt, albeit in different cell types (Coltella et al., 2003; Kakazu et al., 2004). However, in our hands, the application of HGF did not change the phosphorylation status of the Akt in 4D4 cells (Figure 7). One possibility is that in the HEK cells, the activation of Akt may already be high, and therefore the lack of inducibility of Akt in response to HGF might have been attributable to a "ceiling effect". This means that the expression of the $GABA_A$ receptor on cell surface through the Akt/PI3K pathway may be at the maximum. Thus, if we interpret that the "ceiling effect" is due to maximal, or at the very least abundant, levels of $GABA_A$ receptors at the plasma membrane, then it may be reasonable to not see too much additional potentiation of current or surface expression of $GABA_A$ receptors. This is consistent with the fact that U0126 resulted in a relatively small (10-15%) increase in GABA-gated currents. Arguably, a strong activator of the ERK/MAPK pathway may yield a more readily recordable decrease in GABA-gated currents or surface expression of the GABA receptors. The problem in our attempts to use HGF as the activator of the ERK/MAPK pathway is that HGF may not be the "cleanest" activator of the ERK/MAPK pathway since other pathways, that we did not monitor and that may have influences on GABA-gated currents, may have also been activated.

The MEK1/2 inhibitor, U0126, has some inhibitory effect on PKC, and its EC_{50} is around 10 μM, which was the concentration used in this study to inhibit the ERK/MAPK pathway (Favata et al., 1998). Since PKC is reported to be a negative regulatory factor for $GABA_A$

receptor function, the anticipated U0126-induced potentiation of GABA-gated currents might have missed due to the loss of negative regulatory input from the PKC pathway.

The ERK/MAPK pathway and the Akt/PI3K pathway have shown to crosstalk in several cell lines. In HEK cells, expression of Akt inhibited the epidermal growth factor-induced B-Raf activity, and conversely, inhibition of Akt by LY294002 up-regulated B-Raf activity (Guan et al., 2000). In the human breast cancer cell line, MCF-7, concomitant application of insulin-like growth factor (IGF) and Akt inhibitor, LY294002, resulted in increase in p-ERK level (Moelling et al., 2002). Constitutively active Akt resulted in inhibition of the ERK phosphorylation (Dai et al., 2009). Further, our own data showed that the inhibitor of PI3K pathway, LY294002, inhibited GABA-gated currents (Supplemental figure 6). Since the Akt/PI3K pathway may work in opposition to the ERK/MAPK pathway, coupled with the likelihood of high basal Akt/PI3K activity in the HEK cells, the use of the Akt/PI3K pathway inhibitor (LY294002) combined with the activation of the ERK/MAPK pathway may be needed to better focus on the effect of the ERK/MAPK pathway alone on the $GABA_A$ receptor. We assert that by inhibiting the Akt/PI3K pathway input on the trafficking of the $GABA_A$ receptor, the role of the ERK/MAPK pathway may be more specifically investigated on the regulation of the $GABA_A$ receptor.

The PKC activator, PMA, is known to also activate the ERK/MAPK pathway in addition to the activation of PKC (Moelling et al., 2002), showing the cross talk between PKC and ERK/MAPK pathways. Therefore, the PMA-induced internalization of $GABA_A$ receptors observed in the flow cytometric analysis might be partially due to the activation of the ERK/MAPK pathway. Since PMA has also been shown to inhibit Akt activity, albeit in a different cell line (Liu et al., 2006), it is possible that the more pronounced reduction in surface

expression of the $GABA_A$ receptor was due to simultaneously activating ERK while inhibiting Akt activity.

Thr 375 of the α1 subunit

Our studies also showed that Thr 375 within α1 subunit was not phosphorylated by the activation of the ERK/MAPK pathway. The models used in this study were HEK-t cells transfected with the rat $GABA_A$ receptor. The amino acid sequences between human and rat α1, β2, and γ2 subunits are nearly identical. It is therefore quite unlikely that interspecies differences contributed to the inability to detect phosphorylation of the α1 subunit. Therefore, based on our experiments, the data suggest that Thr 375 within α1 subunit was not a direct target of ERK1/2.

Interestingly, the mutation of Thr 375 of the α1 subunit to Ala showed unexpected inhibition of the GABA-gated current when the inhibitor of the ERK/MAPK pathway was used. As such, it would be worth determining the consequences of ERK inhibition on the trafficking of this mutant receptor. If the Thr 375 within α1 subunit was also a target of Akt, this may solve the mystery of the unexpected results from this mutant. Use of U0126 will completely inhibit ERK1/2. It may have some inhibitory effect on PKC but not complete inhibition; thus, PKC is still basally active to produce negative regulation of $GABA_A$ receptor. Due to the inhibition of ERK1/2, Akt is reciprocally active to produce more positive regulatory effect on GABA-gated currents, which was seen in wildtype (Figure 1). However, due to the elimination of Thr 375 (by mutating it to Ala), Akt cannot show the positive regulatory effect on $GABA_A$ receptor at all. WE know that Akt targets the β2 subunit in regulating the expression of the receptors on cell surface. However, here, we suggest that the Thr 375 of α1 subunit may also be a functionally relevant target of Akt.

Future directions

The focus of this study was on the α1 subunit of the $GABA_A$ receptor since this is the most ubiquitously expressed subunit of the $GABA_A$ receptor in the mature mammalian brain. The α1 subunit has a minimal consensus phosphorylation site for ERK1/2 (S/T-P) within the intracellular loop between TM3 and TM4. Meanwhile the α6 subunit contains a full consensus phosphorylation amino acid sequence (P-x-S/T-P). α6 subunits are highly expressed in cerebellum although not all α subunits in cerebellum are α6. BDNF induces the internalization of the $GABA_A$ receptor through activation of the Trk B receptor in cerebellum. However, the specific cell signaling pathways involved in this internalization have not been discovered. It might be interesting to see if HGF-induced activation of the ERK/MAPK pathway in HEK-t cells expressing α6β2γ2-configuration of $GABA_A$ receptor would mimic the internalization effect of BDNF on $GABA_A$ receptors.

MEK1/2 inhibitor, U0126, also inhibits MEK5, and subsequently inhibits ERK5. As such, an additional and worthwhile future direction may be to define the role of ERK5 in the regulation of GABA-gated currents. One potential experiment that can be conducted is to use the ERK1/2 selective inhibitor, PD184352, to tease out the role of ERK1/2 specifically on $GABA_A$ receptor function.

The mechanism of the ERK/MAPK pathway regulation of the $GABA_A$ receptor function was explored in this study since steroidal hormones and neurotrophic factors often induce the activation of this pathway. Previous studies from the Singh and Dillon labs suggested that the ERK/MAPK pathway was involved in the negative regulation of the α1β2γ2 configuration of $GABA_A$ receptor function in HEK-t cells. In this dissertation, however, I showed that while

inhibition of the ERK/MAPK pathway led to the potentiation of GABA-gated currents, the activation of the ERK/MAPK pathway did not influence the GABA-gated current peak amplitude. Activation or inhibition of the ERK/MAPK pathway also did not influence the receptor density at cell surface. And further, the consensus ERK phosphorylation site identified within the α1 subunit, which was involved in the ERK/MAPK pathway inhibition-induced potentiation of the GABA-gated current, was not a direct target for the ERK/MAPK pathway activation.

The Need for Future Studies

GABAergic neurotransmission system is involved in anxiety-depression related mood disorders in addition to other neurotransmission systems such as serotonin. Steroid hormones are often considered as having a major influence on those mood disorders as illustrated in PMS, PMDD, postpartum depression and psychosis, and menopausal mood swings in women where the hormonal levels show large fluctuations and are unstable. Women also show three times higher rate of having major depression than men. $GABA_A$ receptor is regulated in many ways, such as different expression of the subunits, trafficking of the receptor, phasic and tonic hyperpolarization of neurons, gating of the receptor, and phospho-regulation of the receptor. It is possible that steroidal hormones influence $GABA_A$ receptor directly or indirectly through the regulation of cell signaling pathways, or regulating the expression of various factors, including BDNF, in brain. Only through a better and more thorough understanding of the how the $GABA_A$ receptor is regulated will we be able to define better means of treating such disorders as depression and/or anxiety, leading ultimately to providing a higher quality of life.

CHAPTER 6

SUPPLEMENTAL ILLUSTRATIONS

Supplemental figure 1. Results summary

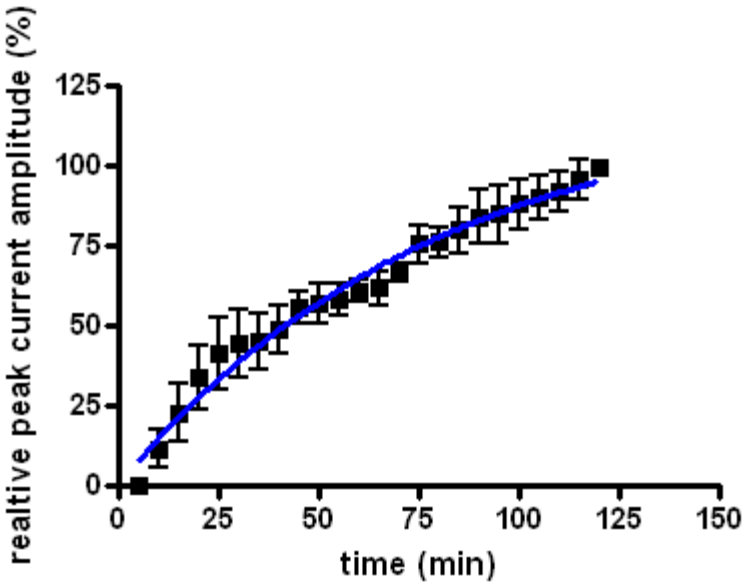
The results of the experiments using 4D4 cells were summarized below.

	p-ERK1/2	p-Akt	GABA-gated current	surface GABA-A R
U0126	↓	N.D.	↑	→*
HGF	↑	→	→	→*
LY294002	N.D.	N.D.	↓	N.D.
PMA	N.D.	N.D.	N.D.	↓*

N.D. = Not determined, * = HEK-t cells

Supplemental figure 2. 2.5 μg/mL gramicidin control

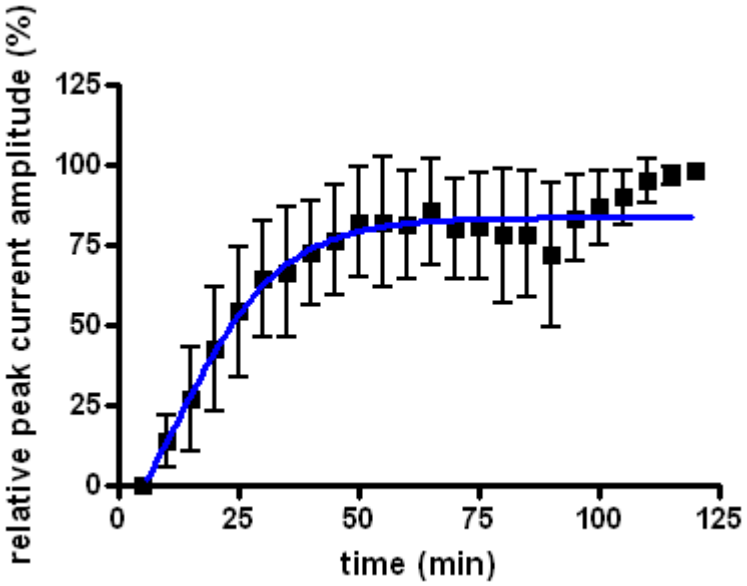
Final concentration of 2.5 μg/mL gramicidin was added to the pipette solution for the perforation (n = 4). Two hours recording with 5 minutes interval of 5 μM GABA (EC_{30}) did not reach its plateau.



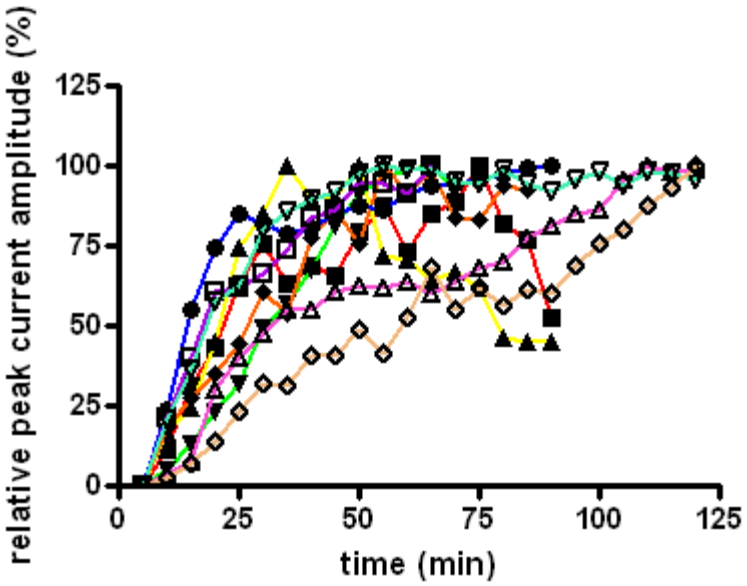
Supplemental figure 3. 5.0 μg/mL gramicidin control

Final concentration of 5.0 μg/mL gramicidin was added to the pipette solution for the perforation (n = 9) (A). Although this concentration reached its plateau, the S.E. was large due to the data sets that did not follow stable perforation establishment (B).

(A) Summary of all of the data collected (n = 9)



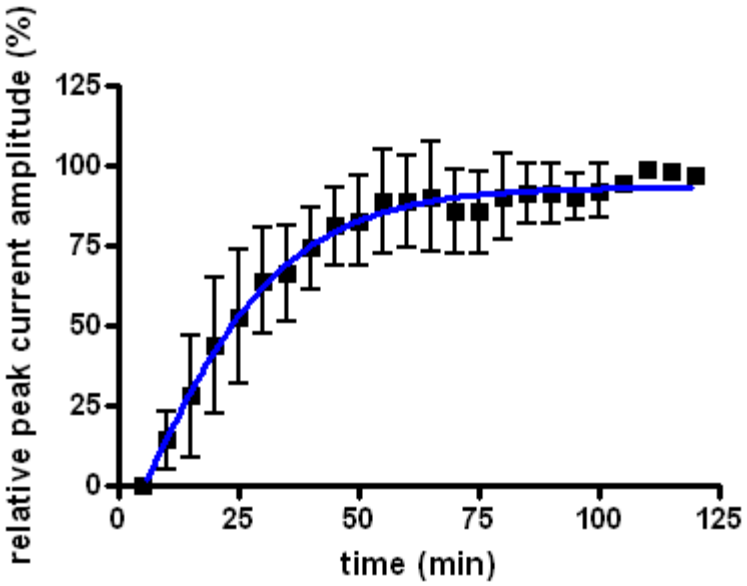
(B) Individually plotted



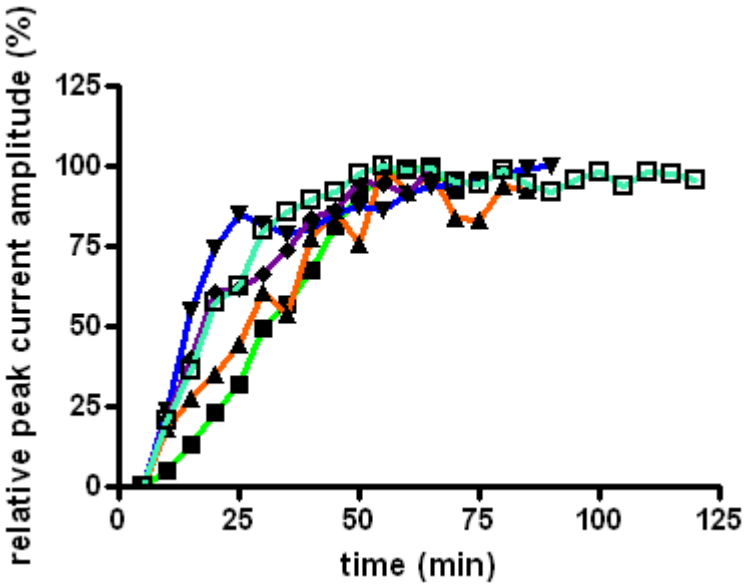
Supplemental figure 3. 5.0 μg/mL gramicidin control

When the data set showing unstable perforation was eliminated, it showed more stable perforation establishing pattern (n = 5).

(C) Summary of n = 5 without unstable perforation patterns



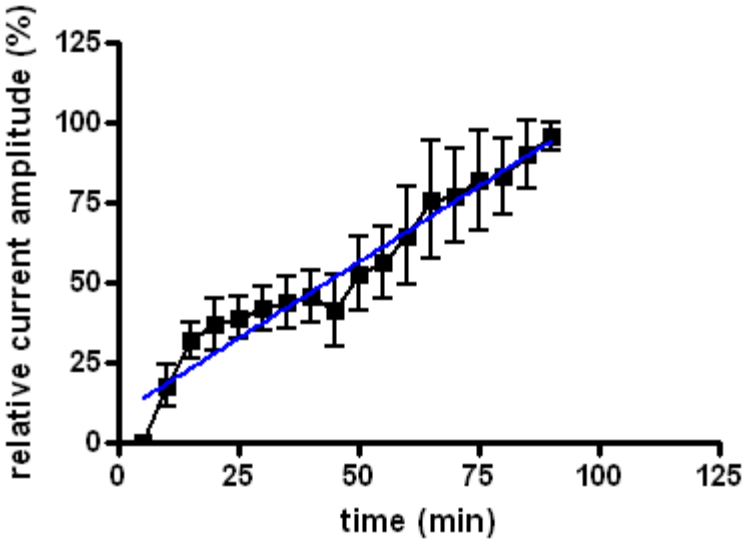
(D) Individual data sets that were included in the graph above



Supplemental figure 4. 10.0 μg/mL gramicidin control

10.0 μg/mL gramicidin did not reach its plateau. The S.E. was relatively large, and this concentration could not be used (n = 3).

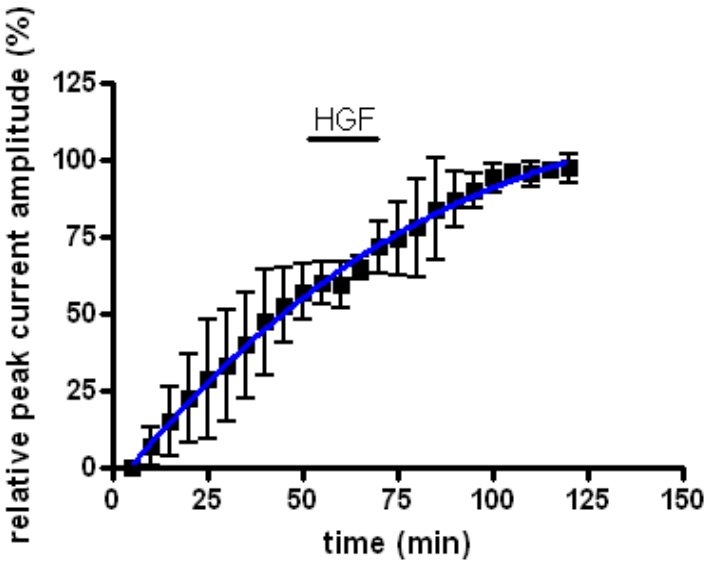
(A)



Supplemental figure 5. It is critical to reach its plateau before HGF application

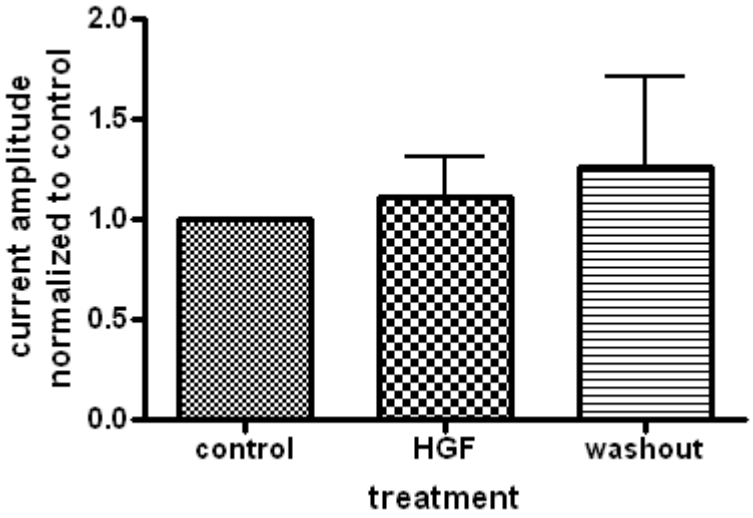
Experimentally HGF was applied for 20 minutes at room temperature (n = 3) while 2.5 μg/mL gramicidin was used for the perforation. As it showed below, the perforation continued, and HGF treatment didn't appear to affect the peak current amplitude.

(A) Summary of the HGF treated 4D4 cells with 2.5 μg/mL gramicidin perforation



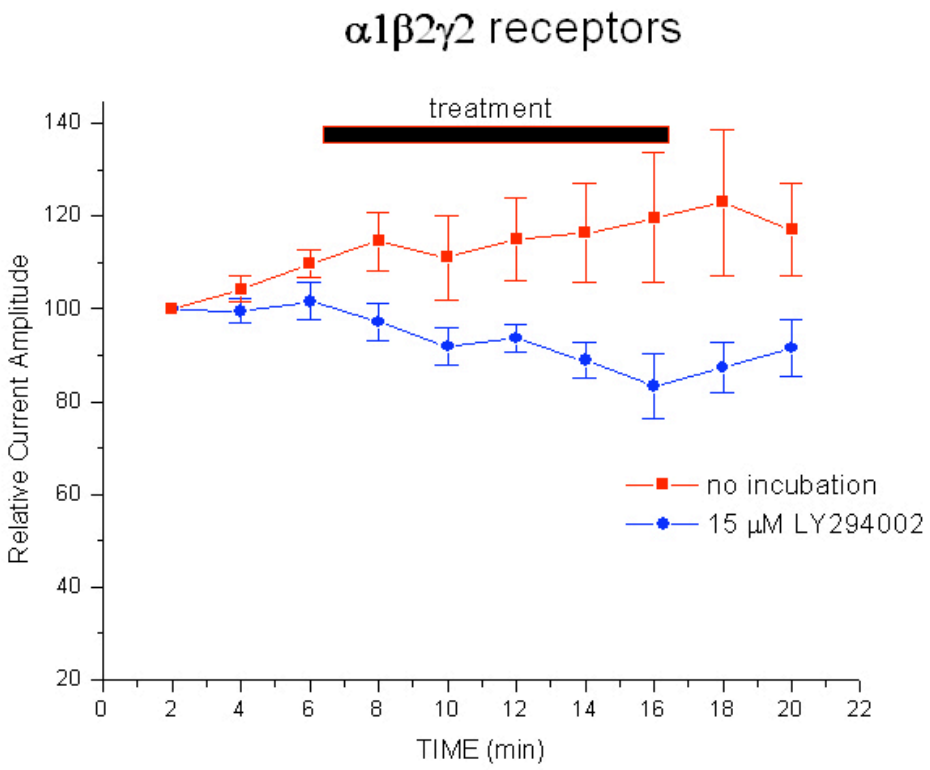
Supplemental figure 5. It is critical to reach its plateau before HGF application

(B) The summary graph comparing the HGF effect to its control. The control was set from two control currents right before the HGF application.



Supplemental figure 6. Inhibition of Akt/PI3K pathway by LY294002 inhibits GABA-gated current (Adapted from Dillon lab)

4D4 cells were treated with 15 μM LY294004 and GABA-gated currents were recorded every 2 minutes with whole cell patch technique. Compared to control, the LY294002 treated cells produced progressively smaller currents.



BIBLIOGRAPHY

Allan AM, Gallaher EJ, Gionet SE, Harris RA (1988) Genetic selection for benzodiazepine ataxia produces functional changes in the gamma-aminobutyric acid receptor chloride channel complex. Brain Res 452:118-126.

Angelotti TP, Uhler MD, Macdonald RL (1993) Enhancement of recombinant gamma-aminobutyric acid type A receptor currents by chronic activation of cAMP-dependent protein kinase. Mol Pharmacol 44:1202-1210.

Baccarini M (2005) Second nature: biological functions of the Raf-1 "kinase". FEBS Lett 579:3271-3277.

Bach B, Meudec E, Lepoutre JP, Rossignol T, Blondin B, Dequin S, Camarasa C (2009) New insights into {gamma}-aminobutyric acid catabolism: Evidence for {gamma}-hydroxybutyric acid and polyhydroxybutyrate synthesis in Saccharomyces cerevisiae. Appl Environ Microbiol 75:4231-4239.

Barnard EA (1992) Receptor classes and the transmitter-gated ion channels. Trends Biochem Sci 17:368-374.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ (1998) International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. Pharmacol Rev 50:291-313.

Bateson AN, Lasham A, Darlison MG (1991) gamma-Aminobutyric acidA receptor heterogeneity is increased by alternative splicing of a novel beta-subunit gene transcript. J Neurochem 56:1437-1440.

Belelli D, Lambert JJ (2005) Neurosteroids: endogenous regulators of the GABA(A) receptor. Nat Rev Neurosci 6:565-575.

Belelli D, Lan NC, Gee KW (1990) Anticonvulsant steroids and the GABA/benzodiazepine receptor-chloride ionophore complex. Neurosci Biobehav Rev 14:315-322.

Belelli D, Pistis M, Peters JA, Lambert JJ (1999) The interaction of general anaesthetics and neurosteroids with GABA(A) and glycine receptors. Neurochem Int 34:447-452.

Belelli D, Casula A, Ling A, Lambert JJ (2002) The influence of subunit composition on the interaction of neurosteroids with GABA(A) receptors. Neuropharmacology 43:651-661.

Bell-Horner CL, Dohi A, Nguyen Q, Dillon GH, Singh M (2006) ERK/MAPK pathway regulates GABAA receptors. J Neurobiol 66:1467-1474.

Bhave SV, Ghoda L, Hoffman PL (1999) Brain-derived neurotrophic factor mediates the anti-apoptotic effect of NMDA in cerebellar granule neurons: signal transduction cascades and site of ethanol action. J Neurosci 19:3277-3286.

Bingley PJ, Christie MR, Bonifacio E, Bonfanti R, Shattock M, Fonte MT, Bottazzo GF, Gale EA (1994) Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. Diabetes 43:1304-1310.

Blair LA, Levitan ES, Marshall J, Dionne VE, Barnard EA (1988) Single subunits of the GABAA receptor form ion channels with properties of the native receptor. Science 242:577-579.

Bormann J (1988) Electrophysiology of GABAA and GABAB receptor subtypes. Trends Neurosci 11:112-116.

Boulton TG, Yancopoulos GD, Gregory JS, Slaughter C, Moomaw C, Hsu J, Cobb MH (1990) An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science 249:64-67.

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.

Browning MD, Bureau M, Dudek EM, Olsen RW (1990) Protein kinase C and cAMP-dependent protein kinase phosphorylate the beta subunit of the purified gamma-aminobutyric acid A receptor. Proc Natl Acad Sci U S A 87:1315-1318.

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.

Brunig I, Penschuck S, Berninger B, Benson J, Fritschy JM (2001) BDNF reduces miniature inhibitory postsynaptic currents by rapid downregulation of GABA(A) receptor surface expression. Eur J Neurosci 13:1320-1328.

Burt DR, Kamatchi GL (1991) GABAA receptor subtypes: from pharmacology to molecular biology. FASEB J 5:2916-2923.

Callachan H, Cottrell GA, Hather NY, Lambert JJ, Nooney JM, Peters JA (1987) Modulation of the GABAA receptor by progesterone metabolites. Proc R Soc Lond B Biol Sci 231:359-369.

Campbell EM, Peterkin D, O'Grady K, Sanson-Fisher R (1997) Premenstrual symptoms in general practice patients. Prevalence and treatment. J Reprod Med 42:637-646.

Chaudhry FA, Reimer RJ, Bellocchio EE, Danbolt NC, Osen KK, Edwards RH, Storm-Mathisen J (1998) The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons. J Neurosci 18:9733-9750.

Cheng Q, Yeh HH (2003) Brain-derived neurotrophic factor attenuates mouse cerebellar granule cell GABA(A) receptor-mediated responses via postsynaptic mechanisms. J Physiol 548:711-721.

Coltella N, Manara MC, Cerisano V, Trusolino L, Di Renzo MF, Scotlandi K, Ferracini R (2003) Role of the MET/HGF receptor in proliferation and invasive behavior of osteosarcoma. FASEB J 17:1162-1164.

Concas A, Mostallino MC, Porcu P, Follesa P, Barbaccia ML, Trabucchi M, Purdy RH, Grisenti P, Biggio G (1998) Role of brain allopregnanolone in the plasticity of gamma-aminobutyric acid type A receptor in rat brain during pregnancy and after delivery. Proc Natl Acad Sci U S A 95:13284-13289.

Costrini NV, Kalkhoff RK (1971) Relative effects of pregnancy, estradiol, and progesterone on plasma insulin and pancreatic islet insulin secretion. J Clin Invest 50:992-999.

Crawley JN, Glowa JR, Majewska MD, Paul SM (1986) Anxiolytic activity of an endogenous adrenal steroid. Brain Res 398:382-385.

Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR, et al. (1991) Cloning of the gamma-aminobutyric acid (GABA) rho 1 cDNA: a GABA receptor subunit highly expressed in the retina. Proc Natl Acad Sci U S A 88:2673-2677.

Dai R, Chen R, Li H (2009) Cross-talk between PI3K/Akt and MEK/ERK pathways mediates endoplasmic reticulum stress-induced cell cycle progression and cell death in human hepatocellular carcinoma cells. Int J Oncol 34:1749-1757.

Davies PA, Wang W, Hales TG, Kirkness EF (2003) A novel class of ligand-gated ion channel is activated by Zn2+. J Biol Chem 278:712-717.

Davies PA, Hoffmann EB, Carlisle HJ, Tyndale RF, Hales TG (2000) The influence of an endogenous beta3 subunit on recombinant GABA(A) receptor assembly and pharmacology in WSS-1 cells and transiently transfected HEK293 cells. Neuropharmacology 39:611-620.

Deng L, Nielsen M, Olsen RW (1991) Pharmacological and biochemical properties of the gamma-aminobutyric acid-benzodiazepine receptor protein from codfish brain. J Neurochem 56:968-977.

Draguhn A, Axmacher N, Kolbaev S (2008) Presynaptic ionotropic GABA receptors. Results Probl Cell Differ 44:69-85.

Duan HF, Wu CT, Lu Y, Wang H, Liu HJ, Zhang QW, Jia XX, Lu ZZ, Wang LS (2004) Sphingosine kinase activation regulates hepatocyte growth factor induced migration of endothelial cells. Exp Cell Res 298:593-601.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661-665.

Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, Tobin AJ (1991) Two genes encode distinct glutamate decarboxylases. Neuron 7:91-100.

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

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273:18623-18632.

Finn DA, Sinnott RS, Ford MM, Long SL, Tanchuck MA, Phillips TJ (2004) Sex differences in the effect of ethanol injection and consumption on brain allopregnanolone levels in C57BL/6 mice. Neuroscience 123:813-819.

Fodor L, Biro T, Maksay G (2005) Nanomolar allopregnanolone potentiates rat cerebellar GABAA receptors. Neurosci Lett 383:127-130.

Gibbs RB (1999) Treatment with estrogen and progesterone affects relative levels of brain-derived neurotrophic factor mRNA and protein in different regions of the adult rat brain. Brain Res 844:20-27.

Gonzalez Deniselle MC, Garay L, Gonzalez S, Saravia F, Labombarda F, Guennoun R, Schumacher M, De Nicola AF (2007) Progesterone modulates brain-derived neurotrophic factor and choline acetyltransferase in degenerating Wobbler motoneurons. Exp Neurol 203:406-414.

Greco S, Storelli C, Marsigliante S (2006) Protein kinase C (PKC)-delta/-epsilon mediate the PKC/Akt-dependent phosphorylation of extracellular signal-regulated kinases 1 and 2 in MCF-7 cells stimulated by bradykinin. J Endocrinol 188:79-89.

Guan KL, Figueroa C, Brtva TR, Zhu T, Taylor J, Barber TD, Vojtek AB (2000) Negative regulation of the serine/threonine kinase B-Raf by Akt. J Biol Chem 275:27354-27359.

Gunn-Moore FJ, Williams AG, Toms NJ, Tavare JM (1997) Activation of mitogen-activated protein kinase and p70S6 kinase is not correlated with cerebellar granule cell survival. Biochem J 324 (Pt 2):365-369.

Halbreich U (2003) The etiology, biology, and evolving pathology of premenstrual syndromes. Psychoneuroendocrinology 28 Suppl 3:55-99.

Harrison NL, Simmonds MA (1984) Modulation of the GABA receptor complex by a steroid anaesthetic. Brain Res 323:287-292.

Herbert TP, Kilhams GR, Batty IH, Proud CG (2000) Distinct signalling pathways mediate insulin and phorbol ester-stimulated eukaryotic initiation factor 4F assembly and protein synthesis in HEK 293 cells. J Biol Chem 275:11249-11256.

Herd MB, Belelli D, Lambert JJ (2007) Neurosteroid modulation of synaptic and extrasynaptic GABA(A) receptors. Pharmacol Ther 116:20-34.

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.

Herzog AG (2009) Hormonal therapies: progesterone. Neurotherapeutics 6:383-391.

Herzog AG, Frye CA (2003) Seizure exacerbation associated with inhibition of progesterone metabolism. Ann Neurol 53:390-391.

Hettema JM, An SS, Neale MC, Bukszar J, van den Oord EJ, Kendler KS, Chen X (2006) Association between glutamic acid decarboxylase genes and anxiety disorders, major depression, and neuroticism. Mol Psychiatry 11:752-762.

Higuchi T, Orita T, Katsuya K, Yamasaki Y, Akiyama K, Li H, Yamamoto T, Saito Y, Nakamura M (2004) MUC20 suppresses the hepatocyte growth factor-induced Grb2-Ras

pathway by binding to a multifunctional docking site of met. Mol Cell Biol 24:7456-7468.

Hylan TR, Sundell K, Judge R (1999) The impact of premenstrual symptomatology on functioning and treatment-seeking behavior: experience from the United States, United Kingdom, and France. J Womens Health Gend Based Med 8:1043-1052.

Ives JH, Drewery DL, Thompson CL (2002) Differential cell surface expression of GABAA receptor alpha1, alpha6, beta2 and beta3 subunits in cultured mouse cerebellar granule cells influence of cAMP-activated signalling. J Neurochem 80:317-327.

Kakazu A, Chandrasekher G, Bazan HE (2004) HGF protects corneal epithelial cells from apoptosis by the PI-3K/Akt-1/Bad- but not the ERK1/2-mediated signaling pathway. Invest Ophthalmol Vis Sci 45:3485-3492.

Kalueff AV (2007) Mapping convulsants' binding to the GABA-A receptor chloride ionophore: a proposed model for channel binding sites. Neurochem Int 50:61-68.

Karege F, Vaudan G, Schwald M, Perroud N, La Harpe R (2005) Neurotrophin levels in postmortem brains of suicide victims and the effects of antemortem diagnosis and psychotropic drugs. Brain Res Mol Brain Res 136:29-37.

Karege F, Perret G, Bondolfi G, Schwald M, Bertschy G, Aubry JM (2002) Decreased serum brain-derived neurotrophic factor levels in major depressed patients. Psychiatry Res 109:143-148.

Kasler HG, Victoria J, Duramad O, Winoto A (2000) ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. Mol Cell Biol 20:8382-8389.

Kato K (1990) Novel GABAA receptor alpha subunit is expressed only in cerebellar granule cells. J Mol Biol 214:619-624.

Keir WJ, Kozak CA, Chakraborti A, Deitrich RA, Sikela JM (1991) The cDNA sequence and chromosomal location of the murine GABAA alpha 1 receptor gene. Genomics 9:390-395.

Khrestchatisky M, MacLennan AJ, Chiang MY, Xu WT, Jackson MB, Brecha N, Sternini C, Olsen RW, Tobin AJ (1989) A novel alpha subunit in rat brain GABAA receptors. Neuron 3:745-753.

Kirkness EF, Bovenkerk CF, Ueda T, Turner AJ (1989) Phosphorylation of gamma-aminobutyrate (GABA)/benzodiazepine receptors by cyclic AMP-dependent protein kinase. Biochem J 259:613-616.

Klatzkin RR, Morrow AL, Light KC, Pedersen CA, Girdler SS (2006) Histories of depression, allopregnanolone responses to stress, and premenstrual symptoms in women. Biol Psychol 71:2-11.

Knip M, Siljander H (2008) Autoimmune mechanisms in type 1 diabetes. Autoimmun Rev 7:550-557.

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.

Korpi ER, Mihalek RM, Sinkkonen ST, Hauer B, Hevers W, Homanics GE, Sieghart W, Luddens H (2002) Altered receptor subtypes in the forebrain of GABA(A) receptor delta subunit-deficient mice: recruitment of gamma 2 subunits. Neuroscience 109:733-743.

Kotzka J, Lehr S, Roth G, Avci H, Knebel B, Muller-Wieland D (2004) Insulin-activated Erk-mitogen-activated protein kinases phosphorylate sterol regulatory element-binding Protein-2 at serine residues 432 and 455 in vivo. J Biol Chem 279:22404-22411.

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.

Lee JD, Ulevitch RJ, Han J (1995) Primary structure of BMK1: a new mammalian map kinase. Biochem Biophys Res Commun 213:715-724.

Letinic K, Zoncu R, Rakic P (2002) Origin of GABAergic neurons in the human neocortex. Nature 417:645-649.

Levitan ES, Schofield PR, Burt DR, Rhee LM, Wisden W, Kohler M, Fujita N, Rodriguez HF, Stephenson A, Darlison MG, et al. (1988) Structural and functional basis for GABAA receptor heterogeneity. Nature 335:76-79.

Liang SL, Carlson GC, Coulter DA (2006) Dynamic regulation of synaptic GABA release by the glutamate-glutamine cycle in hippocampal area CA1. J Neurosci 26:8537-8548.

Liu H, Qiu Y, Xiao L, Dong F (2006) Involvement of protein kinase Cepsilon in the negative regulation of Akt activation stimulated by granulocyte colony-stimulating factor. J Immunol 176:2407-2413.

Lo WY, Botzolakis EJ, Tang X, Macdonald RL (2008) A conserved Cys-loop receptor aspartate residue in the M3-M4 cytoplasmic loop is required for GABAA receptor assembly. J Biol Chem 283:29740-29752.

Lolait SJ, O'Carroll AM, Kusano K, Muller JM, Brownstein MJ, Mahan LC (1989) Cloning and expression of a novel rat GABAA receptor. FEBS Lett 246:145-148.

Lommatzsch M, Hornych K, Zingler C, Schuff-Werner P, Hoppner J, Virchow JC (2006) Maternal serum concentrations of BDNF and depression in the perinatal period. Psychoneuroendocrinology 31:388-394.

Luddens H, Pritchett DB, Kohler M, Killisch I, Keinanen K, Monyer H, Sprengel R, Seeburg PH (1990) Cerebellar GABAA receptor selective for a behavioural alcohol antagonist. Nature 346:648-651.

Lundgren P, Stromberg J, Backstrom T, Wang M (2003) Allopregnanolone-stimulated GABA-mediated chloride ion flux is inhibited by 3beta-hydroxy-5alpha-pregnan-20-one (isoallopregnanolone). Brain Res 982:45-53.

Machu TK, Dillon GH, Huang R, Lovinger DM, Leidenheimer NJ (2006) Temperature: an important experimental variable in studying PKC modulation of ligand-gated ion channels. Brain Res 1086:1-8.

Madsen KK, Larsson OM, Schousboe A (2008) Regulation of excitation by GABA neurotransmission: focus on metabolism and transport. Results Probl Cell Differ 44:201-221.

Maguire J, Ferando I, Simonsen C, Mody I (2009) Excitability changes related to GABAA receptor plasticity during pregnancy. J Neurosci 29:9592-9601.

Majewska MD, Harrison NL, Schwartz RD, Barker JL, Paul SM (1986) Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. Science 232:1004-1007.

Malherbe P, Draguhn A, Multhaup G, Beyreuther K, Mohler H (1990a) GABAA-receptor expressed from rat brain alpha- and beta-subunit cDNAs displays potentiation by benzodiazepine receptor ligands. Brain Res Mol Brain Res 8:199-208.

Malherbe P, Sigel E, Baur R, Persohn E, Richards JG, Mohler H (1990b) Functional expression and sites of gene transcription of a novel alpha subunit of the GABAA receptor in rat brain. FEBS Lett 260:261-265.

Maljevic S, Krampfl K, Cobilanschi J, Tilgen N, Beyer S, Weber YG, Schlesinger F, Ursu D, Melzer W, Cossette P, Bufler J, Lerche H, Heils A (2006) A mutation in the GABA(A) receptor alpha(1)-subunit is associated with absence epilepsy. Ann Neurol 59:983-987.

Mannella P, Brinton RD (2006) Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. J Neurosci 26:9439-9447.

Martin DL, Rimvall K (1993) Regulation of gamma-aminobutyric acid synthesis in the brain. J Neurochem 60:395-407.

McDonald BJ, Moss SJ (1994) Differential phosphorylation of intracellular domains of gamma-aminobutyric acid type A receptor subunits by calcium/calmodulin type 2-dependent protein kinase and cGMP-dependent protein kinase. J Biol Chem 269:18111-18117.

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.

McKernan RM, Whiting PJ (1996) Which GABAA-receptor subtypes really occur in the brain? Trends Neurosci 19:139-143.

Mizoguchi Y, Ishibashi H, Nabekura J (2003) The action of BDNF on GABA(A) currents changes from potentiating to suppressing during maturation of rat hippocampal CA1 pyramidal neurons. J Physiol 548:703-709.

Mody N, Leitch J, Armstrong C, Dixon J, Cohen P (2001) Effects of MAP kinase cascade inhibitors on the MKK5/ERK5 pathway. FEBS Lett 502:21-24.

Moelling K, Schad K, Bosse M, Zimmermann S, Schweneker M (2002) Regulation of Raf-Akt Cross-talk. J Biol Chem 277:31099-31106.

Moss SJ, Doherty CA, Huganir RL (1992a) 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, Smart TG, Blackstone CD, Huganir RL (1992b) Functional modulation of GABAA receptors by cAMP-dependent protein phosphorylation. Science 257:661-665.

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

Mukai H, Tsurugizawa T, Ogiue-Ikeda M, Murakami G, Hojo Y, Ishii H, Kimoto T, Kawato S (2006) Local neurosteroid production in the hippocampus: influence on synaptic plasticity of memory. Neuroendocrinology 84:255-263.

Murinson BB (2004) Stiff-person syndrome. Neurologist 10:131-137.

Nappi RE, Petraglia F, Luisi S, Polatti F, Farina C, Genazzani AR (2001) Serum allopregnanolone in women with postpartum "blues". Obstet Gynecol 97:77-80.

Nusser Z, Ahmad Z, Tretter V, Fuchs K, Wisden W, Sieghart W, Somogyi P (1999) Alterations in the expression of GABAA receptor subunits in cerebellar granule cells after the disruption of the alpha6 subunit gene. Eur J Neurosci 11:1685-1697.

Nutt DJ, Malizia AL (2001) New insights into the role of the GABA(A)-benzodiazepine receptor in psychiatric disorder. Br J Psychiatry 179:390-396.

Obara Y, Horgan AM, Stork PJ (2007) The requirement of Ras and Rap1 for the activation of ERKs by cAMP, PACAP, and KCl in cerebellar granule cells. J Neurochem 101:470-482.

Ogihara T, Watada H, Kanno R, Ikeda F, Nomiyama T, Tanaka Y, Nakao A, German MS, Kojima I, Kawamori R (2003) p38 MAPK is involved in activin A- and hepatocyte growth factor-mediated expression of pro-endocrine gene neurogenin 3 in AR42J-B13 cells. J Biol Chem 278:21693-21700.

Okano Y, Mizuno K, Osada S, Nakamura T, Nozawa Y (1993) Tyrosine phosphorylation of phospholipase C gamma in c-met/HGF receptor-stimulated hepatocytes: comparison with HepG2 hepatocarcinoma cells. Biochem Biophys Res Commun 190:842-848.

Olsen RW, Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. Pharmacol Rev 60:243-260.

Olsen RW, McCabe RT, Wamsley JK (1990) GABAA receptor subtypes: autoradiographic comparison of GABA, benzodiazepine, and convulsant binding sites in the rat central nervous system. J Chem Neuroanat 3:59-76.

Olsen RW, Yang J, King RG, Dilber A, Stauber GB, Ransom RW (1986) Barbiturate and benzodiazepine modulation of GABA receptor binding and function. Life Sci 39:1969-1976.

Ortells MO, Lunt GG (1995) Evolutionary history of the ligand-gated ion-channel superfamily of receptors. Trends Neurosci 18:121-127.

Paul SM, Purdy RH (1992) Neuroactive steroids. FASEB J 6:2311-2322.

Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH (2001) Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 22:153-183.

Poisbeau P, Cheney MC, Browning MD, Mody I (1999) Modulation of synaptic GABAA receptor function by PKA and PKC in adult hippocampal neurons. J Neurosci 19:674-683.

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.

Purdy RH, Morrow AL, Moore PH, Jr., Paul SM (1991) Stress-induced elevations of gamma-aminobutyric acid type A receptor-active steroids in the rat brain. Proc Natl Acad Sci U S A 88:4553-4557.

Ramos F, el Guezzar M, Grenson M, Wiame JM (1985) Mutations affecting the enzymes involved in the utilization of 4-aminobutyric acid as nitrogen source by the yeast Saccharomyces cerevisiae. Eur J Biochem 149:401-404.

Reddy DS, Rogawski MA (2009) Neurosteroid replacement therapy for catamenial epilepsy. Neurotherapeutics 6:392-401.

Regan CP, Li W, Boucher DM, Spatz S, Su MS, Kuida K (2002) Erk5 null mice display multiple extraembryonic vascular and embryonic cardiovascular defects. Proc Natl Acad Sci U S A 99:9248-9253.

Rodriguez Gil DJ, Mitridate de Novara A, Fiszer de Plazas S (2002) Acute hypoxic hypoxia alters GABA(A) receptor modulation by allopregnanolone and pentobarbital in embryonic chick optic lobe. Brain Res 954:294-299.

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.

Rudolph U, Mohler H (2006) GABA-based therapeutic approaches: GABAA receptor subtype functions. Curr Opin Pharmacol 6:18-23.

Sanna E, Talani G, Busonero F, Pisu MG, Purdy RH, Serra M, Biggio G (2004) Brain steroidogenesis mediates ethanol modulation of GABAA receptor activity in rat hippocampus. J Neurosci 24:6521-6530.

Sarup A, Larsson OM, Schousboe A (2003) GABA transporters and GABA-transaminase as drug targets. Curr Drug Targets CNS Neurol Disord 2:269-277.

Schofield PR, Pritchett DB, Sontheimer H, Kettenmann H, Seeburg PH (1989) Sequence and expression of human GABAA receptor alpha 1 and beta 1 subunits. FEBS Lett 244:361-364.

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.

Schrader LA, Birnbaum SG, Nadin BM, Ren Y, Bui D, Anderson AE, Sweatt JD (2006) ERK/MAPK regulates the Kv4.2 potassium channel by direct phosphorylation of the pore-forming subunit. Am J Physiol Cell Physiol 290:C852-861.

Schumacher M, Weill-Engerer S, Liere P, Robert F, Franklin RJ, Garcia-Segura LM, Lambert JJ, Mayo W, Melcangi RC, Parducz A, Suter U, Carelli C, Baulieu EE, Akwa Y (2003) Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. Prog Neurobiol 71:3-29.

Shirayama Y, Chen AC, Nakagawa S, Russell DS, Duman RS (2002) Brain-derived neurotrophic factor produces antidepressant effects in behavioral models of depression. J Neurosci 22:3251-3261.

Shivers BD, Killisch I, Sprengel R, Sontheimer H, Kohler M, Schofield PR, Seeburg PH (1989) Two novel GABAA receptor subunits exist in distinct neuronal subpopulations. Neuron 3:327-337.

Sieghart W (1995) Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. Pharmacol Rev 47:181-234.

Singh M (2001) Ovarian hormones elicit phosphorylation of Akt and extracellular-signal regulated kinase in explants of the cerebral cortex. Endocrine 14:407-415.

Sommer B, Poustka A, Spurr NK, Seeburg PH (1990) The murine GABAA receptor delta-subunit gene: structure and assignment to human chromosome 1. DNA Cell Biol 9:561-568.

Tsai PS, Hayes TB, Licht P (1994) Role of aromatization in testosterone-induced inhibition of luteinizing hormone secretion in female turtles, Trachemys scripta. Biol Reprod 50:144-151.

Tsirogianni A, Pipi E, Soufleros K (2009) Specificity of islet cell autoantibodies and coexistence with other organ specific autoantibodies in type 1 diabetes mellitus. Autoimmun Rev 8:687-691.

Uzunova V, Sheline Y, Davis JM, Rasmusson A, Uzunov DP, Costa E, Guidotti A (1998) Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. Proc Natl Acad Sci U S A 95:3239-3244.

Valenzuela CF, Machu TK, McKernan RM, Whiting P, VanRenterghem BB, McManaman JL, Brozowski SJ, Smith GB, Olsen RW, Harris RA (1995) Tyrosine kinase phosphorylation of GABAA receptors. Brain Res Mol Brain Res 31:165-172.

Vetiska SM, Ahmadian G, Ju W, Liu L, Wymann MP, Wang YT (2007) GABAA receptor-associated phosphoinositide 3-kinase is required for insulin-induced recruitment of postsynaptic GABAA receptors. Neuropharmacology 52:146-155.

Veyrat-Durebex C, Pomerleau L, Langlois D, Gaudreau P (2005) Internalization and trafficking of the human and rat growth hormone-releasing hormone receptor. J Cell Physiol 203:335-344.

Wafford KA, Whiting PJ (1992) Ethanol potentiation of GABAA receptors requires phosphorylation of the alternatively spliced variant of the gamma 2 subunit. FEBS Lett 313:113-117.

Wan Q, Xiong ZG, Man HY, Ackerley CA, Braunton J, Lu WY, Becker LE, MacDonald JF, Wang YT (1997) Recruitment of functional GABA(A) receptors to postsynaptic domains by insulin. Nature 388:686-690.

Wang Q, Liu L, Pei L, Ju W, Ahmadian G, Lu J, Wang Y, Liu F, Wang YT (2003) Control of synaptic strength, a novel function of Akt. Neuron 38:915-928.

Wang QP, Jammoul F, Duboc A, Gong J, Simonutti M, Dubus E, Craft CM, Ye W, Sahel JA, Picaud S (2008) Treatment of epilepsy: the GABA-transaminase inhibitor, vigabatrin, induces neuronal plasticity in the mouse retina. Eur J Neurosci 27:2177-2187.

Wilson-Shaw D, Robinson M, Gambarana C, Siegel RE, Sikela JM (1991) A novel gamma subunit of the GABAA receptor identified using the polymerase chain reaction. FEBS Lett 284:211-215.

Woo TU, Walsh JP, Benes FM (2004) Density of glutamic acid decarboxylase 67 messenger RNA-containing neurons that express the N-methyl-D-aspartate receptor subunit NR2A in the anterior cingulate cortex in schizophrenia and bipolar disorder. Arch Gen Psychiatry 61:649-657.

Yan L, Carr J, Ashby PR, Murry-Tait V, Thompson C, Arthur JS (2003) Knockout of ERK5 causes multiple defects in placental and embryonic development. BMC Dev Biol 3:11.

Ymer S, Draguhn A, Kohler M, Schofield PR, Seeburg PH (1989a) Sequence and expression of a novel GABAA receptor alpha subunit. FEBS Lett 258:119-122.

Ymer S, Schofield PR, Draguhn A, Werner P, Kohler M, Seeburg PH (1989b) GABAA receptor beta subunit heterogeneity: functional expression of cloned cDNAs. EMBO J 8:1665-1670.

Ymer S, Draguhn A, Wisden W, Werner P, Keinanen K, Schofield PR, Sprengel R, Pritchett DB, Seeburg PH (1990) Structural and functional characterization of the gamma 1 subunit of GABAA/benzodiazepine receptors. EMBO J 9:3261-3267.

Zhang J, Walk SF, Ravichandran KS, Garrison JC (2009) Regulation of the Src homology 2 domain-containing inositol 5'-phosphatase (SHIP1) by the cyclic AMP-dependent protein kinase. J Biol Chem 284:20070-20078.

Zhou G, Bao ZQ, Dixon JE (1995) Components of a new human protein kinase signal transduction pathway. J Biol Chem 270:12665-12669.

Zhu D, Lipsky RH, Marini AM (2002) Co-activation of the phosphatidylinositol-3-kinase/Akt signaling pathway by N-methyl-D-aspartate and TrkB receptors in cerebellar granule cell neurons. Amino Acids 23:11-17.

Zhu Y, Rice CD, Pang Y, Pace M, Thomas P (2003) Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. Proc Natl Acad Sci U S A 100:2231-2236.

Zink M, Schmitt A, May B, Muller B, Demirakca T, Braus DF, Henn FA (2004) Differential effects of long-term treatment with clozapine or haloperidol on GABAA receptor binding and GAD67 expression. Schizophr Res 66:151-157.