Identification of serine 867 as new phosphorylation site on the $GABA_B$ receptor: Characterization of physiological effects.

Inauguraldissertation

zur Erlangung der Würde eines Doktors der Philosophie vorgelegt der Philosophisch Naturwissenschaftlichen Fakultät der Universität Basel von

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aus Basel-Stadt

Zürich, 2011

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

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Basel, 8. Dezember 2009

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Dedicated to my parents

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I. Aim of the thesis

The $GABA_B$ receptor is the metabotropic receptor for γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the mammalian central nervous system (CNS). The functional receptor is a heteromer consisting of a $GABA_{B1}$ and $GABA_{B2}$ subunit. Two isoforms from the $GABA_{B1}$ subunit exist; the $GABA_{B1a}$ and the $GABA_{B1b}$ isoform, whose distribution pattern differs throughout the brain. $GABA_B$ receptors manifest their inhibitory action by influencing adenylate cyclase activity, presynaptic voltage sensitive Ca^{2+} channels and postsynaptic rectifying K^+ channels. The large variety of neurological and psychiatric disorders e.g. addiction, epilepsy, nociception and depression caused by $GABA_B$ receptors dysfunctions, highlight the importance for $GABA_B$ regulatory mechanisms. One such regulatory mechanism is phosphorylation which is the principal way to regulate $GABA_B$ receptor functioning. In vivo, two phosphorylation sites on $GABA_B$ receptors were identified; serine 892 and serine 783 and both sites play a role in synaptic plasticity. Phosphorylation of serine 892 prolongs the inhibitory impact of $GABA_B$ receptors activity on the CNS and serine 783 is ascribed for its neuroprotective benefits.

The aim of this thesis was to study the modulatory effect of phosphorylation on $GABA_B$ receptor functioning. The identification of serine 867, a novel physiological relevant phosphorylation site on $GABA_B$ receptors, contrasts with the previous serine sites. Indeed, the serine 867 is positioned on the $GABA_{B1}$ subunit, constrasting with the $GABA_{B2}$ localisation of the serines 892 and 783. Interestingly, serine 867 phosphorylation is mediated by CaMKII, an abundant and relevant kinase in the CNS. Consequently, it is proposed that serine 867 phosphorylation could regulate surface availability of $GABA_B$ receptors under neuronal activation leading to synaptic plasticity modulation.

My thesis is divided in three sections; introduction, results and discussion part. The introduction will provide background information about the structure, distribution and physiological role of $GABA_B$ receptors. The influence of phosphorylation on the $GABA_B$ receptors will be clarified by the itemization of the significant kinases in the CNS and an explanation of $GABA_B$ receptor phosphorylation sites serine 892 and serine 783. The results part includes a manuscript of a potential publication and supplemental results. The final discussion will focus on the consequences of the serine 867 phosphorylation on CNS function.

II. List of abbreviations

$1a^{-/-}$ mice	transgenic $GABA_{B1a}$ subunit isoform deficient mice
$1b^{-/-}$ mice	transgenic $GABA_{B1b}$ subunit isoform deficient mice
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPK	5'AMP-dependent protein kinase
APV	D-(-)-2-Amino-5-phosphonopentanoic acid
Ca^{2+}-CaM	$Ca^{2+}$and Calmodulin
CaMKII	$Ca^{2+}$and Calmodulin-dependent kinase
CID	collision-induced dissociation
Cl^-	chloride
CNS	central nervous system
DAG	diacylglycerol
DAPI	4', 6-Diamidin-2'-phenylindoldihydrochlorid
DIV	day *in vitro*
eGFP	enhanced green fluorescent protein
EPSC	excitatory postsynaptic current
ER	endoplasmic reticulum
ESI-MS	electrospray-ionisation mass spectrometry
GABA	gamma-amino butyric acid
$GABA_A$	gamma-amino butyric acid type A receptor
$GABA_B$	gamma-amino butyric acid type B receptor
$GABAB_C$	gamma-amino butyric acid type C receptor
GDP	guanosine diphosphate
GHB	gamma-hydroxybutyrate
GIRK	G-protein-coupled inwardly-rectifying potassium channels
GPCR	G-protein coupled receptor
GST	glutathione s-transferase
GTP	guanosine-5'-triphosphate
HPLC	high pressure liquid chromatography
IPSC	fast inhibitory postsynaptic current
Da	Dalton
KN-93	N-2-3-4-Chlorophenyl-2-propenyl-methylamino-methyl-phenyl-N-2-hydroxyethyl-4-methoxy-benzene-sulphonamide
LTP	long term potentiation
MCAO	middle cerebral artery occlusion

mGluR	metabotropic glutamate receptor
MS	mass spectrometry
NMDA	N-methyl-D-aspartate
NMDAR	NMDA-receptor
PDE	phosphodiesterase
PKA	cyclic AMP-dependent protein kinase
PKC	protein kinase C
PKI	protein kinase inhibitor
PSD	postsynaptic density
RP-HPLC	reverse-phase high pressure liquid chromatography
SD	standard deviation
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TR-FRET	time-resolved fluorescence resonance energy transfer

III. Introduction

3.1 γ-aminobutyric acid (GABA) – Main inhibitory neurotransmitter in the vertebrate CNS and its receptors

Around 60 years ago, three groups first described GABA in the mammalian brain (Awapara, 1950, Roberts, 1950, Udenfriend, 1950). Four years later, Kuffler (Kuffler, 1954) and Florey (Florey, 1954) discovered the inhibitory activity of GABA on crayfish. Following that discovery, it took less than 10 years for GABA to become generally accepted as the principal inhibitory transmitter, especially in vertebrate brain (Krnjevic, 1963, Krnjevic, 1965, Jasper, 1969, Obata, 1969, Krnjevic, 1967, Dreifuss, 1969, Obata, 1967, Galindo, 1969, Curtis, 1970). There are two major classes of GABA receptors: on one hand, ligand-gated ion channels (ionotropic) $GABA_{A/C}$ receptors and on the other, G-protein-coupled (metabotropic) $GABA_B$ receptors. Ionotropic $GABA_{A/C}$ receptors (Chebib, 1999) directly gate chloride channels; the inward flow of negatively charged chloride ions quickly inhibits the postsynaptic cells. Ionotropic $GABA_A$ and $GABA_C$ receptors (also known as $GABA_{A-\rho}$ receptors) can be discriminated by their sensitivity to pharmacological agents such as barbiturates, benzodiazepines, steroids and bicuculline. Indeed, $GABA_A$ but not $GABA_C$ receptors are sensitive to such agents. Beginning of the 1980s, Hill and Bowery identified, also based on the distinct pharmacological profile for GABA and its analogues (especially bicuculline and baclofen), the second class of GABA receptors (Hill, 1981); the metabotropic $GABA_B$ receptors.

$GABA_B$ receptors function via multistep pathways involving guanine nucleotide binding proteins (G-proteins). Effects of $GABA_B$ receptor activation are slow, long lasting and thus considered as modulatory compared to $GABA_{A/C}$ activation. They even include the induction of long-term changes in synaptic strength. $GABA_B$ receptors generate late inhibitory postsynaptic potentials (IPSPs) which are important for the fine tuning of inhibitory neurotransmission by increasing membrane K^+ conductance. Late IPSPs have a slower onset and a prolonged duration compared with fast IPSPs deriving from $GABA_{A/C}$ receptors. Furthermore, postsynaptic $GABA_B$ receptors modulate neurotransmitter release by depressing Ca^{2+} influx via voltage-activated Ca^{2+} channels, whereas postsynaptic $GABA_B$ receptors mainly couple to inwardly rectifying K^+ channels (Bowery, 2002). $GABA_B$ receptors also inhibit adenylate cyclase; possibly modulating transcription factors (Steiger, 2004) and kinases (Diverse-Pierluissi, 1997, Couve, 2002, Ren, 2003).

3.2 $GABA_B$ receptors - Molecular structure and physiology

In 1997, Kaupmann et al. successfully isolated cDNAs for the two $GABA_{B1}$ subunit isoforms; $GABA_{B1a}$ and $GABA_{B1b}$ (Kaupmann, 1997). Thereupon, $GABA_{B2}$, the other $GABA_B$ receptor subunit was discovered. The $GABA_{B1}$ (either $GABA_{B1a}$ or $GABA_{B1b}$ subunit isoform) functionally heteromerizes with the $GABA_{B2}$ subunits (Bettler, 2004, Calver, 2002, White, 1998). This organization principle was at that time completely novel for G-protein coupled receptors (GPCRs). $GABA_{B2}$ subunits escorts the $GABA_{B1}$ subunit to the cell-surface and appears to be the G-proteins linking component of $GABA_B$ receptors. $GABA_{B1}$ subunits are necessary for agonist binding (Margeta-Mitrovic, 2000, Calver, 2001, Galvez, 2001, Pagano, 2001). Most researchers in the field expected the existence of many $GABA_B$ receptor subtypes due to the various subcellular distributions and different $GABA_B$ pharmacological properties (e.g. different binding properties for the two $GABA_B$ receptor antagonists phaclofen and CGP (Bonanno, 1992, Gemignani, 1994), Cunningham, 1996, Deisz, 1997, Mohler, 1999, Yamada, 1999, Bowery, 2002), similarly to what was shown for the metabotropic glutamate receptors. Additionally, $GABA_B$ receptors structure is homolog to the metabotropic glutamate receptors (mGluRs) structure as both receptors belong to the same GPCR family (Conn, 1997). Nonetheless, there is only one common agreement which is the existence of two $GABA_B$ receptor subtypes; the $GABA_{B(1a,2)}$ heterodimers and the $GABA_{B(1b,2)}$ heterodimers (Bettler, 2004). The broad functional and pharmacological diversity of the $GABA_B$ system emerges from different parameters including the heteromerizing nature of the $GABA_B$ receptors, the homolog but not identical molecular structure of the different subunits and the localization pattern of two $GABA_B$ receptors in different brain regions. The following chapters analyze these parameters. The description of $GABA_B$ receptor localization pattern will be restricted here to the brain, although functional $GABA_B$ receptors can also be found in peripheral organ and tissues (Ong, 1990), e.g. esophageal sphincter (Smid, 2000), uterus, spleen and in rat heart myocytes (Calver, 2000). That restriction was taken in regard to the determined extent of the thesis.

3.2.1 Molecular Structure of $GABA_B$ receptors

3.2.1.1 Heteromerization of $GABA_B$ receptor subunits $GABA_{B1}$ and $GABA_{B2}$

Most experiments with cloned $GABA_{B1}$ and $GABA_{B2}$ subunits expressed in heterologous cells and sympathetic neurons show that individual subunits are functionally inert (Filippov, 2000), a characteristic that differs from other dimeric GPCRs, in which the different subunits are functional when individually expressed (Bouvier, 2001). Active $GABA_B$ receptors assemble into heteromers composed of $GABA_{B1}$ and $GABA_{B2}$ subunits (Marshall, 1999, Mohler, 1999, Bettler, 2004). As mentioned before, $GABA_{B1}$ subunits are involved in the binding of GABA (Kaupmann, 1998a), whereas $GABA_{B2}$ subunits are responsible for escorting $GABA_{B1}$ subunits to the cell-surface and for activating G-proteins (Margeta-Mitrovic, 2000, Margeta-Mitrovic, 2001, Calver, 2002, Galvez, 2001, Robbins, 2001). Mouse genetic studies addressed the question whether cloned subunits guarantee classical $GABA_B$ functions *in vivo*. Mice missing either the $GABA_{B1}$ subunits or $GABA_{B2}$ subunits, from here on referred to as $GABA_{B1}$ deficient or $GABA_{B2}$ deficient, unveil a complete deficit of typical biochemical, electrophysiological and behavioral $GABA_B$ responses (Prosser, 2001, Schuler, 2001, Quéva, 2003, Gassmann, 2004). Additionally, $GABA_B$ subunit knock-out mice reveal the predominantly heteromeric nature of native $GABA_B$ receptors. They show a substantial down-regulation of $GABA_{B2}$ and $GABA_{B1}$ protein in $GABA_{B1}$ deficient and $GABA_{B2}$ deficient mice respectively, supporting that the native interaction between $GABA_{B1}$ and $GABA_{B2}$ subunits prevents their degradation.

On the other hand $GABA_{B2}$ deficient mice show small G-proteins dependent $GABA_B$ responses whereas $GABA_{B1}$ deficient mice did not (Gassmann, 2004), probably due to the inhibition of constitutively active K^+-channels, contrasting with the normally observed activation of K^+-channels. It is unclear whether the atypical $GABA_{B1}$ responses are of physiological relevance or an artifact of the knockout situation. The debate about the assembly of GPCRs is going towards higher order oligomerization structures than dimerization. Time-resolved fluorescence resonance energy transfer (TR-FRET) even foresights in $GABA_B$ receptors transfected HEK 293 and COS cells, a structural and functional multimeric model for $GABA_B$ receptor organization: “dimers of dimers” (Maurel, 2008), but the *in vivo* proof of a higher order oligomerization is still missing. Nevertheless, the possibility that the classical dimeric organization of the $GABA_B$ will in future extended to a higher degree of organization by means of different ways e.g. unknown receptor interactors, cannot be excluded at that time.

3.2.1.2 Molecular structure of $GABA_B$ receptor subunits $GABA_{B1}$ and $GABA_{B2}$

$GABA_B$ subunits share a high degree of homology; by comparing the amino acid sequence of rat $GABA_{B1b}$ (Kaupmann, 1997) and $GABA_{B2}$ subunit, we find at protein level, a similarity and an identity of 45% and 35% respectively, aligning by pileup program human $GABA_{B2}$ (AF099033) and rat $GABA_{B1b}$ (Y10370) coding regions (Martin, 1999). Many structural features are shared by the two subunits, namely, seven-transmembrane-domains, a major characteristic for the GPCR family, an extracellular chain at the amino terminus and at a long carboxyl-terminal tail. $GABA_{B1}$ and $GABA_{B2}$ subunits interact via the carboxyl-terminal resident coiled-coil domains in (Pagano, 2001). Coiled-coil domains are dimerization motifs found in numerous proteins, such as leucine-zipper transcription factors, K_{ATP} channels (Zerangue, 1999) or *N*-methyl-D-aspartate (NMDA) receptors (Scott, 2001). Proximal to coiled-coil domains, the carboxyl-terminal region of the $GABA_{B1}$ subunit contains an arginine-based endoplasmic reticulum (ER) retention signal RSRR. This domain prevents the escape of unassembled $GABA_{B1}$ subunits from ER then solely restricting surface expression to correctly assembled heteromeric receptors. Coiled-coil interaction of both carboxyl-terminals shields the retention signal then allowing the heteromeric receptor to exit from ER to the surface (Couve, 1998, Margeta-Mitrovic, 2000, Pagano, 2001, Gassmann, 2005). The $GABA_{B2}$ subunit carboxyl-terminal is around 100 aminoacids longer than the one from the $GABA_{B1}$ subunit (Marshall, 1999). Carboxyl-terminals of $GABA_B$ receptor subunits harbor several phosphorylation sites important for regulating receptor signaling or localization (chapter 3.2). $GABA_{B2}$ amino-terminal is comparable to the one found on $GABA_{B1b}$ subunit isoform, but shorter (Martin, 1999). Additional reasons for the diversity in the $GABA_B$ system are the two different isoforms of the $GABA_{B1}$ subunit, $GABA_{B1a}$ and $GABA_{B1b}$ isoforms. Further $GABA_{B1}$ cDNA isoforms than $GABA_{B1a}$ and $GABA_{B1b}$ are not found conserved among different species and the existence of stable protein products different from the $GABA_{B1a}$, $GABA_{B1b}$ and $GABA_{B2}$ could not be demonstrated *in vivo*. The structural difference in both isoforms is based on the exclusive presence of a pair of sushi repeats at the amino-terminal of the $GABA_{B1a}$ isoform (Blein, 2004). Sushi repeats, also known as complement control protein modules, or short consensus repeats, are found in other GPCR as well (Grace, 2004), mediating a wide variety of adhesion protein interactions (Lehtinen, 2004). Differential promoter usage from the $GABA_{B1}$ gene generates $GABA_{B1a}$ and $GABA_{B1b}$ isoforms (Bischoff, 1999, Steiger, 2004).

3.2.1.3 $GABA_{B1}$ and $GABA_{B2}$ subunits distribution in the brain

The $GABA_B$ receptors are distributed throughout the whole brain; their transcripts and binding sites are expressed in the brain in almost all neuronal cell populations, in glial cells they are not or only marginal expressed (Benke, 1999, Bischoff, 1999). The pharmacological difference of the $GABA_B$ responses bases on the differing distribution pattern of the $GABA_B$ subunits. In various brain regions

including cerebellum, ventrobasal thalamus and hippocampus a diversing pre- and postsynaptic localization of the $GABA_B$ receptor has been described (Kulik, 2002, Kulik, 2003); e.g. in hippocampus, $GABA_B$ receptors are found on GABAergic and glutamatergic terminals, mainly extrasynaptical and rarely at presynaptic membranes. Most of the $GABA_B$ receptors are consequently located far from the GABA release sites presumably requiring pooling of synaptical released GABA to be activated. The lack of specific $GABA_{B1}$ subunit antibodies or pharmacological compounds, demanded the generation of the $GABA_{B1a}$ and $GABA_{B1b}$ deficient mice, referred as $1a^{-/-}$ and $1b^{-/-}$ mice respectively (Vigot, 2006). The $1a^{-/-}$ and $1b^{-/-}$ mice have become an essential tool for studying $GABA_B$ receptor localization and function. Quantitative analysis of immunogold labeled $GABA_{B1}$ subunits in wild-type (*WT*) and $1a^{-/-}$ or $1b^{-/-}$ mice unveils a localization of the $GABA_{B1a}$ subunit in the CA1 region of the hippocampus on presynaptic terminal of the glutamatergic synapses (Vigot, 2006, Guetg, 2009), whereas the $GABA_{B1b}$ subunit was more abundant at postsynaptic density (Guetg, 2009). Electrophysiological experiments confirm this distribution, showing that the inhibition of excitatory postsynaptic currents (EPSCs) at glutamatergic synapses was reduced drastically in $1a^{-/-}$ mice compared to *WT* mice (Vigot, 2006), indicating that presynaptic $GABA_B$ responses are mainly $GABA_B$(1a,2) receptor subtype dependent at CA3-to-CA1 synapses. No difference of the inhibitory postsynaptic currents (IPSCs) inhibition was observed for $1a^{-/-}$ and $1b^{-/-}$ compared to *WT* mice. Both $GABA_{B1}$ subunit isoforms seem to contribute equally to $GABA_B$ response on presynaptic GABAergic terminals. The $1b^{-/-}$ mice show a 50% postsynaptic reduction in K^+-currents compared to WT mice, $1b^{-/-}$ mice K^+-currents were similar in WT mice, meaning that the postsynaptic $GABA_B$ response is predominantly mediated by $GABA_{B1b}$ isoform (Vigot, 2006). In layer 5 neocortical pyramidal neurons, inhibition of dendritic Ca^{2+} spikes is believed to be mediated exclusively by $GABA_B$ (1b, 2) receptor subtypes. Presynaptic inhibition of GABA release was mediated on the contrary through $GABA_B$ (1a, 2) receptors (Perez-Garci, 2006). NMDA-independent LTP in lateral amygdale reveals that $GABA_{B1a}$, but not $GABA_{B1b}$ is mainly localized at cortical afferents (Shaban, 2006). Similar functional segregation of $GABA_B$ (1a, 2) and $GABA_B$ (1b, 2) receptor subtypes was also observed in the thalamus (Ulrich, 2007).

3.2.2 $GABA_B$ receptors effector mechanisms

The metabotropic $GABA_B$ receptor acts through G-proteins activation. $GABA_B$ receptors couple to $G_i\alpha$- and $G_0\alpha$-type G-proteins (Asano, 1986, Campbell, 1993, Greif, 2000, Menon-Johansson, 1993, Morishita, 1990). They are present on as well inhibitory as excitatory terminals where they either regulate the release of GABA (*autoreceptors*) or glutamate (*heteroreceptors*). Both, autoreceptors and heteroreceptors, act by inhibiting the presynaptic and voltage sensitive Ca^{2+} channels. At the postsynaptic side, $GABA_B$ receptors inhibitory actions are mediated through the K^+ channels. Both ion channels are modulated through the $G\beta\gamma$ G-protein subunits. $GABA_B$ receptors, finally, also inhibit adenylate cyclase activity, trough $G_i\alpha/G_0\alpha$ G-protein subunits. This chapter discusses the inhibitory mechanisms of $GABA_B$ receptors in more detail.

3.2.2.1 Blocking of Ca^{2+}channels

Presynaptic $GABA_B$ receptors act through voltage-dependent inhibition of high-voltage activated N type (Cav2.2) and P/Q type (Cav2.1) Ca^{2+} channels (Amico, 1995, Cardozo, 1995, Menon-Johansson, 1993, Mintz, 1993, Newberry, 1985, Pfrieger, 1994, Poncer, 1997, Scholz, 1991, Takahashi, 1998). Both Ca^{2+} channel types are shown to trigger neurotransmitter release at presynaptic terminals (Wu, 1997). L-type Ca^{2+} channels seem to get reulated by $GABA_B$ receptors; either inhibitory (Amico, 1995, Maguire, 1989, Marchetti, 1991) or facilitatory (Shen, 1999). However, the effect was shown to be indirect and protein kinase C (PKC) activity dependent. $GABA_B$ receptors also inhibit and disinhibit T-type Ca^{2+} channels (Crunelli, 1991, Futatsugi, 1998, Matsushima, 1993, Scott, 1986, Scott, 1990). The Ca^{2+} channels inhibition can be modulated by the action potential frequency, where strong depolarization relieves Ca^{2+} channels from their G-protein mediated inhibition (Herlitze, 1996, Ikeda, 1996, Zamponi, 1997).

3.2.2.2 Opening of K^+ channels

Through the activation of inwardly rectifying GIRK or Kir3 K^+ channels $GABA_B$ receptors induce late IPSCs (Luscher, 1997, Schuler, 2001). Ba^{2+}, a Kir3 channel blocker, inhibits the $GABA_B$-induced late IPSCs (Jarolimek, 1994, Pitler, 1994, Thompson, 1994). The physiological effect of the activation of Kir3 channels is a K^+ efflux resulting in hyperpolarization. The baclofen-induced outward currents are absent in hippocampal neurons from Kir3.2 and $GABA_{B1}$ deficient mice (Luscher, 1997, Schuler, 2001). The rectifying properties of synaptical evoked late IPSCs differ between studies. On one hand, stimulus-evoked and spontaneous late IPSCs in dopaminergic neurons are inwardly rectifying and similar to the baclofen activated ones (Hausser, 1994). On the other hand, baclofen induces also linear or even outwardly rectifying conductance, suggesting the contribution of channels other than

Kir3, e.g. inactivating voltage gated K^+ channels (Saint, 1990) and a small Ca^{2+} conductance contribute to late IPSCs.

3.2.2.3 Down regulation of adenylate cyclase activity

The $GABA_B$ receptor dependent activation of $G_{i/0}\alpha$ subunits influences the adenylate cyclase activity. Indeed, in brain slices it is shown that $GABA_B$ receptor agonists inhibit basal or forskolin induced adenylate cyclase activity (Xu, 1986, Knight, 1996). The physiological confirmation of this effect comes from cerebral cultures taken from microdialysed living rats' experiments (Hashimoto, 1997). A microdialysis cannula was implanted into the striatum of the rats, trough which forskolin was administrated. The application of GABA and $GABA_B$ receptor agonist baclofen dampens the increase of forskolin stimulated cAMP formation whereas $GABA_B$ receptor antagonist CGP5426 abolishes the reduction in cAMP formation. The main relevance of adenylate cyclase regulation by $GABA_B$ receptors is still unclear. A regulatory role on transcription factors Steiger, 2004) and kinases, especially cAMP-dependent protein kinase A (PKA) or even on cAMP, Ca^{2+} and calmodulin dependent vesicle priming is conceivable (Diverse-Pierluissi, 1997, Couve, 2002, Ren, 2003). Interestingly, few studies claim that $G_{\beta\gamma}$ subunits of the $GABA_B$ receptors activated G-protein can also weakly stimulate adenylate cyclase activity (Bowery, 2002, Calver, 2002).

3.2.3 $GABA_B$ receptor, diseases and drugs

A number of clinical studies suggest that $GABA_B$ might play an important role in alleviating symptoms of different maladies like addiction, epilepsy, nociception and multiple sclerosis. This chapter informs on the current knowledge of $GABA_B$ receptors contribution in regard to addiction, epilepsy and nociception and on baclofen and GHB, two therapeutic relevant $GABA_B$ receptors agonists.

3.2.3.1 Addiction

The $GABA_B$ agonists reduce human and animal craving for different drugs. Preliminary clinical studies with cocaine abusing patients show a reduced demand for cocaine after baclofen administration (Brebner, 2002, Ling, 1998). Animal experiments also support baclofen therapeutic effectiveness against cocaine abuse. Baclofen suppresses intravenous self-administration of cocaine in rodents (Brebner, 2000, Campbell, 1999, Roberts, 1996, Shoaib, 1998). A reduced self-administration after baclofen administration was also observed for heroine (Xi, 1999). $GABA_B$ agonists are also effective in clinical studies against alcoholism (Addolorato, 2002), reducing alcohol intake (Colombo, 2000, Daoust, 1987, Smith, 1999). GHB application shows promising effects against nicotine dependence (Dewey, 1998, Dewey, 1999). The $GABA_B$ receptor acts inhibitory on the dopaminergic cells, especially in the nucleus accumbens. The nucleus accumbens is part of the mesolimbic dopamine system and is believed to be involved in the reward and reinforcement circuitry. Drugs of abuse increase extracellular dopamine levels in the nucleus accumbens. $GABA_B$ receptor activating compounds could block the effect of drugs of abuse, by decrasing the dopamine release in the mesolimbic system, and are then interesting therapeutic candidates for the control of addiction (Xi, 1999).

3.2.3.2 Epilepsy

The $GABA_B$ receptors participate in the generation of absence seizures within the thalamus (Kim, 1997). The exact receptor-mediated mechanism is still unclear. Animal experiments show that $GABA_B$ agonists increase the probability for seizures. The application of $GABA_B$ antagonists on the other hand reduces the risk for seizures. The activation of postsynaptic $GABA_B$ receptors on thalamocortical neurons produces a prolonged neuronal hyperpolarization facilitating the Ca^{2+} spiking. That, in turn, is relayed to the cerebral cortex. One evident phenotype of the $GABA_{B1}$ deficient mice is the appearance of absence seizures (Prosser, 2001, Schuler, 2001). Interestingly, the absence of seizures seen in the $GABA_{B1}$ deficient mice and GAERS rats, a model for absence epilepsy (Marescaux, 1992), are different. Indeed, the seizures in the $GABA_{B1}$ deficient mice are rare and longer in duration which is indicative for “atypical” absence seizures. Conversely, GAERS rats show seizures characterized by

frequent and short EEG bursts. It is conceivable that the different composition of $GABA_B$ receptor in nervous cell system is important in the inherited predisposition to epilepsy (Gambardella, 2003). However, GAERS rats offer no evidence for differences in the $GABA_{B1}$ subunit populations or post-receptor mechanisms. The eventual availability of subtype specific $GABA_B$ receptor antibodies would enable detection of $GABA_B$ receptor variants alterations.

3.2.3.3 Nociception

Clinical trials with the $GABA_B$ agonist baclofen reveal an antinociceptive property of the $GABA_B$ receptor activity on e.g. vagoglossopharyngeal and ophtalmic-postherpetic neuralgias, diabetic neuropathy, and migraine (Bowery, 1993, Fromm, 1989, Hering-Hanit, 1999, Sindrup, 2002). Further animal experiments; rodent models of acute pain, such as tail-flick, hot-plate tests, or chronic pain models in rats, support this antinociceptive effect of baclofen (Balerio, 2002, Przesmycki, 1998, Cui, 1998, Wiesenfeld-Hallin, 1997). The acute pain tests with $GABA_B$ receptor deficient mice confirmed the antinociceptive role (Schuler, 2001). The $GABA_{B1}$ deficient mice display a pronounced hyperalgesia in the hot-plate and tail-flick tests, as well as reduced paw-withdrawal thresholds to mechanical pressure (Bettler, 2004). These two mice models suggest that the absence of functional $GABA_B$ receptors most probably results in an increased central hyperexcitability of spinal nociceptive pathways. Intrathecal application of baclofen, that exerts its effects in the brain and the spinal cord, relieves central pain in patients with spinal lesions or after cerebral strokes (Herman, 1992, Taira, 1995).

3.2.3.4 Depression and anxiety

20 years ago Lloyd et al. (Lloyd, 1985) demonstrated an upregulation in $GABA_B$ binding sites occurring in rat frontal cortex after chronic administration of antidepressant drugs and as a result of electroconvulsive therapy. First these findings were disputed, but nowadays there is no doubt for the contribution of the $GABA_B$ receptor in the etiology of depression (Enna, 2004). The primary question is surely whether the modulation or the antagonism of $GABA_B$ receptors produces antidepressant-like or anxiolytic effects. The $GABA_{B1}$ deficient or $GABA_{B2}$ deficient mice are more anxious but exhibit also an antidepressant-like behavior (Mombereau, 2005), suggesting that the loss or blockade of $GABA_B$ receptor functions produce antidepressant-like effects. Several studies performed with $GABA_B$ receptor antagonists in a variety of animal models support that statement (Cryan, 2005). However, until clinical studies are undertaken, it will not be clear whether antidepressant activity will emerge from an action at the $GABA_B$ receptor. Activation of the $GABA_B$ receptor in the dorsal periaqueductal grey of rats impairs one-way escape in the elevated T maze test, which is consistent with an

anxiolytic or panicolytic effect. In addition, baclofen has been reported to reduce the incidence of panic attacks in patients following the systemic administration.

3.2.3.5 Baclofen (Lioresal™)

Several centrally acting neuronal inhibitory drugs, including benzodiazepines, mediate their effects by the $GABA_A$ receptors activation. In contrast, the only compound in current clinical use, baclofen (β p-chlorophenyl-GABA), mediates its effects directly through the activation of $GABA_B$ receptors. Baclofen, a common antispastic medicament, was first prescribed in 1972 for therapeutic use (Faigle, 1972), even before $GABA_B$ receptors discovery. 30 years later baclofen is still the only $GABA_B$ receptor specific agonist, although the receptors structure was described already a decade ago (Jones, 1998, Kaupmann, 1998a, White, 1998), acclaiming a clinical poor penetration of the blood-brain barrier. Its beneficial effects on epilepsy, nociception, depression, anxiety and drug addiction have been discussed already. Muscle-relaxant properties of baclofen are well established clinically, making it the drug of choice in the treatment of spasticity associated with cerebral palsy, multiple sclerosis, stiff-man syndrome and tetanus. Still, the large doses that have to be administered in order to compensate for the poor blood brain barrier diffusion are rarely tolerated by the patients. Indeed, the adverse effects of large orally intake of baclofen include dizziness, nausea, sedation and hallucinations. That has been largely overcome by the intrathecal administration of baclofen using an indwelling pump. Since this form of administration is locally, it reduces the incidence of adverse effects and the risk of drug tolerance. Finally it was shown that baclofen suppresses cognitive behavior in animals (McNamara, 1996). Although this property, baclofen is of little consequence to clinical medicine, there is the chance that $GABA_B$ receptor antagonists might provide a novel opportunity for treating cognitive impairment. Already some animal models for learning and memory retention have been established (Bowery, 2002) and the first clinical trials of $GABA_B$ receptor antagonist have started recently (Helm, 2005).

3.2.3.6 γ-hydroxybutyric acid (Xyrem™)

The γ-hydroxybutyric acid (GHB) acts mainly through the $GABA_B$ receptors (Kaupmann, 2003). GHB is a naturally occurring, is a short-chain fatty acid related to GABA, rapidly producing effects likened to a combination of alcohol (euphoria, reduced anxiety, drowsiness, loss of motor control and ecstasy (enhanced sensuality, emotional warmth) (Galloway, 2000). GHB is used recreationally at raves, or to heighten sexual pleasure. GHB has also been used as a "health product" for its soporific activity. Body builders make use of GHB as steroid replacement, although no anabolic effects have yet been convincingly reported in animals (Nicholson, 2001). The clinical evidence shows GHB abuse produces severe dependence and withdrawal symptoms. Two large clinical studies where GHB was prescribed

as part of treatment program for alcoholism and heroine abuse, showed that 10–15% of patients became dependent and addicted to GHB (Gallimberti, 2000). GHB combination with alcohol leads to severe coma. It is unfortunately also used by criminals who are taking advantage of the anterograde amnesia, muscle relaxation and the fast disinhibition caused by GHB intake, to abuse their unaware victims.

3.3 Phosphorylation regulates $GABA_B$ receptor function

Considering that $GABA_B$ receptors play a crucial role in synaptic inhibition it is important to understand the mechanisms involved in their modulation and the impacts of such modulations on the synaptic function. One major mechanism regulating GPCRs function is phosphorylation of their intracellular domains by protein kinases (Pitcher, 1998). For the most GPCRs, phosphorylation results in the reduction of the effector coupling and receptor removal from cell-surface (Tsao, 2000). Both effects lead to the phenomena called desensitization. Desensitization is described as the decrease of a receptor response over time despite the continuous presence of the agonist. Ferguson wrote in 2001 (Ferguson, 2001) about GPCR desensitization: "The waning of GPCR responsiveness to agonist with time represents an important physiological feedback mechanism that protects against both acute and chronic over stimulation of GPCRs". They are two major types of receptor desensitization: the homologous and the heterologous desensitization.

The homologous desensitization results from a receptor phosphorylation by G-protein coupled receptor kinases (GRKs), leading to the binding of cytoplasmic inhibitory proteins known as arrestins (Craft, 1995). The binding of arrestins prevents receptor-dependent activation of its associated G-proteins and, therefore, its effectors. Furthermore, binding of arrestins to GRK phosphorylated receptors is believed to initiate GPCRs endocytosis, or sequestration (Ferguson, 1996), into the recycling endosomes (von Zastrow, 1992). In the resensitization the dephosphorylation by a membrane associated phosphatase returns the internalized receptors to cell-surface (Pitcher, 1995).

On the other hand, the heterologous desensitization means phosphorylation of GPCRs by second messenger–dependent kinases, e.g. PKA, calcium/calmodulin-dependent kinase II (CaMKII), protein kinase c (PKC) or 5'-AMP activated protein kinase (AMPK). Receptor phosphorylation by these kinases impairs receptor-dependent stimulation of their G-proteins (Benovic, 1985, Pitcher, 1992, Hosey, 1999).

The following chapter focuses on the main modulatory kinases in the mammalian brain, being the serine/threonine kinases CaMKII, PKA, PKC and AMPK. It highlights too their central role in neuronal processes including LTP. In the second part of this chapter the current knowledge of the phosphorylation sites on $GABA_B$ receptors and their actual physiological relevance, especially in regard to their regulatory effect on desensitization mechanisms, are explained. Only, the phosphorylation site serine 883 on the $GABA_{B2}$ subunits, revealed by phosphoproteomic analysis of cortical human synaptosomes (DeGiorgis, 2005), will not be included in this discussion due to the absence of its physiological relevance.

3.3.1 Central kinases in the mammalian brain

3.3.1.1 CaMKII

The calcium/calmodulin-dependent kinase II (CaMKII) is very abundant in the CNS (Erondu, 1985) and is implicated in a wide variety of neurobiological processes (Braun, 1995, Lisman, 2002, Hook, 2001). CaMKII is encoded by four genes in mammals: α, β, γ, and δ; each of the genes encodes a protein having an amino-terminal kinase domain, followed by a regulatory region with an autoinhibitory sequence and a calmodulin binding site. The carboxyl-terminal is usually called association domain and is responsible for the subunit assembly into large (from 8 to 14 subunits) multimers. Structural differences between CaMKII isozymes are primarily the consequence of differences within their variable regions, localized between the regulatory region and association domain (Hudmon, 2002). All four genes also undergo alternative splicing in these variable regions. In CNS α and β CaMKII isozymes are predominant (Miller, 1985). The γ and δ isozymes are found at low levels in all tissues but are enriched in non-neuronal tissues (Tobimatsu, 1989). For instance, the δ CaMKII is found in the heart whereas α, β and δ in skeletal muscles. The CaMKII is activated by the Ca^{2+}-CaM binding to its regulatory domain, resulting in the relief of the catalytic domain, from its inhibition by autoregulatory sequences proximal to CaM binding site. That allows CaMKII to phosphorylate itself on the autophosphorylatory site threonine 286 and to phsophorylate the CaMKII substrates. As aforementioned, the CaMKII plays a central role in neuronal processes which is outlined by its abundant localization in the nervous cell system. The CaMKII is found in dendritic spines and listed as a major constituent of the postsynaptic density, PSD (Kennedy, 1983). The CaMKII is strongly involved in the regulation of synaptic plasticity. The PSD is a tiny, amorphous structure located beneath the postsynaptic membrane of the synapses in the CNS, visible under the electron microscope as tight complexes of post-synaptic junctional proteins.

The glutamatergic excitatory synaptic transmission in the brain is mediated trough NMDA and AMPA glutamate receptor activation. Activity-dependent changes in AMPA receptor signaling represent a key mechanism for the brain plasticity and are the basis of learning and memory formation. The LTP in hippocampal pyramidal cells is the best established model for plasticity. Although the molecular mechanisms for LTP are not entirely defined; it is well accepted that the Ca^{2+} influx through NMDA receptors initiates the changes seen during LTP (Malenka, 1999, Lisman, 2003, Malenka, 2004, Collingridge, 2004). The intracellular increases in Ca^{2+} level activate CaMKII that in turn phosphorylates the AMPA receptors, leading to their recruitment to the spines PSD (Xie, 2007). The α-CaMKII deleted mice show impaired CA1 LTP and spatial learning (Silva, 1992a, Silva, 1992b). The same phenotype is observed in mice with a point mutation preventing autophosphorylation at threonine 286 (Giese, 1998). The CaMKII even modulates the morphology of dendritic spines through

binding and bundling of F actin (Okamoto, 2007) or through the phosphorylation and activation of guanine-nucleotide exchange factor Rac-1 GEF kalirin-7 (Xie, 2007). That makes of CaMKII a kinase of primer interest in the CNS research.

3.3.1.2 PKA

The adenosine-3', 5'-cyclic monophosphate (cAMP) was the first second messenger to be identified. During the past 30 years, a large number of studies have elucidated the fundamental role of cAMP in cellular responses to hormones and neurotransmitters. The cAMP activates protein kinase A (PKA). The PKA consists of a complex of two regulatory ©- and two catalytic ©- subunits. R-subunits are encoded by four genes Riα, Riβ, RIIα and RIIβ, whereas C-subunits are encoded by three; Cα, Cβ and Cγ. The cAMP binding to PKA R-subunits induces their dissociation from the C-subunits. The PKA activity is important for the induction of long-term synaptic, physiological and behavioral changes and can be seen as another key player in the learning and long-term memory consolidation (de Toledo-Morrell, 1984, Ardenghi, 1997, Barad, 1998, Bach, 1999, Barros, 2000). The PKA has also a crucial role in the higher CNS functions; regulatory mechanisms are present in order to control the PKAs activity. For example, the PKA activity downregulation is achieved through endogenous protein kinase inhibitors (PKIs). The PKIs are shown to disrupt LTP formation in hippocampus and/or to impair the long-term memory consolidation. Interestingly, with Rp-cAMPS, the inactive enantiomer of cAMP, the same effects are observed (Barad, 1998, Spencer, 2002, Hyman, 2001). Application of the Sp-cAMPS, PKA activators, induces the LTP and enhances memory consolidation (Bach, 1999, Ardenghi, 1997).

The perfusion of a constitutively active isoform of the PKA catalytic subunit into the CA1 pyramidal neurons is sufficient to induce persistent, long-lasting synaptic facilitation (Duffy, 2003). Consistently, evidence from the literature proves that PKA activation enhances the long-term memory function dependent on the hippocampus, the brain region which is known, from studies of amnesic patients and experimental animals, to be of eminent importance for long-term memory formation. The PKA activation has been shown to reverse long-term memory deficits and the extended PKA activity, by systemic administration of phosphodiesterase (PDE) nhibitors, preventing the cAMP breakdown, improving the hippocampal function in aged rats (de Toledo-Morrell, 1984). The application of rolipram, a selective PDE4 inhibitor, or PKA activators (e.g. dopamine receptor agonists) in aged mice with memory deficits improved their long-term memory consolidation and facilitated LTP (Bach, 1999, Barad, 1998). The enhancing effects of PKA on memory consolidation also appear to extend to interconnected cortical regions, such as entorhinal and posterior parietal cortex (Ardenghi, 1997). PKA activation enhanced retrieval in these cortical regions, whereas inhibition of PKA blocked memory retrieval. That was seen in rats, where PKA activators have been infused bilaterally into the

CA1 of rats (Barros, 2000, Barros, 2001).Thus, the activation of PKA in several interconnected cortical and hippocampal circuits enhance consolidation and possibly the retrieval of memory. Hence, it is not surprising that PKA is considered as an interesting compound for developing effective therapeutics for cognitive disorders such as age related cognitive decline, post-traumatic stress disorder and drug abuse (Arnsten, 2005).

3.3.1.3 PKC

The protein kinase C (PKC) influences the neurite outgrowth or several neuronal functions, for instance; alcohol actions, ischemic preconditioning and pain The PKC family is separated in three subgroups; the conventional PKC (cPKC), the novel PKC (nPKC) and the atypical PKC (aPKC). The PKC consists of, again, an R- and a C-domain tethered together by a hinge region. The C-region is highly conserved among the different isoforms and to a lesser degree, among the C-region of other serine/threonine kinases. The second messenger requirement for the activation of PKC differs according to the isoforms as a result of differences between the R-regions of a same class. The cPKC isoforms PKCα, PKCβ and PKCγ are diacylglycerol (DAG) and Ca^{2+} responsive, through the archetypal C2 domains. The nPKC isoforms PKCδ, PKCε and PKCη are DAG sensitive but Ca^{2+} insensitive, as their do not retain Ca^{2+} at their C2 domains. Finally, the aPKC isoforms PKCζ and PKCι/λ have altered C2 domains and are DAG and Ca^{2+} insensitive. Their regulation is mediated through their amino-terminal PB1 domains. The PKCε is expressed at higher levels in the brain, compared to the other tissues (Shirai, 2008), suggesting its meaningful role in the nervous system. In the PKCε null mice, a higher sensitivity to the behavioral effects of ethanol is observed conjointly to a reduced rate of ethanol self-administration. Conditional expression of PKCε in basal forebrain, amygdale and cerebellum of these mice rescues hypersensitivity and restores the ethanol intake. The hypersensitivity and avoidance of ethanol in PKCε KO mice appears to be mediated by $GABA_A$ activation, since the allosteric $GABA_A$ activators such as pentobarbital and diazepam increase the PKCε KO mice locomotor activity compared to WT mice (Hodge, 1999, (Choi, 2002).

As mentioned, CaMKII is an essential player for LTP formation. The PKCε, conversely, although necessary is not sufficient to induce the LTP. In the hippocampus two different types of LTP exist; the LTP in Schaffer collaterals-CA1 pathway, and the one in the Mossy fibers to CA3 pathway. The first one is Ca^{2+}-dependent and involves postsynaptic NMDA receptor phosphorylation by PKCγ (Saito, 2002) whereas the second takes place in the presynaptic neurons, where PKCε is enriched consistent with its role in LTP at this synapse (Koide, 1992, Saito, 1993). Indeed, the presence of PKCε at nerve terminals is involved in phorbol ester-induced enhancement of glutamate exocytosis and in phorbolester-induced synaptic potentiation (Saitoh, 2001, Dumuis, 1988). The by transcardial perfusion applied phorbolester translocated PKCε toward the synaptic side of the nerve terminal. It is

thought that PKCε activated by phorbol ester might interact with the microfilament F-actin and change its conformation, thereby increasing transmitter release. Therefore it is expected that PKCε contributes to Mossy fiber LTP by increasing the presynaptic neurotransmitter release. However the mechanism by which PKCε is activated presynaptically during LTP is not fully understood. One potential mechanism involves the contribution of arachidonic acid as a retrograde messenger produced by the postsynaptic neuron following NMDA receptor activation. Indeed, the diffusion of arachidonic acid to the Mossy fiber terminal would then allow presynaptic PKCε activation and would cause a persistent potentiation of evoked responses (Dumuis, 1988, Kasahara, 1995). The PKCε has been reported to be also involved in nocioceptor sensitization (Premkumar, 2000, Tominaga, 2001). Indeed, PKCε directly phosphorylates TRPV1, a capsaicin receptor. TRPV1 is involved in the sensation of thermal and inflammatory pain (Davis, 2000). Through the PKCε phosphorylation the desensitization or potentiation of TRPV1 is possible (Mandadi, 2006, Dai, 2004), appointing PKCε as potential therapeutic target for pain regulation.

Additionally, the PKCε and its specific activators and inhibitors seem to be involved in neuronal preconditioning and were investigated in hippocampal slices (Raval, 2003, Lange-Asschenfeldt, 2004) and primary cultured neurons (Wang, 2004, Di-Capua, 2003). The phenomenon “preconditioning” refers to sub-leathal and mild ischemic insults promoting tolerance against more severe subsequent ischemic insults in organs such as the heart and the brain. According to these studies, NMDA and adenosine receptor-mediated preconditioning requires the PKCε activity. Altough the mechanism of PKCε mediated neuronal preconditioning is not fully understood, these findings suggest that PKCε may have a protective role in apoplexy. Recently, Shimomura et al. demonstrated a decrease of PKCε levels at the core of focal cerebral ischemia, and interestingly this decrease was prevented by hypothermia, a well known neuroprotective mechanism (Shimomura T, 2007). How hypotheramia exactly alters PKCε levels is currently unknown. In the electron microscopy the PKCε localization at presynaptic terminals is revealed at nerve fibers (Saito, 1993 Tanaka, 1994). There, it was also shown that nerve growth factor induced neurite outgrowth is improved by PKCε overexpression and accordingly, downregulation of PKCε expression inhibits that effect (Hundle, 1995). The PKCε induced neurite outgrowth is blocked by RhoA, a member of the Ras homolog gene family, indicating an involvement of RhoA in the PKCε induced neurite outgrowth (Ling, 2004).

3.3.1.4 AMPK

The nervous cell system requires a big part of total body energy, and neurons are particularly vulnerable to energy deficits as their metabolism is rather inflexible and their nutrient storage capacity is poor. The adenosine monophosphate (AMP)-activated protein kinase AMPK was known for the last 35 years as a simple homologue of a yeast non-fermenting gene. Only recently AMPK has

been recognized as a central player in the maintenance of balanced cellular and body energy levels. The AMPK is unsurprisingly highly expressed in the CNS (Turnley, 1999, Culmsee, 2001). During the high metabolic activity or pathological states of anoxia and ischemia the decrease in cellular ATP levels rapidly activates AMPK coinciding with AMP increase. The AMPK then increases ATP generation by increasing cellular glucose uptake and the biogenesis of glucose transporter 4 (Winder, 2001).

The AMPK has a heterotrimeric structure consisting of a catalytic α subunit (α1 and α2) and two non-catalytic regulatory subunits; β subunit (β1, β2. And β3) and γ subunit (γ1, γ2 and γ3) (Hardie, 2007). All the three subunits are required for the formation of a stable and fully functional AMPK complex. Each subunit possesses unique structural components facilitating the characterization of their specific roles in regulating activity and AMPK functioning in mammalian cells. The catalytic subunits have a highly conserved amino-terminal domain including the activating phosphorylation site threonine 172 and an autoinhibitory site.

The AMPK β subunit is a scaffold/docking subunit that contains an amino-terminal myristoylation site responsible for targeting the AMPK to membranes. Additionally the β subunit is an internal glycogen-binding domain and a carboxyl-terminal α- and γ-subunit binding domain, essential for the formation of stable AMPK heterotrimers (Woods, 1996, Iseli, 2005, Towler, 2007, Turnley, 1999). The β subunits contain several regulatory phosphorylation sites implicated in nuclear localization of AMPK as well as regulation of AMPK catalytic activity. The γ subunits possess variable amino-terminal region, followed by highly conserved cystathionine-b-synthase sequence motifs, forming the two functional Bateman domains. The Bateman domain 1 and Bateman domain 2 are the structures responsible for binding adenine nucleotides, such as AMP or ATP. The decrease of the ATP/AMP ratio, caused by enhanced metabolic activity, anoxia, or ischemia, activates the AMPK. AMP binds to the Bateman domains, induces a conformational change in the heterotrimeric AMPK structure, increasing the AMPK's activity.

Under physiological conditions the AMPK signaling, especially in hypothalamus, plays an important role in the mammalian feeding behavior (Kim, 2004). The majority of identified AMPK substrates are either enzymes or transcription factors controlling carbohydrate and lipid metabolism (Kahn, 2005). Pathophysiological regulation of AMPK signaling, e.g. ischemia or anoxia, leads to excitotoxcity, oxidative and metabolic stresses. The application of AMPK activators protects hippocampal neurons or astrocytes from the excitoxicity (Culmsee, 2001, Blasquez, 2001). Oxygen and glucose deprived hippocampal slice cultures show an increase of the activated AMPK (McCullough, 2005). Focal stroke models where mice are subjected to middle cerebral artery occlusion (MCAO), confirm that increase *in vivo* (Murphy, 2003). However, there is some discrepancies in literature, as it is reported that MCAO subjected mice show decrease of the behavioral deficits after stroke (McCullough, 2005), or

even provide sustained neuroprotection for days after the stroke (Li, 2007) following the AMPK inhibitor treatments. The differences in cell-type and conditions might contribute to the divergent findings. The AMPK remains an interesting target for the development of medication for stroke injuries or diseases involving the energy deregulation.

3.4 $GABA_B$ phosphorylation sites: Identification and physiological relevance

3.4.1 PKA site: serine 892 on the $GABA_{B2}$ subunit

The phosphorylation of GPCRs generally results in a reduction in the receptor activity, an increase in the desensitization or the removal of the receptor from the cell-surface (Tsao, 2000). In 2002, Couve et al. described a controversial effect of the $GABA_B$ receptor phosphorylation by the PKA in neurons. They showed that PKA phosphorylation of the $GABA_{B2}$ subunits at serine 892 (S892) leads to a reduction of $GABA_B$ receptors desensitization. Also, they provided the preliminary evidence of a potential participation of $GABA_B$ receptors in the well documented phenomenon of β-adrenergic-dependent facilitation of GABAergic transmission in cerebral cortex and cerebral Purkinje cells (Sessler, 1995, Saitow, 2000).

Using whole-cell patch clamp recording in primary cultures of rat hippocampal neurons, they showed that application of baclofen induces K^+ channel activation. Short repeated applications of baclofen result in a clear reduction of the amplitude of the $GABA_B$ evoked K^+ currents. It is also known that the $GABA_B$ mediated K^+ response desensitizes and that intracellular perfusion of cAMP significantly reduces the agonist and time-dependent desensitization. Consistently, the PKA inhibitors largely prevent this effect of cAMP perfusion. Following the cAMP perfusion, baclofen induces K^+ currents desensitization, indistinguishably from control neurons and this already from the beginning of the perfusion. That implies a cAMP-dependent regulation of $GABA_B$ receptor mediated K^+ responses trough the activation of PKA. The purified PKA phosphorylates glutathione-S-transferase (GST) fusion proteins containing the complete carboxyl-terminal domain of the $GABA_{B2}$ subunit, termed GST-CR2. Moreover, the detergent-solubilized brain extracts also phosphorylates GST-CR2 proteins suggesting that endogenous brain PKA is active in brain extracts. The increase of cAMP concentration on brain extracts augments phosphorylation and Walsh peptide application, a PKA inhibitor, diminishes the phosphorylation signal. The carboxyl-terminal domain of the $GABA_{B2}$ subunit contains a strong consensus site for PKA at S892 (Kennelly, 1991). The GST-CR2 fusion proteins with an alanine mutation at S892, the GST-CR2 S892A, no longer get phsophorylated by endogenous brain PKA. The PKA phosphorylation of the complete $GABA_B$ receptor is observed in COS cells, transfected with $GABA_{B1}$ and $GABA_{B2}$ complementary DNA (cDNA). After metabolic labeling with [^{32}P] orthophosphate, immunoprecipitation of a phosphorylated $GABA_{B2}$ subunit from COS cells lysate was found. However, no significant phosphorylation of $GABA_{B1}$ subunit was observed. Interestingly, forskolin treated COS cells show an increase in $GABA_{B2}$ subunit phosphorylation, indicating a PKA activity. Consistently, transfection of alanine substituted $GABA_{B2}$ subunits S892A cDNA in COS cells show only a weak basal phosphorylation of the immunoprecipitated $GABA_{B2}$ subunits. The [^{35}S] methionine labeling and

immunofluorescence analysis of WT and S892A exclude differences in protein expression, stability or membrane targeting, other expressing systems like the HEK 293 or the CHO cells confirmed that result. The comparison of the tryptic maps from the $GABA_{B2}$ WT and $GABA_{B2}$ S892A show the loss of major, by the [^{32}P] orthophosphate labeled SDS-PAGE gel and trypsin digested phosphorylation peptides in the map of $GABA_{B2}$ S892A, accrediting PKA phosphorylation of the recombinant whole $GABA_{B2}$ subunit at S892. To investigate the possible functional effect of S892 phosphorylation by PKA on K^+ currents, HEK 293 cells stably expressing Kir3.1 and Kir3.2 K^+ channel subunits and transiently expressing $GABA_{B1}$ and $GABA_{B2}$ subunits, were analyzed by whole-cell recordings after the administration of GABA. In agreement with the results from hippocampal neurons, short-pulses of GABA application result in K^+ current desensitization. The same results were obtained with the $GABA_{B2}$ WT and the $GABA_{B2}$ S892A expressing neurons. The application of cAMP reduces the desensitization for the WT form, but not for the S892A form, confirming that the S892 phosphorylation is responsible for the $GABA_B$ receptor amelioration of the desensitization after PKA activation. There are many possible ways how S892 modulates K^+ response desensitization. The concentration-response curves for $GABA_{B2}$ and $GABA_{B2}$ S892A show no differences in the potency of GABA on $GABA_B$ receptors in presence or absence of cAMP. To study possible effects of cAMP on K^+ channels, K^+ currents were analyzed in HEK 293 cells expressing Kir3.1 and Kir3.2 K^+ channels along with $GABA_{B1}$ and either the $GABA_{B2}$ WT or the $GABA_{B2}$ S892A. The chord conductance for GABA induced current is increased in the presence of cAMP for hyperpolarized holding potentials of WT $GABA_B$ receptors consistent with the assumed role of PKA phosphorylation on the $GABA_B$ receptor desensitization reduction. However, the S892A $GABA_B$ receptors show overlaying current-voltage relationships in the presence or absence of cAMP, in accordance with the lack of cAMP effect on desensitization of mutant receptors. From all the current-voltage relationships, it can be observed that the reversal potential for the GABA-activated K^+ currents is unchanged by cAMP, indicating that properties of inwardly rectifying K^+ channels are unaffected by PKA activation.

The K^+ response desensitization might also be regulated by a decreased coupling of $GABA_B$ receptors to $G\alpha_{i/o}$ subunits. The [^{35}S] GTPγS binding assay within the CHO cells stably transfected with $GABA_{B1}$ and $GABA_{B2}$ offers a good mean to solve this question However, there were no differences in the GABA induced EC_{50} or the maximal [^{35}S] GTPγS binding between membranes derived from cells exposed to control medium or to a forskolin containing medium. Attenuation of the K^+ desensitization response by the PKA caused by different amounts of functional cell-surface $GABA_{B1}$ or $GABA_{B2}$ heterodimers was excluded using the whole-cell enzyme-linked immunesorbent assay of HEK 293 cells transiently transfected with the $GABA_{B1}$ and the $GABA_{B2}$ subunits before and after PKA activation. The controls are untransfected cells or cells expressing MYC-tagged $GABA_{B1}$ alone that are sequestered within the ER show no cell-surface binding of the MYC antibody. The signal was

observed in MYC-tagged $GABA_{B1}$ transfected cells after membrane permeabilization. A robust cell-surface expression of $GABA_{B1}$ was detected after coexpression of $GABA_{B2}$ and the $GABA_{B1}$ amount expressed on cell-surface. The cell-surface stability of receptor heterodimers was verified in cells incubated with the MYC antibody. Loss of the cell-surface antibody was analyzed after the incubation at 37°C. During the short-term treatment baclofen showed no significant effect on receptor internalization. The PKA activation, despite the significantly reduced detriment of cell-surface heterodimers containing $GABA_{B2}$, had only little effect on the $GABA_{B2}$ S892A containing dimers. Altogether, these results imply that phosphorylation of the $GABA_{B2}$ S892 by PKA strengthens $GABA_B$ receptors at the cell-surface, enhancing the effector coupling of $GABA_B$ receptors. In immunoblots of crude brain membranes anti-pS892, recognized at the S892 phosphorylated $GABA_{B2}$ subunits. That detection could be blocked by adding of the chemically phosphorylated synthetic peptides (the ones used for immunization); confirming specificity of the anti-pS892 antibody and showing S892 phosphorylation in the mammalian CNS. The colocalization studies demonstrate that $GABA_{B2}$ S892 phosphorylated $GABA_B$ receptors are enriched in the periphery of the cell body and abundant within the edges of neuronal projections. This indicates that a significant pool of phosphorylated $GABA_{B2}$ subunits is located in vicinity of the cell-surface. In cultured hippocampal cells it was studied with anti-pS892 antibody whether the native $GABA_{B2}$ S892 phosphorylation is in consequence of the active PKA in the CNS. The phosphorylation was studied in the presence or the absence of 8-Br-SpcAMP, a stable (and membrane permeable) activator of the PKA. The 8-Br-SpcAMP leads to a significant increase in the $GABA_{B2}$ S892 phosphorylation, but there are no changes in total amount of the $GABA_B$ receptors. To investigate the role of $GABA_B$ receptor activation on $GABA_{B2}$ S892 phosphorylation, cultured cortical neurons were exposed to baclofen or CGP62349, a $GABA_B$ receptor antagonist, blocking the effect of baclofen. Baclofen decreases basal phosphorylation of the S892, CGP62349 inhibits that reduction. The baclofen treatment had no effect on total amounts of $GABA_B$ subunits. The activation of $G\alpha i$ through $GABA_B$ receptors results in a reduction of cAMP concentrations, diminishing the PKA activity and the S892 phosphorylation. The physiological implications of S892 phosphorylation were recessed using a signaling cAMP level increasing pathway, which was done through β-adrenergic receptor activation. The noradrenaline application to the cultured cortical cells, cotransfected with the $GABA_{B2}$ and β2 adrenergic receptors, shows an increase of the S892 phosphorylation. The phosphorylation of S892 was monitored after the incubation of forskolin or isoproterenol (a β-adrenergic agonist) plus 8-Br-Rp-cAMP. The forskolin stimulates as expected S892 phosphorylation in the cultured neurons, the S892 phosphorylation was also stimulated on the isoproterenol treatment. The 8-Br-Rp-cAMP pretreatment reduced the enhancement of the S892 phosphorylation by the β-adrenergic stimulation; the control protein amounts are unchanged. They also indicate that the signaling pathways modulating cAMP

concentration and modify the PKA activation have significant affects on $GABA_B$ receptors S892 phosphorylation in cortical and hippocampal neurons. In conclusion, $GABA_{B2}$ S892 phosphorylation underlies enhanced coupling of $GABA_B$ receptors to inwardly rectifying K^+ channels after PKA activation in hippocampal neurons.

3.4.2 AMPK sites: serine 783 on the $GABA_{B2}$ subunit and serine 917 on the $GABA_{B1}$ subunit

Most of the identified AMPK substrates are involved in the control of carbohydrate and lipid metabolism. Kuramoto et al. showed the first neuroprotective target of AMPK activity, namely, the $GABA_B$ receptors (Kuramoto, 2007). A yeast two-hybrid screen of a rat brain library using the $GABA_{B1}$ cytoplasmic tail identified the AMPK α1 subunit as interacting protein. The coprecipitation analysis of endogenous proteins in rat brain extracts confirmed the result. The α1 and α 2 AMPK isoforms and the $GABA_{B1}$ and the $GABA_{B2}$ subunits were found to be associated together. The fluorescence microscopy in neuronal processes of cultured hippocampal neurons reveals that colocalization, especially in neuronal processes or in the nucleus. The GST-fusion proteins of carboxyl-terminal tails of both $GABA_B$ receptor subunits; GST-CR1 and GST-CR2, were phosphorylated by the endogenous AMPK in rat brain extracts and by purified AMPK. The two major phosphorylation sites were identified combining high pressure liquid chromatography and masspectrometry; the serine 917 (S917) on $GABA_{B1}$ subunits and the serine 783 (S783) on $GABA_{B2}$ subunits.

In the HEK 293 cells transiently expressing $GABA_{B1,}$ $GABA_{B2}$ subunits and the K^+ channels Kir 3.1 and 3.2, a time-dependent rundown of K^+ currents was observed. The rundown was caused by a GABA internal perfusion via a patch pipette, containing ATP and GTP. The application of AMP reduced the rundown. The transfection of the $GABA_{B2}$ subunits serine 783 alanine substitution mutants (S783A) inhibits even that rundown. The activation of the cultured hippocampal neurons, where metformin activated the AMPK, still shows in the $GABA_{B1}$ S917A mutants the rundown after baclofen administration. It suggests the S783 phosphorylation has a stabilizing effect on the K^+ channels activation by $GABA_B$ receptors. The phosphospecific antibody, anti-pS783, recognizes the AMPK phosphorylated $GABA_{B2}$ subunits, but not the S738A mutant. Phenformin, an AMPK activating drug, increases in transfected HEK 293 as well cultured hippocampal cells the phosphorylation of the S783. The immunofluorescence experiences using anti-pS783 antibody reveal a minority of cellular $GABA_{B2}$ subunits is phosphorylated at S783. To assess whether the AMPK is necessary for S783 phosphorylation, an inactive AMPK mutant was transfected into cultured hippocampal neurons. The S783 phosphorylation signal was significantly decreased in the hippocampal regions CA3 and dentate gyrus (DG), only on the injured side of the brain; the anti-pS783 immunoreactivity was enhanced. The

total $GABA_{B2}$ amount was unaltered in response to ischemia. To address the question whether the S783 phosphorylation exerts a neuroprotective effect cultured hippocampal neurons underlying the catabolism inhibition (using deoxyglucose plus azide) and the glycolysis, causing the AMPK to activate and the $GABA_{B2}$ S783 to get phosphorylated, were studied. The expression of the WT or the S783A mutant of $GABA_{B2}$ subunits in these cells causes significant decreases in survival after the anoxic insults in cells expressing the mutant. Kuramoto et al. support with these results previous findings of the neuroprotective effect of AMPK during metabolic insults (Culmsee, 2001). But they did not address whether the coupling of the $GABA_B$ receptors to the Ca^{2+} channels was also affected. If phosphorylation works by promoting coupling between the receptor and G-proteins, like it seems to be the case, AMPK activation would not only accentuate hyperpolarization in response to postsynaptic action of GABA, it would promote also its presynaptic effects inhibiting glutamate release.

IV. Results

The results chapter is divided in two parts. First, the actual manuscript for a potential publication concerning the identified phosphorylation site serine 867 (S867), located on the $GABA_{B1}$ subunits. Second, supplemental results are added to explain and actualize the previous findings. This includes the results from the preliminary sequence analysis showing the first phosphorylation experiments and indicating the phosphorylation of S867. That part also contains the latest results showing the predominant $GABA_{B1b}$ subunit isoforms S867 phosphorylation *in vivo*. Additionally, a potential NMDA receptor dependent mechanism regulating S867 phosphorylation is presented. Finally the electrophysiological findings proving the regulatory effect of S867 phosphorylation on the $GABA_B$ receptor function. Some chapters of the second part will flow into the next manuscript.

4.1 NMDA receptor activation decreases surface $GABA_B$ receptors by CaMKII-mediated phosphorylation of $GABA_{B1}$ at serine 867 (Manuscript)

4.1.1 Abstract

$GABA_B$ receptors are G-protein-coupled receptors for gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain. Depending on their subcellular localization $GABA_B$ receptors exert distinct regulatory effects on synaptic transmission. While presynaptic $GABA_B$ receptors inhibit the release of neurotransmitters, postsynaptic $GABA_B$ receptors inhibit neuronal excitability by gating Kir3-type K^+ channels. In hippocampal pyramidal neurons postsynaptic $GABA_B$ receptors are abundant around excitatory synapses and their cell surface availability was reported to be controlled by glutamate. However, the mechanism by which glutamate regulates surface $GABA_B$ receptor levels has not been studied. Here, we report that in primary hippocampal neurons activation of N-methyl-D-aspartic acid (NMDA) receptors decreases $GABA_B$ cell surface levels by promoting their internalization. This internalization is dependent on Ca^{2+} influx and can be blocked by inhibiting CaMKII. We show that serine 867 in the carboxyl-terminus of the $GABA_{B1}$ subunit ($GABA_{B1}$S867) is phosphorylated by CaMKII. Preventing phosphorylation of the $GABA_{B1}$ carboxyl-terminus by mutation of $GABA_{B1}$S867 to alanine renders $GABA_B$ receptors refractory to NMDA-mediated internalization. These results indicate that NMDA receptor activation controls the availability of surface $GABA_B$ receptors through CaMKII-mediated phosphorylation of $GABA_{B1}$S867. The regulation of $GABA_B$ receptor surface levels may provide a mechanism that increases the time-window of postsynaptic neuronal excitability upon NMDA receptor activation.

4.1.2 Introduction

The modulation of synaptic transmission and strength depends on mechanisms regulating the number of neurotransmitter receptors at the plasma membrane and consequently their availability to neurotransmitters. Surface trafficking of the ionotropic neurotransmitter receptor in response to synaptic activity has been extensively studied (Moss, 2001, Inoue, 2003). In contrast, little is known about the mechanisms that regulate the trafficking of $GABA_B$ receptors in response to synaptic activity. $GABA_B$ receptors are the metabotropic receptors for γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain. They exist as obligate heteromers composed of $GABA_{B1}$ and $GABA_{B2}$ subunits. Both subunits are required for normal receptor functioning (Kaupmann, 1998a, Prosser, 2001, Schuler, 2001, Gassmann, 2004). The $GABA_{B1}$ subunit binds GABA, whereas the $GABA_{B2}$ subunit is responsible for escorting $GABA_{B1}$ to the cell surface and for G-protein coupling (Bettler, 2004). Depending on their subcellular localization $GABA_B$ receptors exert distinct regulatory effects on synaptic transmission (Couve, 2000, Bowery, 2002, Ulrich, 2007). Presynaptic $GABA_B$ receptors inhibit the release of GABA (autoreceptors) and other neurotransmitters (heteroreceptors) (Scanziani, 1992, Jarolimek, 1997, Yamada, 1999). Postsynaptic $GABA_B$ receptors inhibit neuronal excitability by activating Kir3-type K^+ channels that shunt excitatory currents and induce membrane hyperpolarization (Luscher, 1997). Two different $GABA_{B1}$ subunit isoforms exist, $GABA_{B1a}$ and $GABA_{B1b}$, giving rise to the receptor subtypes $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$. These receptor subtypes are differentially distributed in neurons (Kaupmann, 1997, Bischoff, 1999). In hippocampal pyramidal neurons $GABA_{B1a}$ is most abundant presynaptically, whereas $GABA_{B1b}$ is predominant postsynaptically (Vigot, 2006, Guetg, 2009).

Basal endocytosis of $GABA_B$ receptors can be observed in heterologous cells (Grampp, 2007, Wilkins, 2008) as well as in neurons (Fairfax, 2004, Grampp, 2008), where it is confined to the somatodendritic compartment (Vargas, 2008). Upon basal endocytosis $GABA_B$ receptors are delivered via dynamin- and clathrin-dependent pathways to lysosomes for degradation (Grampp, 2007, Vargas, 2008).

Contradicting results were found regarding the regulation of surface $GABA_B$ receptor levels upon exposure to agonist. While some reported agonist accelerated endocytosis (Ramoino, 2006, Laffray, 2007, Grampp, 2008), others did not see $GABA_B$ receptor internalization upon GABA application (Couve, 2002, Fairfax, 2004, Grampp, 2007, Vargas, 2008). Interestingly, a recent study demonstrated that exposure of cortical neurons to glutamate, but not GABA, decreases surface $GABA_B$ receptors by promoting their degradation via proteosomes (Vargas, 2008). Due to the prominent localization of postsynaptic $GABA_B$ receptors in spines opposite glutamatergic boutons (Kulik, 2003, Lopez-Bendito, 2004, Kulik, 2006) the modulation of surface $GABA_B$ receptor levels by glutamate could be of

functional relevance. However, the glutamate receptors involved as well as the signaling mechanisms downstream of receptor activation have not been identified. Glutamate release from presynaptic neurons activates ionotropic as well as metabotropic glutamate receptors. Ionotropic glutamate receptors are divided into AMPA, NMDA and kainate receptors based on pharmacological and structural criteria (Collingridge, 1989). AMPA receptors primarily function as Na^+ channels, with rapid activation and deactivation kinetics. They serve as mediators of fast excitatory neurotransmission. NMDA receptors additionally flux Ca^{2+} ions and have slower kinetics than AMPA receptors. A unique property of NMDA receptors is their voltage dependent activation, a result of ion channel block by extracellular Mg^{2+} ions. Under certain conditions, the Ca^{2+}-influx through NMDA receptors promotes the activation of Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) (Malenka, 1989a, Malinow, 1989, Lisman, 2002).

In the current study, we focused on unraveling the mechanism by which glutamate regulates surface $GABA_B$ receptor levels in primary hippocampal neurons, using a pharmacological approach. We report that NMDA receptor activation decreases surface $GABA_B$ receptor levels and provide evidence that this is at least in part due to increased internalization. Moreover, we show that this mechanism is CaMKII dependent and involves direct phosphorylation of serine 867 in the carboxyl-terminus of $GABA_{B1}$. The regulation of $GABA_B$ surface levels by NMDA receptor activation may provide a mechanism to regulate neuronal excitability and synaptic strength.

4.1.3 Experimental Procedures

4.1.3.1 Neuronal cultures and transfection

High-density primary hippocampal neuronal cultures were prepared from 18.5-day old rat embryos and cultured in Neurobasalmedium supplemented with B27 (Invitrogen) at a density of ~750 cells/mm^2 on poly-L-lysine coated coverslips as described previously (Brewer, 1993, Goslin, 1998). At day in vitro (DIV) 7, neurons were co-transfected with $GABA_{B1b}$ (HA-GB1b-eGFP or HA-GB1bS867A-eGFP) and $GABA_{B2}$ (Myc-GB2) expression vectors using Lipofectamin 2000 (Invitrogen). The HA- and Myc-tags were located at the very N-terminus of the extracellular domain of $GABA_{B1b}$ and $GABA_{B2}$, respectively. Gene expression was under control of the neuron specific *synapsin-1* promoter (gift from T. Oertner/K. Svoboda).

4.1.3.2 Treatment protocols for neuronal culture

All treatments were performed in conditioned medium at 37°C / 5% CO_2. Control neurons were incubated for 30 min with 5 µM glycine (Sigma-Aldrich). Glutamate treatment: neurons were incubated for 30 min with 50 µM glutamate (Sigma-Aldrich) and 5µM glycine. NMDA treatment:

neurons were incubated for 3 min with 75 µM N-Methyl-D-aspartic acid (NMDA, Sigma-Aldrich) and 5 µM glycine, and returned to conditioned medium for 27 min (adapted from (Kim, 2007). APV, KN-93 and EGTA treatment were applied prior to glutamate or NMDA treatment. Treatment procedures were as follows: APV treatment: neurons were preincubated for 2 h in 100 µM D-(-)-2-Amino-5-phosphonopentanoic acid (APV, Tocris); KN-93 treatment: neurons were preincubated in 10 µM. N-[2-[[[3-(4-Chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulphonamide (KN-93, Tocris) for 2 h; EGTA treatment: neurons were preincubated in 2 mM Ethylene glycol-bis-(2-aminoethyl)-N,N,N', N'-tetraacetic acid (EGTA, Sigma-Aldrich) for 10 min (adapted from (Faure, 2007)).

4.1.3.3 Immunocytochemistry and quantification

Neurons were fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) in phosphate buffered saline (PBS) containing 120 mM sucrose for 20 min, permeabilized with 0.25% Triton-X-100 in PBS for 10 min and blocked for 2 h in 10% normal goat serum (NGS, Invitrogen) in PBS. Primary antibodies were incubated for 2 h in 10% NGS/PBS; secondary antibodies were incubated for 1 h in 10% NGS/PBS. Stained neuronal cultures were mounted in FluorSave Reagent (Calbiochem) and viewed on a Leica TCS SPE confocal microscope. Digital pictures were captured with Leica Software (LAS AF) and identically processed with ImageJ software (Abramoff, 2004). In order to label surface HA-GB1b-eGFP protein, neurons were stained with mouse anti-HA antibodies (1:500, Covance) prior to permeabilization. Total HA-GB1b-eGFP protein was labeled with rabbit anti-eGFP antibodies (1:500, Molecular Probes) following permeabilization. Internalized HA-GB1b-eGFP protein was labeled as follows: HA-GB1b-eGFP protein at the cell surface of living neurons was labeled prior to application of the treatment protocol by incubation with rabbit anti-HA antibodies (1:50, Immunology Consultants Laboratory) for 30 min at 37°C / 5% CO_2. Following application of the treatment protocol, anti-HA antibodies were removed from HA-GB1b-eGFP remaining at the cell surface by an acid wash (2 min wash in Neurobasalmedium pH 2.0 on ice, followed by intensive washing with Neurobasalmedium pH 7.4 on ice). Following fixation and permeabilization, anti-HA antibodies bound to internalized HA-GB1b-eGFP were labeled with fluorophore-conjugated secondary anti-rabbit antibodies. An appropriate number of neurons were stained with secondary anti-rabbit antibodies without permeabilization as a control for the acid wash. Secondary antibodies were: Alexa Fluor 488 goat anti-mouse, Alexa Fluor 568 goat anti-mouse, Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 568 goat anti-rabbit (1:500; Molecular Probes). All steps were performed at room temperature unless indicated differently. The fluorescent intensity of labeled HA-GB1b-eGFP proteins was measured on the soma of transfected neurons and visualized in single optical planes using ImageJ software. For quantification of surface HA-GB1b-eGFP protein levels, fluorescent intensity of surface

HA-GB1b-eGFP was normalized to the total HA-GB1b-eGFP fluorescent intensity. Statistical analysis was performed with Graph Pad Software. For each statistical analysis independent sample groups were analyzed.

4.1.3.4 Co-Immunoprecipitation

Brain membranes from BALB/c mice were prepared as follows. Whole brains were polytron-homogenized in HEPES buffer (4 mM HEPES pH 7.4, 320 mM sucrose, 1 mM EDTA, 1 mM EGTA) containing Complete Protease Inhibitor Cocktail (Roche) and centrifuged for 10 min at 1000 x g. Samples were kept at 4°C throughout the procedure. Supernatants were collected and ultracentrifuged for 30 min at 48000 x g. The resulting pellets (P2) containing the membrane fraction were solubilized in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate) and were centrifuged for 30 min at 10000 x g to remove insoluble material. Supernatants were precleared by 3 h incubation with protein G-agarose beads (Roche). Anti-$GABA_{B1}$ antibody 174.1 (Malitschek, 1998) and anti-$GABA_{B2}$ antibody (Chemicon) respectively, were added to the precleared lysates and incubated for 1 h followed by overnight incubation with protein G-agarose beads. Rabbit and guinea pig serum were used as a control. Immunoprecipitated complexes were washed extensively with RIPA buffer, separated by SDS-page and probed with an anti-CaMKIIα antibody (1:1000, Santa Cruz).

4.1.3.5 GST-fusion proteins

To generate GST-GB1 the full-length carboxyl-terminus of $GABA_{B1}$ (amino acids 857–960) was amplified by PCR from rat $GABA_{B1}$ cDNA (Kaupmann, 1997), digested with *BamH*I and *Xho*I, and subcloned in-frame into pGEX-4T-1 fusion vector (GE Healthcare). GST-fusion protein expression vectors containing alanine substitutions of serine or threonine residues in the $GABA_{B1}$ carboxyl-terminus (GST-GB1S867A, GST-GB1T869A, GST-GB1T872A and GST-GB1T869A/T872A) were generated by overlapping PCR from GST-GB1. To generate GST-GB2 the full-length carboxyl-terminus of $GABA_{B2}$ (amino acid 745-940) was amplified by PCR from rat $GABA_{B2}$ cDNA (Kaupmann, 1998b), digested with *Bam*HI- and *EcoR*I and subcloned in-frame into pGEX-4T-1. GST-fusion proteins were expressed in *E. coli* BL21 (DE3) competent cells. Expression of the fusion-proteins was induced by 1mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 4 h. Cells were lysed by sonication and GST-fusion proteins were purified by incubation with Glutathione Sepharose 4B (Amersham Biosciences) overnight at 4°C and washed with PBS.

4.1.3.6 Pull-Down assay

Whole-brain lysates from BALB/c mice were prepared by polytron-homogenization in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% Triton X-100) containing Complete Protease Inhibitor Cocktail. Lysates were cleared by centrifugation at 15700 x g for 30 min at 4°C and incubated for 4 h with GST-fusion proteins immobilized on sepharose beads. Following extensive washing in RIPA buffer, isolated proteins were eluted by boiling in Laemmli buffer, separated by SDS-page and probed with anti-CaMKII antibody (1:5000, BD Biosciences).

4.1.3.7 In vitro kinase assay

GST-fusion proteins immobilized on sepharose beads were phosphorylated with recombinant CaMKII (New England Biolabs) or the cytosolic fraction (S2) of whole-brain extracts prepared from BALB/c mice by polytron-homogenization in HEPES buffer (brain membrane preparation above). Recombinant CaMKII was activated in phosphorylation buffer for 10 min at 30°C, according to the manufacturer's instructions. For whole-brain extracts the following phosphorylation buffer was used: 20 mM HEPES pH7.4, 1.7 mM $CaCl_2$, 0.1 M dithiothreitol, 10 mM $MgCl_2$, 1.6 mM cold ATP. For each phosphorylation reaction, 20 µg GST fusion-protein was incubated with recombinant CaMKII (500 units) or whole-brain extract (50 µg) in the appropriate phosphorylation buffer and in the presence of 1 µl [γ-^{32}P]-ATP (3000Ci/mmol) for 30 min at 30°C. Free [γ-^{32}P]-ATP was removed by extensive washing with ice-cold phosphorylation buffer. To inhibit CaMKII activity, whole-brain extracts were preincubated with 10 µM KN-93 for 20 min at 4°C. Phosphorylated GST-fusion proteins were either separated by SDS-page and subjected to autoradiography or subsequently used for RP-HPLC and ESI-MS/MS analysis.

4.1.3.8 Reverse phase-high pressure liquid chromatography (RP-HPLC)

For HPLC analysis, 20 µg of phosphorylated GST-fusion protein was digested with the endoproteinase Lys C (Wako Chemicals) followed by a second digestion with trypsin (Promega). Digestion was stopped by adding TFA to 0.1% (v/v) final concentration (TFA, Applied Biosystems). Insoluble material was removed by centrifugation (12,000 rpm, 5 min) and the supernatant was subjected to RP-HPLC on Vydac C18 reverse-phase columns (218TP52, 2.1 x 250 mm; Grace Vydac) connected to a Hewlett Packard 1090 HPLC system. Bound peptides were eluted at 150 l/min with a linear gradient from 0.1% TFA/2% acetonitrile to 0.09% TFA/75% acetonitrile during 60 min. The effluent was monitored at 214 nm. Fractions were collected at 1 min intervals. The phosphorylated peptides in the fractions were located by liquid scintillation counting.

4.1.3.9 Electro spray ionization mass spectrometry (ESI-MS/MS)

The radioactively labeled peptides were analysed by capillary liquid chromatography tandem MS (LC/MS/MS) using a set up of a trapping 300SB C-18 column (0.3x50mm) (Agilent Technologies) and a separating column (0.1x100mm) that had been packed with Magic 300Å C18 reverse-phase material (5 µm particle size, Michrom Bioresources Inc.). The columns were connected on line to an Orbitrap FT hybrid instrument (Thermo Finnigan). A linear gradient from 2 to 80% solvent B (0.1% acetic acid and 80% acetonitrile in water) in solvent A (0.1% acetic acid and 2% acetonitrile in water) in 85 min was delivered with a Rheos 2200 pump (Flux Instruments) at a flow rate of 100 µl/min. A pre-column split was used to reduce the flow to approximately 100 nl/min. The eluting peptides were ionized at 1.7 kV. The mass spectrometer was operated in a data-dependent fashion. The precursor scan was done in the Orbitrap set to 60,000 resolutions, while the fragment ions were mass analysed in the LTQ instrument. A top five method was run so that the five most intense precursors were selected for fragmentation. The MS/MS spectra were then searched against the NCBI non-redundant databank using TurboSequest or Mascot software (Perkins, 1999, Gatlin, 2000).

4.1.4 Results

4.1.4.1 Glutamate-induced decrease of cell surface GABAB receptors is NMDA receptor dependent

It was recently described that glutamate controls the availability of cell surface $GABA_B$ receptors in cortical neurons by promoting degradation of endocytosed receptors (Vargas, 2008). However, identification of the glutamate receptors and signaling cascades involved in this regulation has been lacking. We addressed the mechanism by which glutamate decreases the level of cell surface $GABA_B$ receptors in cultured neurons using a pharmacological approach. Abundant cell surface expression of $GABA_{B1b}$ was observed following co-transfection of primary hippocampal neurons with $GABA_{B1b}$ and $GABA_{B2}$ expression constructs (HA-GB1b-eGFP and Myc-GB2, respectively). Surface expression was monitored by staining living cells with antibodies directed against the N-terminal HA-tag prior permeabilization (Figure 1*A*). Total HA-GB1b-eGFP levels were detected by anti-eGFP immunostaining following permeabilization of transfected cultures (Figure 1*B*). To quantify the level of surface $GABA_{B1b}$ between different treatments, surface HA-GB1b-eGFP staining was normalized to total HA-GB1b-eGFP. Upon glutamate treatment (50 µM for 30 min in the presence of 5 µM glycine), surface $GABA_{B1b}$ levels were significantly reduced to 41% of control (Figure 1*A* and *B*). Preincubation of neurons with the NMDA receptor antagonist APV (100 µM for 2 hours) prevented this glutamate-induced decrease in surface $GABA_{B1b}$ (Figure 1*A* und *B*). This indicates that activation of NMDA receptors is critical for decreasing surface $GABA_{B1b}$ levels. We next tested whether direct activation of

NMDA receptors is sufficient to decrease surface $GABA_{B1b}$ levels. Following a short pulse of NMDA application (75µM NMDA for 3 min in the presence of 5µM glycine) and recovery in conditioned medium for 27 min, surface $GABA_{B1b}$ levels were significantly reduced to 28% of control levels (Figure 1*A* and *B*). We examined whether the decrease of surface $GABA_{B1b}$ levels after glutamate and NMDA treatment is caused by the removal of receptors from the cell surface. While basal endocytosis of surface $GABA_{B1b}$ was detectable under control conditions, the pool of endocytosed $GABA_{B1b}$ was visibly increased upon glutamate and NMDA treatment. Endocytosis was prevented by preincubation with APV (Figure 1*C*). In conclusion, these findings indicate that glutamate reduces the availability of cell surface $GABA_B$ receptors by promoting internalization and that this process is dependent on the activation of NMDA receptors.

4.1.4.2 NMDA receptor-induced decrease of cell surface $GABA_B$ receptors is mediated through CaMKII

We assessed whether Ca^{2+}-influx through NMDA receptors is critical for the regulation of surface $GABA_B$ receptor levels by glutamate. In the presence of the extracellular Ca^{2+}-chelator EGTA, glutamate failed to significantly reduce $GABA_{B1b}$ surface levels in transfected hippocampal neurons (normalized surface $GABA_{B1b}$: glutamate, 34 ± 6.4% of control, n = 10, ***p < 0.001; glutamate + EGTA, 79 ± 6.0% of control, n = 10, p > 0.05; mean ± SEM, one-way ANOVA followed by Dunnet post-hoc test). CaMKII can be activated by the Ca^{2+} elevation following NMDA receptor activation in the postsynaptic cell. We therefore investigated whether CaMKII activation is required for the NMDA receptor induced decrease in cell surface $GABA_B$ receptors. Upon preincubation of primary hippocampal neuronal cultures with the CaMKII inhibitor KN-93, glutamate (Figure 2*A*) and NMDA (Figure 2*B*) failed to reduce surface $GABA_{B1b}$ levels. These experiments demonstrate that CaMKII activation by NMDA receptors is involved in controlling $GABA_B$ receptor levels at the cell surface.

4.1.4.3 CaMKII binds and phosphorylates $GABA_B$ receptors

We next investigated whether CaMKII can associate with $GABA_B$ receptors. We found that rabbit anti-$GABA_{B1}$ or guinea pig anti-$GABA_{B2}$ antibodies co-immunoprecipitated CaMKII from purified mouse brain membranes. As a control, rabbit and guinea pig sera did not precipitate CaMKII (Figure 3*A*). Furthermore GST pull-down assays were used to demonstrate the association of CaMKII with $GABA_B$ receptors. As the carboxyl-termini of $GABA_{B1}$ and $GABA_{B2}$ are relatively large domains, in contrast to the intracellular loops, they represent the most likely targets for modification by kinases (Fairfax, 2004). GST-fusion proteins containing the entire carboxyl-terminus of $GABA_{B1}$ (GST-GB1) or $GABA_{B2}$ (GST-GB2) were generated. CaMKII from whole-brain lysates was predominantly binding to GST-GB1, and to a lesser extent to GST-GB2 (Figure 3*B*). *In vitro* phosphorylation assays were

performed to further evaluate the phosphorylation of $GABA_B$ receptor carboxyl-termini by CaMKII. The GST-fusion proteins were incubated with [γ-^{32}P]-ATP and recombinant CaMKII for 30 min at 30°C and resolved by SDS-PAGE. CaMKII-dependent phosphorylation was detected by autoradiography and was observed on GST-GB1 but not GST-GB2 or GST alone (Figure 3*C*). The degradation of the GST-GB2 fusion protein seen by Coomassie blue staining is consistent with earlier reports from other laboratories (Couve, 2002). Our findings indicate that CaMKII is associated with heteromeric $GABA_B$ receptors in the brain, and specifically phosphorylates the carboxyl-terminus of $GABA_{B1}$.

4.1.4.4 Identification of a CaMKII phosphorylation site within the carboxyl-terminal of $GABA_{B1}$

According to phosphorylation prediction programs, the carboxyl-terminus of $GABA_{B1}$ contains a number of putative CaMKII phosphorylation sites (Pearson, 1985). To identify residues that are phosphorylated by CaMKII, proteolytic digests of in vitro phosphorylated GST-GB1 were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC) combined with electro spray ionization mass spectrometry (ESI-MS/MS). GST-GB1 was phosphorylated by recombinant CaMKII in the presence of [γ-^{32}P]-ATP and digested by LysC and trypsin. Resulting peptides were separated by RP-HPLC analysis (Figure 4*A*, upper panel) and fractions collected at 1 min intervals. The majority of the radiolabel eluted in a single peak in fraction 54 (Figure 4*A*, lower panel), which was subjected to ESI-MS/MS analysis. An abundant phosphorylated peptide corresponding to residues 863-884 of the $GABA_{B1}$ carboxyl-terminus was identified (GEWQpSETQDTMK) and phosphorylation was assigned to serine 867 (Figure 4*B*). Mutational analysis of the GST-GB1 fusion protein revealed that serine 867 is the only phosphorylation site in the carboxyl-terminus of $GABA_{B1}$ that is phosphorylated by recombinant CaMKII (Figure 4*C*). Specifically, no phosphorylation was observed with GST-GB1S867A, which contains a S867 to alanine. Moreover, substitution of threonine 869 and/or threonine 872 with alanine (GST-GB1T869A, GST-GB1T872A and GST-GB1T869A/T872A) did not prevent phosphorylation of the GST fusion proteins. By combining different approaches, we have shown that recombinant CaMKII is specifically phosphorylating $GABA_{B1}$867.

4.1.4.5 Endogenous CaMKII phosphorylates serine 867

Next we tested whether serine 867 within the carboxyl-terminus of $GABA_{B1}$ is phosphorylated by kinases in brain extractions. GST-GB1 was incubated with the cytosolic fraction of whole brain extracts in the presence of [γ-^{32}P]-ATP and proteolytic peptides were separated by RP-HPLC (Figure 5). Similar to phosphorylation by recombinant CaMKII, the majority of radiolabel eluted in a single peak following from the reverse-phase column (Figure 5*A*). ESI-MS/MS analysis demonstrated that the $GABA_{B1}$ carboxyl-terminal peptide GEWQSETQDTMK was a major constituent of the radiolabeled

fraction. Due to low phosphorylation stoechiometry obtained with endogenous brain kinases direct phosphorylation of serine 867 could not be demonstrated. However, in phosphorylation experiments with brain extracts and GST-GB1S867A, the RP-HPLC fraction containing the mutated peptide GEWQAETQDTMK did not reveal any radiolabel (Figure 5*B*). Moreover, when GST-GB1 was phosphorylated with brain extract in the presence of the CaMKII inhibitor KN-93, no radiolabel was detected in the RP-HPLC fraction containing peptide GEWQSETQDTMK (Figure 5*C*). Thus, our findings indicate that CaMKII in whole brain extracts phosphorylates serine 867 in the carboxyl-terminus of $GABA_{B1}$.

Finally, we addressed whether phosphorylation of serine 867 participates in the reduction of surface $GABA_B$ receptors upon NMDA receptor activation. Primary hippocampal neurons were transfected with a $GABA_{B1b}$ mutant containing a substitution of serine 867 with alanine (HA-GB1bS867A-eGFP) together with Myc-GB2. Surface expression was monitored by staining living cells with antibodies directed against the N-terminal HA-tag prior to permeabilization and normalized to total $GABA_{B1b}$S867A levels detected by anti-eGFP immunostaining following permeabilization (Figure 6*A*). Quantitative analysis revealed that neither glutamate nor NMDA application was able to promote $GABA_{B1b}$S867A internalization (Figure 6*B*). In conclusion, our findings indicate that NMDA receptor activation controls surface $GABA_B$ receptor levels through CaMKII-dependent phosphorylation of serine 867 within the carboxyl-terminus of the $GABA_{B1}$ subunit.

4.1.5 Discussion

The regulation of synaptic transmission depends on the availability of neurotransmitter-receptors at the cell surface. Therefore it is important to understand the mechanisms that regulate the trafficking of receptors to and from the plasma membrane. Contradicting results have been reported regarding the regulation of surface $GABA_B$ receptor levels. Using different cell culture systems, it was shown that basal endocytosis and recycling takes place (Grampp, 2007, Vargas, 2008, Wilkins, 2008). However, although the rate of recycling was reported to be increased in the presence of agonist, the total amount of cell surface $GABA_B$ receptors stayed unchanged (Grampp, 2007, Grampp, 2008, Vargas, 2008). More importantly, new evidence suggested that surface $GABA_B$ receptor levels were decreased in the presence of glutamate (Vargas, 2008). In the present work, we aimed to identify the glutamate receptor type responsible for regulating surface $GABA_B$ receptor levels as well as to characterize the underlying molecular mechanism.

Our results confirm previous findings which describe reduction of surface $GABA_B$ receptors upon application of glutamate. Further, we identified the NMDA receptor as the glutamate receptor type

responsible for decreasing surface $GABA_B$ receptor levels. We demonstrate that $GABA_B$ receptors are internalized upon stimulation with glutamate or NMDA. In a recent report it was shown that glutamate stimulation may lead to proteolytic degradation of constitutively internalized $GABA_B$ receptors (Vargas, 2008). However, in contrast to the findings presented in our study, the authors did not detect increased levels of internalized receptors. This discrepancy could be caused by different experimental conditions.

Preincubation of neurons with an NMDA receptor antagonist prevented the glutamate-induced reduction of surface $GABA_B$ receptor levels, ruling out that other glutamate receptors directly regulate surface $GABA_B$ receptor levels. However, other glutamate receptors, in particular AMPA receptors, are likely to contribute to the NMDA receptor-mediated effect. NMDA receptor activation is voltage dependent, requiring removal of the Mg^{2+} block by depolarization. Under physiological conditions this is primarily mediated by AMPA receptor activation. It is expected that there is sufficient basal activity in primary hippocampal neuronal cultures allowing for direct NMDA receptors activation by NMDA. In conclusion, our results demonstrate that NMDA receptor activation is essential and sufficient to reduce $GABA_B$ surface receptors, however modulatory roles of other glutamate receptors cannot be ruled out.

NMDA receptors are localized at the postsynaptic density (PSD) of dendritic spines where their activation allows Ca^{2+} and Na^{+} to enter the spine. Glutamate failed to reduce surface $GABA_B$ receptors in the presence of extracellular EGTA, which implicates a crucial role for the Ca^{2+} influx following NMDA receptor activation. CaMKII is an established target downstream of NMDA receptor activation. CaMKII is one of the most abundant serine/threonine kinases in the brain and is enriched in the PSD. It is activated by binding of Ca^{2+}/Calmodulin (Bennett, 1983, Kennedy, 1983) and subsequent autophosphorylation (Malenka, 1989b, Bayer, 2001). The $GABA_B$ receptor is known to be localized predominantly perisynaptically (Kulik, 2006, Guetg, 2009) and therefore in the vicinity of PSD associated proteins. Since CaMKII is coupled to the activation of NMDA receptors in many physiological processes, it was tested whether CaMKII plays a critical role in NMDA induced surface reduction of $GABA_B$ receptors. Indeed, preincubation with a CaMKII inhibitor failed to decrease surface $GABA_B$ receptors. We show evidence that reduction of surface receptors is mediated trough phosphorylation of the $GABA_B$ receptor by CaMKII. Recent studies show that, upon glutamate application or LTP induction, increased levels of activated CaMKII are restricted to single spines and do not incorporate neighboring spines (Zhang, 2008, Lee, 2009) Therefore, glutamate- and NMDA-induced reduction of surface $GABA_B$ receptors most likely occurs postsynaptically on glutamatergic synapses.

We identified serine 867 within the carboxyl-terminus of $GABA_{B1}$ ($GABA_{B1}$S867) as a novel phosphorylation site that can be phosphorylated by recombinant as well as endogenous CaMKII.

Interestingly, the sequence around $GABA_{B1}$S867 is not conform to the consensus sequence for CaMKII phosphorylation (Pearson, 1985). $GABA_{B1}$S867 is located in the juxtamembrane domain which is recognized as a regulatory region for many transmembrane proteins, in particular receptor tyrosine kinases. However a similar regulatory role for the juxtamembrane domain in GPCRs has not been established yet. Although we cannot exclude the existence of additional CaMKII phosphorylation sites within other domains of $GABA_{B1}$ or on $GABA_{B2}$, mutational analysis demonstrates that $GABA_{B1}$S867 is essential for glutamate-induced reduction of surface $GABA_B$ receptor levels. Two other phosphorylation sites on the $GABA_B$ receptor are described; first, serine 892 on the $GABA_{B2}$ subunit ($GABA_{B2}$S892) was shown to be phosphorylated by cyclic AMP-dependent protein kinase (PKA) (Couve, 2002) and secondly, serine 783 on the $GABA_{B2}$ subunit ($GABA_{B2}$S783) by 5'AMP-dependent protein kinase (AMPK) (Scott, 2002, Hardie, 2007, Kuramoto, 2007).

Functionally, both $GABA_{B2}$S892 and $GABA_{B2}$S783 phosphorylation lead to stabilization of surface $GABA_B$ receptors and enhance $GABA_B$ receptor functions (Couve, 2002, Fairfax, 2004, Kuramoto, 2007). In contrast, our results demonstrate that phosphorylation of $GABA_{B1}$S867 reduces surface $GABA_B$ receptor levels. Similar to CaMKII, AMPK is activated in response to Ca^{2+}-influx following neuronal activity. However in contrast to CaMKII activation, which is directly mediated by calmodulin/Ca^{2+}, AMPK activation requires the activation of calmodulin-dependent kinase kinases, in particular CaMKKβ. Moreover, AMPK is activated in response to metabolic stresses that deplete neurons of ATP and increase ADP levels (Hawley, 2005, Witters, 2006, Hardie, 2007). Thus the availability of $GABA_B$ receptors at the plasma membrane is differentially modulated by the carboxyl-terminal phosphorylation.

While another report showed that stimulation with glutamate stabilizes $GABA_A$ receptors at the cell surface thereby promoting inhibition (Marsden, 2007) our results suggest that NMDA-induced decrease of $GABA_B$ surface receptors strengthens excitatory signals. The bidirectional effect of glutamate may operate on the fast acting inhibitory $GABA_A$ receptor to prevent overexcitation and neurotoxicity, whereas decrease of slow acting surface $GABA_B$ receptor may support memory formation and contribute to long term potentiation.

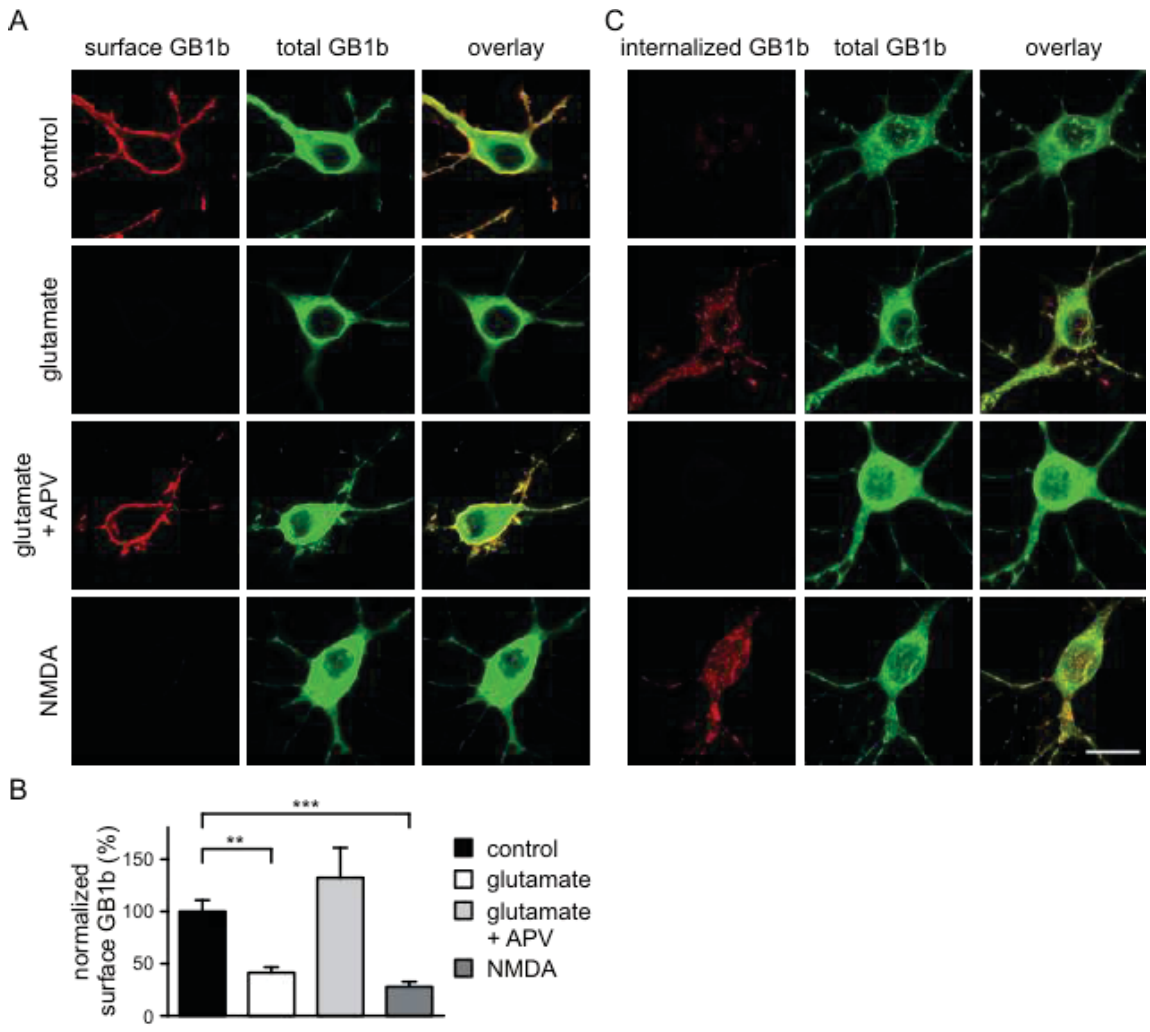

Figure 1. Glutamate-induced decrease of $GABA_B$ surface receptors is NMDA receptor dependent. *A*, Rat hippocampal neuronal cultures were cotransfected with HA-GB1b-eGFP and Myc-GB2 expression vectors and analyzed at DIV14. Surface $GABA_{B1b}$ (GB1b) proteins were labelled with anti-HA antibodies prior to permeabilization. Total GB1b protein was labelled with anti-eGFP antibodies after permeabilization. Treatment with 50 µM glutamate in the presence of 5 µM glycine for 30 min decreased surface $GABA_{B1b}$ levels. This decrease of surface $GABA_{B1b}$ was abolished when neurons were preincubated for 2 hours with 100 µM APV, a NMDA receptor antagonist. Specific activation of NMDA receptors with 75µM NMDA and 5µM glycine for 3 min followed by recovery in conditioned medium for 27 min was sufficient to reduce surface $GABA_{B1b}$ levels. Neurons were visualized with confocal microscopy and represented as single optical planes. *B*, Quantification of surface $GABA_{B1b}$ levels expressed as surface fluorescent intensity relative to total fluorescent intensity. Values for individual treatments were normalized to control. Normalized surface $GABA_{B1b}$ levels were significantly reduced after glutamate and NMDA treatment. No significant change was observed with glutamate treatment after preincubation with APV (control: 100 ± 11%, n = 10; glutamate: 41 ± 5%, n = 10; glutamate + APV: 132 ± 29%, n = 10; NMDA: 28 ± 5, n = 10). Data are presented as mean ± SEM. For statistical analysis one-way ANOVA, followed by Dunnet post-hoc test was performed; $^{**}p < 0.01$, $^{***}p < 0.001$. *C*, To assess internalization of surface $GABA_{B1b}$, live cells were incubated with anti-HA antibodies for 30 min at 37°C, washed and left untreated for another 30 min (control) or incubated with, glutamate, glutamate + APV or NMDA. Basal constitutive internalization was observed under control conditions. After glutamate treatment the rate of $GABA_{B1b}$ internalization was visibly increased. Preincubation with APV prevented glutamate-induced internalization of $GABA_{B1b}$ above control levels. Specific activation of NMDA receptors with NMDA was sufficient to increase internalization of $GABA_{B1b}$. Maximum projections of representative neurons visualized with confocal microscopy are shown. Scale bar = 15µm.

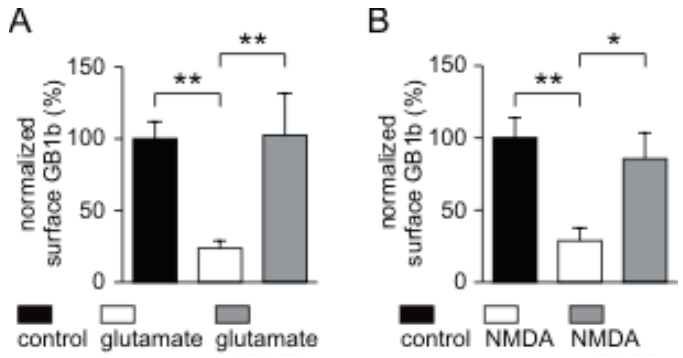

Figure 2. CAMKII is required for the NMDA receptor-induced decrease of surface $GABA_B$ receptors. Rat hippocampal neuronal cultures were co-transfected with HA-GB1b-eGFP and Myc-GB2 expression vectors and analyzed at DIV14. Surface $GABA_{B1b}$ (GB1b) labelled with anti-HA antibodies prior to permeabilization were assessed relative to total $GABA_{B1b}$ expression. Values for individual treatments were normalized to control. Preincubation of neurons with the CaMKII inhibitor KN-93 (10 µM) for 2 h prevented the reduction in surface $GABA_{B1b}$ levels following treatment with glutamate (control: 100 ± 12, *n* = 10; glutamate 24 ± 5, *n* = 10; glutamate + KN-93: 102 ± 29; *n* = 6;) or NMDA (control: 100 ± 14, *n* = 10; NMDA: 29 ± 9, *n* = 10; NMDA + KN-93: 86 ± 18, *n* = 6). Data were represented as mean ± SEM. For statistical analysis one-way ANOVA, followed by Tuckey post-hoc test was performed; **p* < 0.05, ***p* < 0.01.

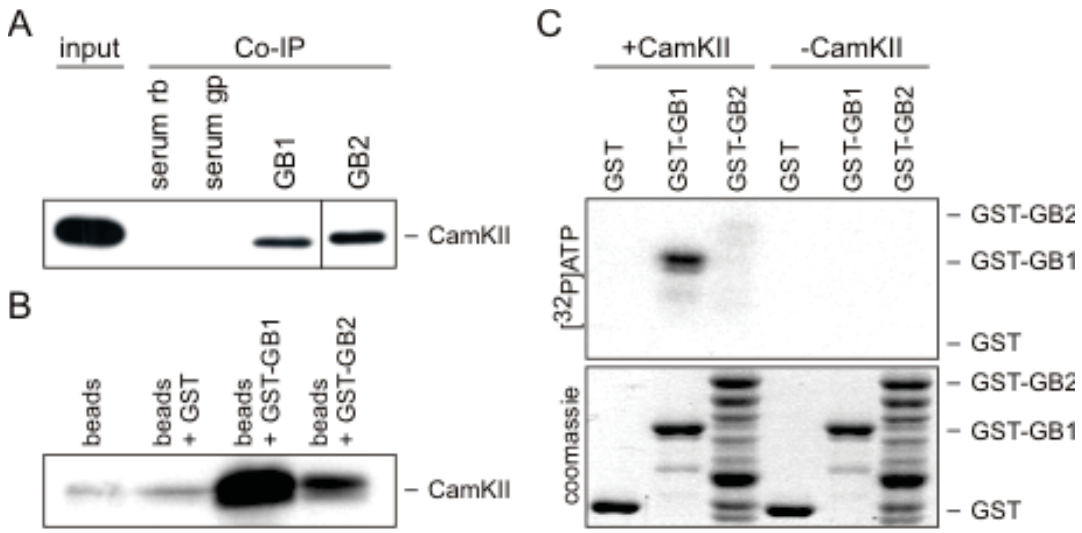

Figure 3. CaMKII is associated with $GABA_B$ receptors and phosphorylates residues within the carboxyl-terminal domain of $GABA_{B1}$. *A*, Anti-$GABA_{B1}$ (GB1) and anti-$GABA_{B2}$ (GB2) antibodies co-immunoprecipitated CaMKII from purified mouse brain membranes. Normal rabbit serum (serum rb) and normal guinea pig (serum gp) were used as control. *B*, Pull down assays with GST-fusion protein containing the entire carboxyl-terminus of $GABA_{B1}$ (GST-GB1) or $GABA_{B2}$ (GST-GB2). Immobilized GST-fusion proteins were incubated with whole brain lysates and washed in RIPA buffer. Interacting proteins were pulled-down by centrifugation, separated by SDS-PAGE and probed for CaMKII by Western blotting. Pull-down assays with glutathione beads alone or together with GST were used as a control. CaMKII was predominantly observed in samples pulled-down with GST-GB1 and to some extend also with GST-GB2. *C*, *In vitro* phosphorylation assays of GST-fusion proteins with recombinant CaMKII. GST, GST-GB1 and GST-GB2 were incubated with [γ-^{32}P]-ATP in the presence or absence of recombinant CaMKII. Phosphorylated proteins were separated by SDS-PAGE and exposed to autoradiography. Coomassie blue staining of the gel was used as loading control. Recombinant CaMKII specifically phosphorylated GST-GB1 but not GST-GB2 or GST alone. This demonstrates that residues within the carboxyl-terminal domain of $GABA_{B1}$ are accessible for phosphorylation by CaMKII.

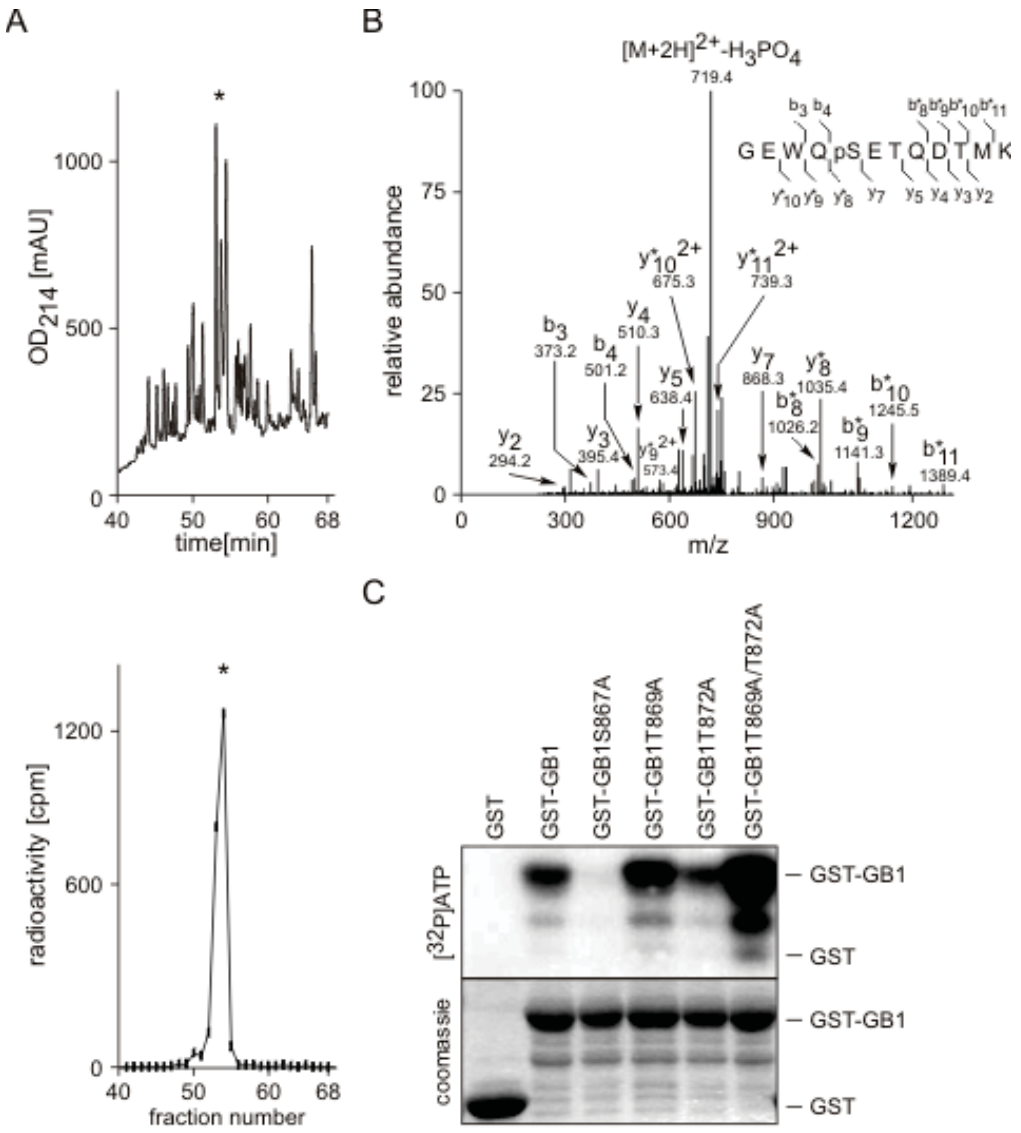

Figure 4. Identification of serine 867 as a CaMKII phosphorylation site on $GABA_{B1}$. *A*, Reverse-phase high-performance liquid chromatography (RP-HPLC) analysis of GST- $GABA_{B1}$ carboxyl-terminus fragment (GST-GB1) that had been phosphorylated by recombinant CaMKII. GST-GB1 and digested with endoproteinase LysC and trypsin. GST-GB1 elution of peptides monitored at 214 nm (*A*) and and liquid cintillation counting of the fractions collected during chromatography (*C*). The asterisk marks the elution of the phosphopeptide in fraction 54. *B*, Fragmentation spectrum of the doubly charged 768.29 Da precursor from the radioactively labelled peptide of fraction 54 of GST-GB1. The fragmentation pattern is in accordance with the predicted MS/MS spectrum for the phosphopeptide GEWQ***pS***^{867}ETQDTMK. The y- and b-ions that match the GEWQ***pS***^{867}ETQDTMK sequence are labelled. Phosphorylated ions are marked by an asterisk. *C, In vitro* phosphorylation of GST fusion proteins with recombinant CaMKII in the presence of [γ-^{32}P]-ATP. Samples were separated by SDS-page and exposed to autoradiography. Substitution of serine 867 with alanine in GST-GB1S867A prevented phosphorylation by CaMKII. In contrast, GST fusion proteins with substitutions of other phosphorylable residues in proximity of serine 867 were still phosphorylated (GST-GB1T869A, GST-GB1T872A and GST-GB1T869A/T872A). Coomassie blue stain was used as loading control.

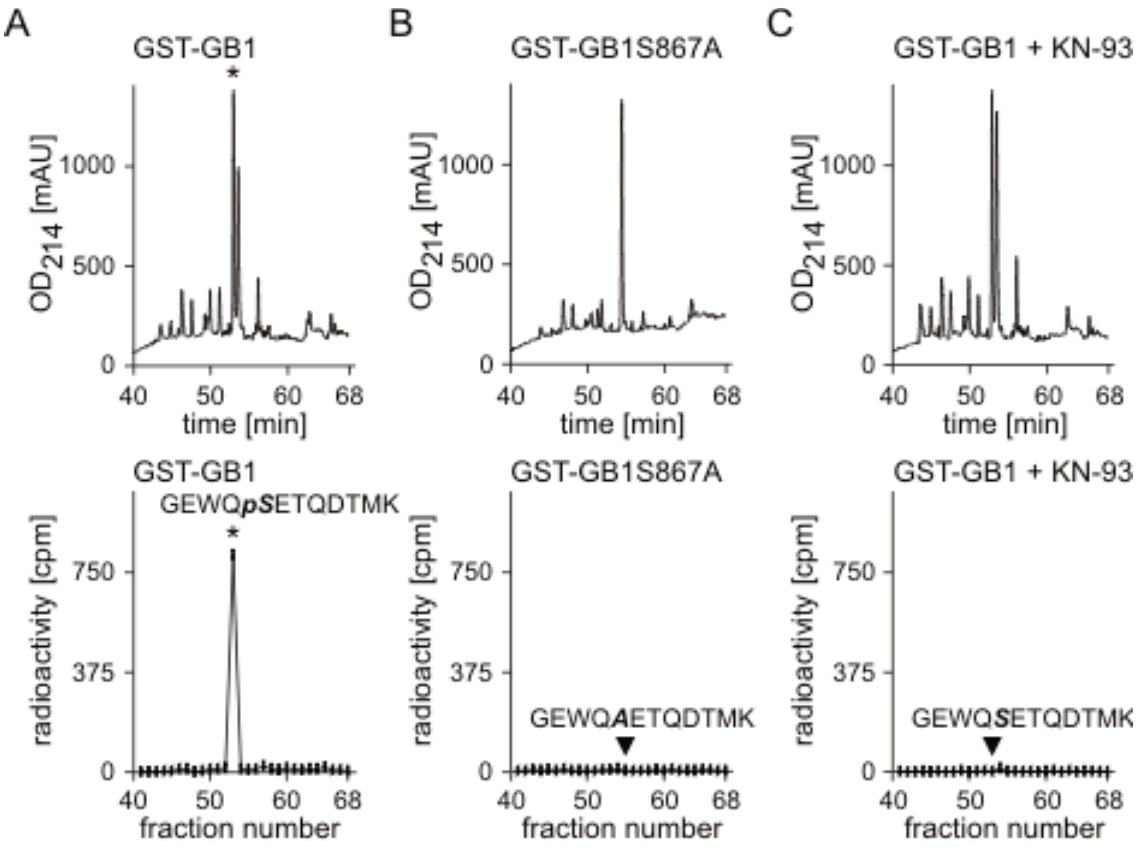

Figure 5. Endogenous CaMKII phosphorylates $GABA_{B1}$S867. (*A-C*) GST-fusion proteins were incubated with the cytosolic fraction of whole mouse brain extracts in the presence of [γ-^{32}P]-ATP and analyzed by RP-HPLC. Elution profiles are shown on the top and scintillation graphs on the bottom. The carboxyl-terminal $GABA_{B1}$ peptide GEWQ***S***ETQDTMK was identified by ESI-MS/MS as a major constituent of a highly phosphorylated fraction (asterisk) of the GST-GB1 effluent (*A*). The corresponding fraction of the GST-GB1S867A effluent (arrow head) containing peptide GEWQ***A***ETQDTMK with a substitution of alanine for serine 867 did not get phosphorylated (*B*). Following phosphorylation of GB1-GST in the presence of KN-93 no radiolabel was detected in the fraction containing GEWQ***S***ETQDTMK (arrow head) (C).

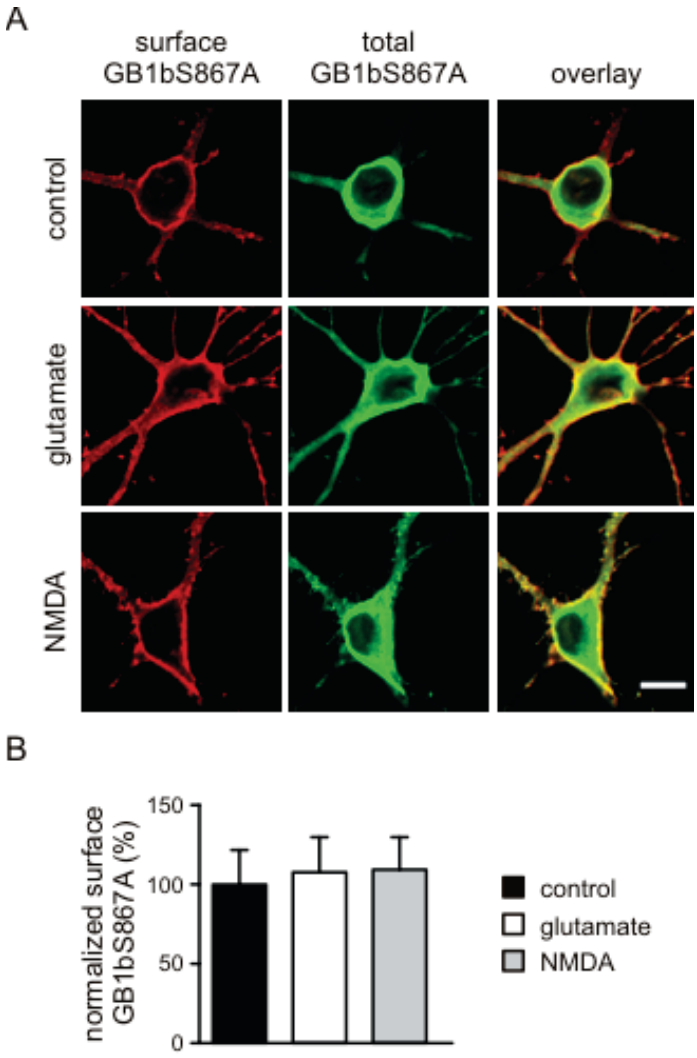

Figure 6. Mutation of serine 867 in $GABA_{B1}$ prevents NMDA receptor-induced down-regulation. *A*, Rat hippocampal neuronal cultures were cotransfected with HA-GB1bS867A-eGFP and Myc-GBR2 expressing vectors. At DIV14, neurons were treated with glutamate (50 µM for 30 min at 37°C in the presence of 5µM glycine) or NMDA (75 µM for 3 min at in the presence of 5µM glycine, followed by recovery in conditioned medium for 27 min). Surface $GABA_{B1b}$S867A protein was labelled prior to permeabilization with anti-HA antibodies. Total $GABA_{B1b}$S867A protein was labelled after permeabilization with anti-eGFP antibodies. Neurons were visualized with confocal microscopy and represented as single optical planes. Scale bar = 15µm. *B*, Glutamate as well as NMDA did not induce any significant changes in surface levels of $GABA_{B1b}$S867A. (control: 100 ± 22, n = 10; glutamate: 108 ± 22, n = 10; NMDA: 109 ± 21, n = 9). Data were represented as mean ± SEM. For statistical analysis one-way ANOVA, followed by Dunnet post-hoc test was performed.

Statement of personal contribution to the manuscript:

- Planning and generation of the GST-fusion proteins.
- Design and accomplishement of the pull down assays
- Design and accomplishement of all presented phosphorylation experiments with the purified and native kinases.
- Design and accomplishement of all HPLC experiments.
- Preparation of the samples for MS analysis.
- Preparation and analysis of the scintillation data.
- Identification of the phosphorylation site S867.
- Writing of the material method part of the phosphorylation eperiments, HPLC, scintillation and MS analysis.
- Writing of the results part of the phosphorylation eperiments, HPLC, scintillation and MS analysis.
- Contribution to the finalization of the manuscript.

4.2 Supplemental results

4.2.1 Analysis of the $GABA_{B1}$ subunit carboxyl-terminus for phosphorylation targets

Carboxyl-terminals are the most promising intracellular target domains for $GABA_B$ receptor phosphorylation. In the case of mGluRs, which are closely related to $GABA_B$ receptors, a growing body of literature suggests that phosphorylation happens at the carboxyl-terminal region and regulates the mGluRs function. Schaffhauser et al. discovered that mGluR2 phosphorylation by PKA at serine 843 inhibits its function, probably due to coupling of mGluR2 to GTP-binding proteins (Schaffhauser, 2000). Later, the same group stated that PKA inhibits the function of multiple mGluR subtypes by a similar mechanism (Cai, 2001). Other groups have rather attributed that effect to the PKC activity (Macek, 1998, Kim, 2005). However, all these works have as as common ground phosphorylation sites located in the carboxyl-terminal region of the mGluRs and an effect on the mGluRs function.

The carboxyl-termini of the $GABA_B$ receptor subunits are large domains and therefore, most likely include possible phosphorylation targets (Fairfax, 2004). The $GABA_{B1}$ carboxyl-terminal starts after the methionine 856, covering the amino acids from the arginine 857 (R857) up to the lysine 960 (K960) (Kaupmann, 1997). In Table 1, the predicted phosphorylation sites for the $GABA_{B1}$ carboxyl-terminal are summarized. There are six serines, four threonines and no tyrosine. None of the predicted sites was shown to be phosphorylated, but the serine 923 appeared to influence the S917 phosphorylation (Couve, 2002). The preliminary analysis of that carboxyl-terminal was done using many consensus motifs from literature (Table 2). Consensus motifs are the conserved kinase recognition sequences. The findings were validated further by state-of-the-art phosphorylation site prediction programs (Table 3). The phosphorylation site prediction has proven, due to its accuracy, to be of great importance for phosphorylation site research. The predicted sites are equally distributed and not concentrated at a certain region of the $GABA_{B1}$ carboxyl-terminal. Therfore, there is no specific region on which to focus thus the first *in vitro* phosphorylation experiments have to be done with the full-length $GABA_{B1}$ carboxyl-terminal GST-fusion protein.

$GABA_{B1}$ carboxyl-terminus

R857-RRLITRGEWQ SETQDTMKTG SSTNNNEEEK SRLLEKENRE LEKIIAEKEE RVSELRHQLQ

SRQQLRSRRH PPTPPDPSGG LPRGPSEPPD RLSCDGSRVH LLYK-K960

Table 1. Summary of the literature and phosphorylation programs predicted phosphorylation targets in the $GABA_{B1}$ carboxyl-terminus. Predicted threonine phosphorylation sites (T) are highlighted in red and serines (S) in blue. The coiled-coil domains are underlined.

Kinase	Published consensus motif	Reference
AMPK	M or L/F/I/V-$(X)_2$-S^*/T^*-$(X)_3$-M/L/F/I/V	Scott, 2002
CaMKII	R-$(X)_2$- S^*/T^*	Hanson, 1989
GSK3	S^*-$(X)_2$-S^*, S^*/T^*-$(X)_{3/4}$-S/P/T-P	Ptacek, 2005 , Doble, 2007
PKA	R/K-X- S^*/T^*	Pearson, 1991
PKB	R-X-R-$(X)_2$- S^*/T^*	Fayard, 2005
PKC	S^*/T^*-$(X)_{1/2}$-K, K-$(X)_{1/2}$- S^*/T^*	Wera, 1999

Table 2. Listing of the in literature found diverse kinase consensus motifs.

Program	URL Number	Literature
ELM	1	Puntervoll, 2003
An EXCEL-based method to search for potential Ser/ Thr-phosphorylation sites in proteins	2	Wera, 1999
Motif Scan	3	Liu, 2008
Net Phos 2.0	4	Huang, 2005
Net PhosK 1.0	5	Xue, 2005
PhosphorylationElm	6	Diella, 2004
PhosphorylationSite Plus	7	Rush, 2005
Prediction of phosphorylation sites using SVMs	8	Kim, 2004
Pred Phospho	9	Wong, 2007
Scansite	10	Obenauer, 2003

URL Number	URL Address
1	http://elm.eu.org/links.html
2	http://www.sciencedirect.com/science?_ob=MImg&_imagekey=B6T5J-3V5TKGB-6-1&_cdi=5004&_user=946149&_orig=search&_coverDate=01%2F01%2F1998&_sk=999419998&view=c&wchp=dGLbVzW-zSkzS&md5=e62cdfd15745ad93fac409d29a9fa02b&ie=/sdarticle.pdf
3	http://hits.isb-sib.ch/cgi-bin/PFSCAN
4	http://www.cbs.dtu.dk/services/NetPhos/
5	http://www.cbs.dtu.dk/services/NetPhosK/
6	http://phospho.elm.eu.org/
7	http://www.phosphosite.org/homeAction.do
8	http://www.ncbi.nlm.nih.gov/pubmed/15231530?ordinalpos=3&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum
9	http://phosphovariant.ngri.go.kr/predphospho.htm?PHPSESSID=0e390b13ed30d0beaf5a2d42896c993e
10	http://scansite.mit.edu/

Table 3. Phosphorylation programs register and their citation in literature (upper part), the URL addresses from the phosphorylation programs are listed in the lower part.

4.2.2 Glutathione S-tranferase fusion system

The glutathione s-transferase (GST) fusion system is profiled as the optimal model to study phosphorylation of the $GABA_B$ receptors at the $GABA_{B1}$ carboxyl-terminal. The GST fusion system expresses, purifies, and detects recombinant fusion proteins produced in *E. coli*. It relies on inducible, high-level gene expression of gene fragments as fusion proteins with a *Schistosoma japonicum* GST-tag (Table 4.a). A fusion protein contains the GST-tag localized at the amino terminal and the protein of interest at the carboxyl-terminal. The GST fusion proteins are purified from bacterial lysates by means of affinity chromatography using immobilized glutathione (glutathione coated sepharose beads) while the impurities are removed by washing. Boiling the samples in Laemmli buffer or under mild, non-denaturing conditions using reduced glutathione elutes the GST fusion proteins from the immobilized glutathione. The GST fusion system has been used successfully in many applications including molecular immunology (Toye, 1990), the production of vaccines (Fikrig, 1990, Johnson, 1989) and studies involving protein-protein (Kaelin, 1991) and DNA-protein (Chittenden, 1991) interactions. In this work, the GST-GB1 protein, including the full-length carboxyl-terminal of $GABA_{B1}$ (amino acids 857–960, see Table 4.b) was used.

A first important hint, that a phosphorylation site on the $GABA_{B1}$ carboxyl-terminal is located at a juxtramembrane site was given from the first *in vitro* phosphorylation experiments using the GST-GB1 fusion protein and its truncations. These GST-GB1 fusion proteins were gradually truncated from their carboxyl-terminal. They were phosphorylated using native and purified kinases. GST-GB1Δ1 (Table 4.c) and GST-GB1Δ2 (Table 4.d), the two truncated forms were generated by PCR from GST-GB1. The sense primer (5`-GTTCCGCGTGGATCCCGCAGG-3') is the same for GST-GB1Δ1 and GST-GB1Δ2. Conversly, the antisense is different for GST-GB1Δ1 (5`-TTTCTCGAGCTACTTCTCTTCCTCGTTGT T-3') and GST-GB1Δ2 (5'-TTTCTCGAGCTATGGGGGGTGGCGCCG TGA -3'). The generated PCR fragments were digested with *BamHI*- and *XhoI* and subcloned in-frame into the pGEX-4T-1 plasmid. The truncated GST-fusion proteins were expressed, induced and lysed as previously described for the GST-GB1 fusion proteins.

The GST fusion system enabled the discovery of a new phosphorylation site on the GABAB receptors by in vitro phosphorylation and HPLC-MS analysis. On the other hand, the interaction between the responsible kinase with the GABAB receptor can be showed in pull-down assay. But one must keep in mind that the GST fusion system is artificial and a simplifying model. Indeed, this technique does not take into account phosphorylation influencing factors like folding of the carboxyl-termini or interaction with binding partners. Nevertheless, the GST fusion system enables the generation of large amounts (important for further analysis steps like high pressure liquid chromatography) of a model protein comprising most of the phosphorlylation sites of the native GABAB1carboxyl-terminal.

a) GST-tag:
MSPILGYWKI KGLVQPTRLL LEYLEEKYEE HLYERDEGDK WRNKKFELGL EFPNLPYYID GDVKLTQSMA IIRYIADKHN MLGGCPKERA EISMLEGAVL DIRYGVSRIA YSKDFETLKV DFLSKLPEML KMFEDRLCHK TYLNGDHVTH PDFMLYDALD VVLYMDPMCL DAFPKLVCFK KRIEAIPQID KYLKSSKYIA WPLQGWQATF GGGDHPPKSD LVPRGS
Total protein length: 226 amino acids, 27 kDa long

b) GST-GB1:
GST-RRLITRGEWQ SETQDTMKTG SSTNNNEEEK SRLLEKENRE LEKIIAEKEE RVSELRHQLQ SRQQLRSRRH PPTPPDPSGG LPRGPSEPPD RLSCDGSRVH LLYK
Total protein length: 330 amino acids, 35 kDa long

c) GST-GB1Δ1:
GST-RRLITRGEWQ SETQDTMKTG SSTNNNEEEK SRLLEKENRE LEKIIAEKEE RVSELRHQLQ SRQQLRSRRH PP
Total protein length: 298 amino acids, 33 kDa long

d) GST-GB1Δ2:
GST-RRLITRGEWQ SETQDTMKTG SSTNNNEEEK
Total protein length: 256 amino acids, 28 kDa long

Table 4): Sequences of the GST-fusion protein; a) GST-Tag alone (GST-) from *Schistosoma japonicum*, b) GST-GB1: Full length $GABA_{B1}$ carboxyl-terminal (R857-K960) fused to the GST-tag, c) GST-GB1Δ1: The first fusion protein from the GST-GB1 protein with a 3' end truncated carboxyl-terminal (R857-P928) and d) the second one; GST-GB1Δ2 (R857-K887).

4.2.3 In vitro phosphorylation using purified or endogenous kinases

In vitro phosphorylation enables the study of kinase activity under experimental conditions. The target protein GST-GB1 mimics the $GABA_{B1}$ carboxyl-terminal in the *in vitro* phosphorylation assay. The GST-GB1 is bound to the immobilized glutathione. The kinases can be purified and easily purchased whereas active native kinases can be obtained from native tissue. In the following work started with the native kinases obtained from mouse brain lysate. Importantly, in order to properly activate the native kinases, the right buffer conditions are fundamental. Indeed, they ought to activate as many kinases as possible and block as few as possible. Kim et al. published 2005 data on a buffer that was able to activate PKC (Kim, 2005). This buffer contains basic kinase buffer components; $CaCl_2$, $MgCl_2$ and ATP where ATP does not only activate the kinase but also primarly serves as the phosphate group source. The total concentration of ATP in an *in vitro* kinase essay is around 1.6 mM. The application of only of 1 µl [γ-^{32}P]-ATP (3000Ci/mmol) is enough to radioactively tag the phosphorylated protein. To get rid of the unnecessary not incorporated [γ-^{32}P]-ATP and to finally stop the phosphorylation reaction, the immobilized beads were washed with the ice-cold phosphorylation buffer. Afterwards, the samples were eluted by boiling in Laemmli buffer containing phosphatase inhibitors. The samples were next separated by SDS-PAGE. The phosphorylated proteins were revealed on X-ray film exposed to the dried polyacrylamide gel. *In vitro* phosphorylation experiments with native kinases show a clear phosphorylation of GST-GB1 proteins, whereas the GST-tag alone stays unphosphorylated (Figure 7). To get an idea on the localization of the phsophorylation sites, the GST-GB1 carboxyl-terminal truncated forms GST-GB1Δ1 and GST-GB1Δ2, described above were used. The truncated-GST-GB1Δ2 containing the $GABA_{B1}$ carboxyl-terminal first

30 amino acids (coming from the amino terminus) and the GST-GB1Δ2 already showing a clear phosphorylation signal were both phosphorylated by native kinases (Figure 7). This experiment thus gave the first hint for the juxtramembrane position of a phosphorylation site on the $GABA_{B1}$ carboxyl-terminal. The protein loading amount was normalized by Coomassie blue staining of the gel (not shown).

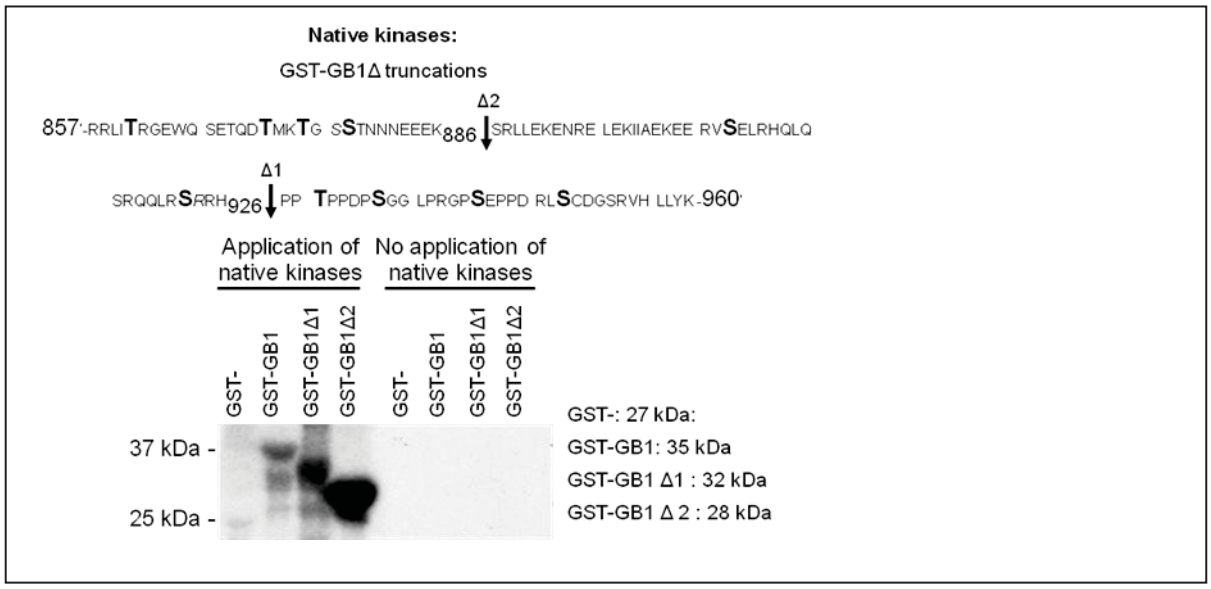

Figure 7. The *in vitro* phosphorylation of the GST-GB1 truncations GST-GB1Δ1 and GST-GB1Δ2 with native kinases. The truncations are indicated in the GST-GB1 sequence. As expected, GST-GB1 gets phosphorylated. Both truncations also get phsophorylated. The first 30 amino acids of the $GABA_{B1}$ C-terminus were sufficient to get a phosphorylation signal.

In order to identify the active native kinases involved in the phosphorylation, the work was carried on with purified kinases as they yield to high phosphorylation levels. The alternative would have been to use kinase inhibitors althought problems with the inhibitors specificity can always emerge. For the exact identification of the phosphorylation site by HPLC and MS, the usage of purified kinases then seemed to be the better option. The purified kinases CaMKII, PKC and PKA were chosen due to their important role in the CNS. Repeating the *in vitro* phosphorylation of the GST-GB1 fusion protein using the three kinases unveiled an interesting finding. The $GABA_{B1}$ carboxyl-terminal can be phosphorylated by the three kinases (Figure 8). Interestingly, the GST-GB1Δ1 and GST-GB1Δ2 truncations also were phosphorylated by the purified CaMKII, PKA and PKC (not shown). This observation indicates that native $GABA_{B1}$ carboxyl-terminals contain phosphorylation sites for CaMKII, PKA and PKC and for the first two kinases the phosphorylation sites are positioned at the juxtamembrane. It cannot be excluded, although highly unlikely, that these phosphorylatation sites overlap, especially due to their different consensus motifs.

The identification of several sites for instance by making alanine substitutions, would have been time consuming and a difficult task to undertake. The considerations of the HPLC/ MS technique are

limited and not suitable for the analysis of all the possible phosphorylation sites. The variety of different possible kinase phosphorylation sites demands the systematic analysis of a single phosphorylation site. That was the most promising and fastest way to find, under the given circumstances, a physiological relevant phosphorylation site. The kinase with the most potential was CaMKII. Indeed, it has a consensus motif appearing in the proteolytic peptide fragments and most importantly, it is very abundant in the CNS. Additionnaly, indications from the literature (Vargas, 2008) and from different unpublished electrophysiological experiments and localization studies predict a physiological relevant connection between CaMKII and GABA receptor. All this information led to the decision of focusing all the efforts on CaMKII actions.

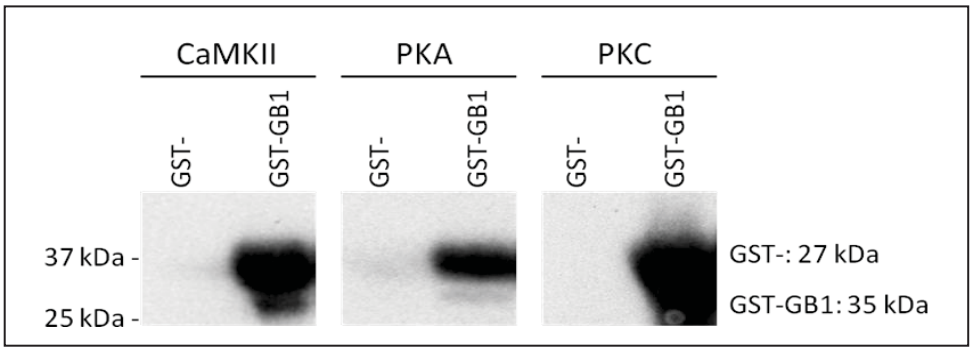

Figure 8. The *in vitro* phosphorylation of GST-GB1 with the purified kinases CaMKII,PKA and PKC. All three kinases phosphosphorylate GST-GB1. This indicates that the $GABA_{B1}$ carboxyl-terminus could be a target for several kinases.

4.2.4 High pressure liquid chromatography (HPLC) and Mass spectrometry (MS)

The mass spectrometry (MS) measures the mass to charge ratio (m/z) of ions. The high pressure liquid chromatography (HPLC, also sometimes called high performance liquid chromatography) is a form of column chromatography used to separate, identify and quantify compounds. The HPLC consists of a column packed with a chromatographic material, generating a specific surface layer (stationary phase) and a pump that moves the analyte containing mobile phase through the column. A detector at the end records the elution of the analytes. The retention time varies depending on the interactions between the stationary phase, the molecules being analyzed and the solvents used.

The combination of liquid chromatography and mass spectrometry allows the detection of analytes in the picomol range. It was made possible by the availability of the electro spray (ES) technology (Issaq, 2009). The electro spray technology is a technique that ejects the ions from a solvent containing the analyte(s) of interest. The electro spray disperses the liquid into a fine aerosol. The ion formation involves extensive solvent evaporation by mixing water with volatile organic compound solvents (e.g. methanol, acetonitrile). The first vacuum stage of a MS samples the aerosol through a

capillary. The solvent evaporates from a charged droplet until it becomes unstable upon reaching its Rayleigh limit; the droplet size decreases, the charge to surface ratio increases. Ions of equal charge repel each other and the droplet deforms and emits charged ions. During this process, the droplet loses a small percentage of its mass (an electron) along with a relatively large percentage of its charge. The ES technique is so mild that labile analytes remain intact after the electro spray ionisation (ESI) procedure (Bothner, 1998). For the analysis of phosphorylated peptides, reverse phase-high pressure liquid chromatography (RP-HPLC) is the chromatography of choice. In RP-HPLC, the solid phase support is modified by long saturated hydrocarbon chains. The analyte binds to the stationary phase via hydrophobic interactions. The analyte is dissolved in aqueous trifluoroacetic acid, the polar solvent. When the concentration of acetonitrile slowly increases during the gradient, adsorbed peptides elute sequentially from the stationary phase with increasing hydrophobicity. The detector records eluting peptides at 214 nm, while at 280 nm the aromatic amino acids containing peptide fragments can be detected.

To increase the stoichiometry of phosphorylation, at least 10µg of the target protein should be phosphorylated with a final ATP concentration of 20 µM. To facilitate the tracking of the phosphorylated peptides, the kinase reaction is carried out by spiking the ATP mixture with 1 µl of radioactive ATP (indicates the specific activity) to reach at least 10% phosphorylation of the protein of interest. In that experiment, however, we could not reach the desired phosphorylation stoichiometry using native kinases. Instead, we used purified, highly active CaMKII, to obtain reasonable phosphorylation. The peptide fragments are generated by a digestion with endoproteinases lys-c and trypsin (Jeno, 1995). The lys-c cleaves at the carboxyl-terminal end of lysine whereas the trypsin cleaves peptide bonds carboxyl-terminal of lysine and arginine. The rate of hydrolysis can be slowed down by acidic residue (glutamic acid or aspartic acid)_on either side of the cleavage site. A proline on the carboxyl-terminal of the cleavage site can even inhibit digestion by trypsin without affecting lys-c cleaving activity. The peptide fragments generated are usually between 5 to 30 amino acids longs. Very short peptides do not bind to the reverse-phase matrix while very long peptides tend to bind irreversibly to the column. In Table 5 the lys-c and trypsin digested peptides obtained from GST-GB1 are summarized.

The liquid scintillation counting of the HPLC fragments unveils a strongly phosphorylated peptide. The MS analysis of this fraction yielded two peptides of 1439.61 Da and 1519.58 Da. The collision-induced dissociation (CID) spectrum and databank searching showed that the two peptides are derived from the juxtamembrane position of GST-GB1 and comprise the unphosphorylated GEWQS$_{867}$ETQDTMK (Figure 9.a) peptide and the phosphorylated peptide GEWQpS$_{867}$ETQDTMK with a phosphorylation at the S867 (Figure 9.b).

GST-Tag: amino acids 0-226

```
            9  2        7          9        8      5/13  2 2 1                    19
0-MSPILGYWKI  KGLVQPTRLL  LEYLEEKYEE  HLYERDEGDK  WRNKKFELGL  EFPNLPYYID  GDVKLTQSMA
   9     5            9  2                    14/16  5       5      6         6      6     5    4/10
  IIRYIADKHN  MLGGCPKERA  EISMLEGAVL  DIRYGVSRIA  YSKDFETLKV  DFLSKLPEML  KMFEDRLCHK
                                                35      5  1 1          9  3  3
  TYLNGDHVTH  PDFMLYDALD  VVLYMDPMCL  DAFPKLVCFK  KRIEAIPQID  KYLKSSKYIA  WPLQGWQATF
          21         6
 GGGDHPPKSD  LVPRGS-226
```

$GABA_{B1}$ C-terminus: amino acids 227-330

```
   3 1    4                    12              10  2    4   3    4/7   5  3      5       6      4  2 1
227-RRLI  TRGEWQS867ETQ  DTMKTGSSTN NNEEEKSRLL  EKENRELEKI  IAEKEERVSE  LRHQLQSRQQ  LRSRRHPPTP
          14        8          7/15    6
  PDPSGGLPRG  PSEPPDRLSC DGSRVHLLYK-330
```

Table 5. The digestion map of GST-GB1 for the combined application of the endoproteinases lys-c and trypsin and the MS spectrum of the phosphorylated S867 containing peptide. In the upper line the generated peptide fragment lengths are indicated. The negative charged amino acids (glutamic acids (**E**) and aspartic acids (**D**) slow down the tryptic hydrolysis rate at an arginine (R), the cursive number stands for the affected fragments. For that reason, the following fragment also contains two length numbers for respresenting uncompleted cleavage. The cleavage sites are indicated by bold letters (**K** and **R)**. The fragment containing the phosphorylated S867, underlined in the sequence, starting with G233 and ending with K244, appeared in the MS analysis.

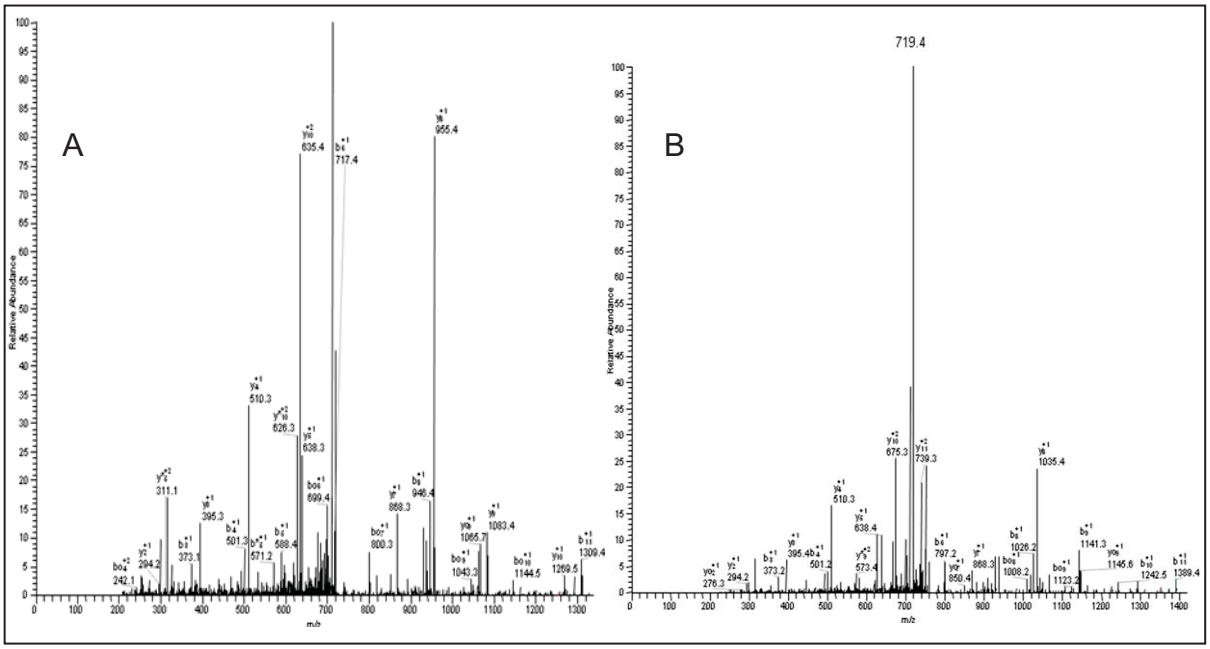

Figure 9. The electrospray ionization mass spectrometry (ESI-MS) spectra from (A) the unphosphorylated GEWQSETODTMK peptide fragment and (B) the phosphorylated GEWQpSETODTMK. A) S867 matches with collisional induced ions y_8 and b_5. All ions before y_8 and b_5 are identical in both spectra. B) The remaining y as well b ions have a 80 Da shift, indicating a phosphorylation at S867. The ESI-MS of the phosphorylated fraction revealed the phosphorylated peptide GEWQpS867ETQDTMK containing the serine 867 (S867) as phosphorylation site for CamKII. The doubly charged precursor (768.29 m/z) was subjected to fragmentation. The present y- and b-ions are match with the identified phosphorylated peptide. Ions showing a mass shift of 80 Da (mass of a phosphate residue).

4.2.5 The phosphospecific pS867 antibody

After showing the phosphorylation of GST-GB1 at S867 by native CaMKII, the next step was to demonstrate that S867 can be phosphorylated in native $GABA_{B1}$ subunits. This was done by generating a phosphospecific antibody pS867. This pS867 antibody specifically recognizes the phosphorylated S867 site. In the following chapter the generation and characterization of the pS867 antibody will be discussed. The interesting findings of native S867 phosphorylation will be highlighted and the mechanism increasing its phosphorylation will be explained. Finally the preliminary immunohistochemical experiments with the pS867 antibody will be outlined.

4.2.5.1 Generation of the pS867 antibody

For the pS867 antibody generation, an S867 phosphorylated peptide ITRGEWQpS867EAQDT (Lamp, 2001) was conjugated to the carrier protein keyhole limpet hemocyanin (KLH) to achieve an increased immunization. The sequence and phosphorylation of the phosphorylated peptide was verified by MS analysis. The phosphorylated peptide-KLH conjugate was emulsified with complete Freund's adjuvant and injected subcutaneously in New Zealand rabbits four times at biweekly intervals. Additional injection of the peptide-KLH conjugate in Freund's incomplete adjuvant boosts the immunization reaction. One week after the boost, the blood is collected from the central ear artery and is allowed to clot and retract at 37°C overnight. The serum is decanted and clarified by centrifugation at 2'500 rpm for 15 minutes. The antibody fraction is then purified by affinity chromatography on beads. For this, beads were equilibrated with the phosphorylated peptide-KLH conjugate dissolved in a PBS and NaCl solution. The beads were washed with PBS. The washed beads were loaded into a plastic column and washed again with PBS until the OD at 280 nm is 0. The elution of the antibodies was done with a mixture of 200 mM glycine at pH 2.8. The immunoglobulin-containing fraction is identified by measuring the absorbance at 280 nm. The fractions were dialyzed against PBS at 4°C overnight. To absorb anti-non-phosphopetide antibody, the generated antibody solution is applied onto a column and equilibrated with the non-phosphopetide-KLH conjugate. The non-phosphorylated peptide ITRGEWQ*S*EAQDT. The purified solution is then collected and its absorbance mesured on more time at 280 nm. The pS867 specificity was finally confirmed by ELISA where the antibody showed a strong affinity towards the phosphorylated peptide.

4.2.5.2 Characterization of the pS867 antibody

Additionally to the ELISA data, the specificity of the pS867 antibody was examined using the GST-GB1 protein and its S867A substitution, phosphorylated by purified CaMKII. The pS867 antibody surprisingly does not recognize the unphosphorylated GST-GB1 proteins (Figure 10a, first lane).but

recognize the phosphorylated form of GST-GB1 protein (Figure 10a, second lane). Finally, in the last lane, a CaMKII phosphorylated GST-GB1S867A alanine mutant was loaded and the the pS867 antibody was unable to recognize it (Figure 10a, third lane). The equal loading of GST-GB1 and GST-GB1S867A protein amounts was ensured by $GABA_{B1}$ antibody staining of the stripped blot (Figure 10a, lower panel). The pS867 antibody fulfils the criteria of sequence and phosphorylation specificity and is then suitable for the further analysis of the S867 phosphorylation.

Unfortunately, phosphorylation sites are often the target of several kinases. Luckily, the pS867 antibody allows getting a quick and easy assessment of S867 phosphorylation by other kinases than CaMKII. Indeed, GST-GB1 phosphorylation by different purified kinases can be straightforwardly tested (Figure 10b). In the first lane, the unphosphorylated GST-GB1 protein was loaded and, as expected, no signal from the pS867 antibody was obtained (Figure 10b, first lane). As previously seen, the purified CaMKII phosphorylates the GST-GB1 at S867 (Figure 10b, second lane). Additionally, the purified PKA also phosphorylates the GST-GB1 protein at S867 (Figure 10b, third lane). However, PKC has been shown to phosphorylate the GST-GB1 protein (Figure 7), but not at the S867 site (Figure 40.b), fourth lane). The loading amount was normalized by $GABA_{B1}$ antibody blotting (lower panel).

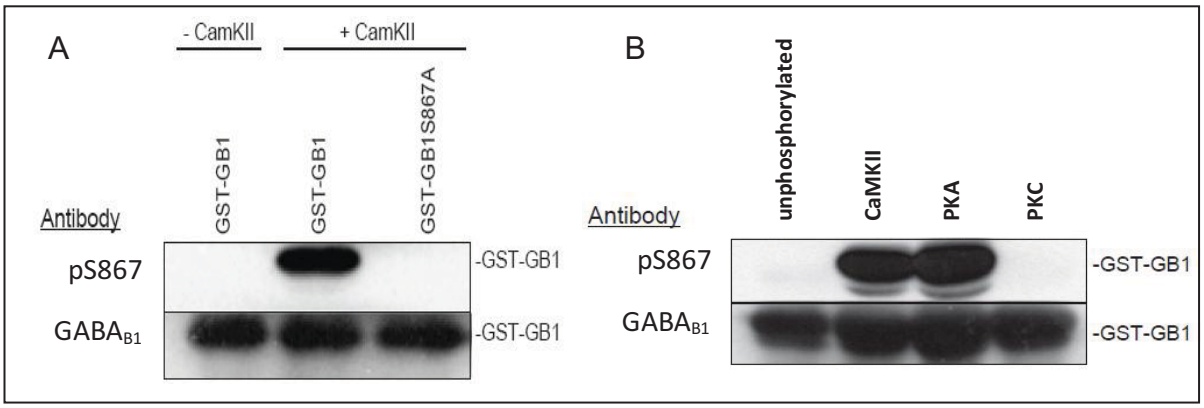

Figure 10. Characterization of the pS867 antibody and the phosphorylation of S867 by CaMKII and PKA. A) To control the phosphorylation specificity of the antibody pS867, GST-GB1 fusion proteins were phosphorylated by CaMKII. The unphosphorylated GST-GB1 proteins are not recognized by the pS867 antibody (first lane). The phosphorylated GST-GB1 proteins get phosphorylated (second lane). Finally the phosphorylated GST-GB1S867A does not get recognized by the pS867 antibody. The loading amount was normalized by staining the blot with a $GABA_{B1}$ antibody (lower panel). B) The pS867 antibody allows determining which kinases could phosphorylate GST-GB1 at S867. The unphosphorylated GST-GB1 protein is not recognized (first lane). CaMKII as previously shown phosphorylates S867 (second lane). PKA also phosphorylates GST-GB1 at S867 (third lane); whereas PKC does not (fourth lane).

4.2.5.3 In vivo phosphorylation of the $GABA_{B1}$ isoform

The results generated with the different versions of the recombinant GST-GB1 fusion proteins, clearly show, that S867 is a phosphorylation target on the $GABA_{B1}$ subunits. The pS867 antibody finally

allows determining if native $GABA_{B1}$ subunits are phosphorylated at S867. For that, an immunoprecipitation of the $GABA_{B1}$ subunit from mouse brain lysate was done.

The mouse brain lysate was prepared from adult wildtype Balb/C (WT). The brains are homogenized in 0.32 M Sucrose-HEPES buffer (0.32 M sucrose, 4 mM HEPES, 1 mM EDTA, 0.5 M EDTA pH 8, 1 mM EDTA, 0.5 M EGTA pH 8, complete protease inhibitor cocktail and the phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich, St. Louis, MO). The lysates are centrifuged at 2900 g for 10min at 4°C to remove nucleic debris. Afterwards, the supernatant is ultracentrifuged for 30 min at 4°C at 48000 g. The pellets are solubilised in 3 ml RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 (IGEPAL), 0.5 % DOC, and complete protease inhibitor cocktail, and the phosphatase inhibitor cocktails 1 and 2). By sonication and centrifugation at 15'000 g the lysates are finally cleared. Loading of the prepared WT mouse brain lysate show no phosphorylation signal (Figure 11 input lanes). Only after immunoprecipitation of the $GABA_{B1}$ subunits a pS867 signal was obtained (Figure 11, $GABA_{B1}$ immunoprecipitation lanes). For negative control, an immunoprecipitation was done with adult $GABA_{B1}$ knock out Balb/C mice ($R1^{-/-}$).

For immunoprecipitation, the mouse brain lysate first had to be precleared with agarose G-protein (Roche) 4°C. Immunoprecipitation is done with protein agarose G, coupled with the mouse polyclonal $GABA_{B1}$ antibody (Abcam).

Comparing the immunoprecipitation with the input lanes, a clear accumulation of both $GABA_{B1}$ subunit isoforms (Figure 11, lower $GABA_{B1}$ blot) is obvious. This accumulation is specific, as suggested by the mouse immunoglobulin control (lanes IgM). The pS867 antibody clearly shows S867 phosphorylation at the immunoprecpitated $GABA_{B1}$ subunits. Moreover, the pS867 antibody unveils that S867 phosphorylation is predominantly at $GABA_{B1b}$ isoforms.

CaMKII was shown to be concentrated at the postsynaptic terminal (Colbran, 2004). Various localization studies claim that the $GABA_{B1b}$ isoforms are postsynaptically located on dendritic spines at the glutamatergic synapses (Vigot, 2006, Ulrich, 2007, Guetg, 2009). It cannot be excluded that $GABA_{B1a}$ isoforms are also phosphorylated by CaMKII at S867, since $GABA_{B1a}$ isoforms, fewer than $GABA_{B1b}$ isoforms, are also found on postsynaptic terminal of glutamatergic synapses (Guetg, 2009). The native S867 phosphorylation was only visible after immunoprecipitation of the $GABA_{B1}$ subunits. The S867 phosphorylation, that will be shown later, is part of a modulatory and reversible process. It is then anticipated that at any time-point, only a small population of native $GABA_{B1}$ isoforms remains in its phosphorylated state. By immunoprecipitation the amount of phosphorylated $GABA_{B1}$ subunits can be accumulated.

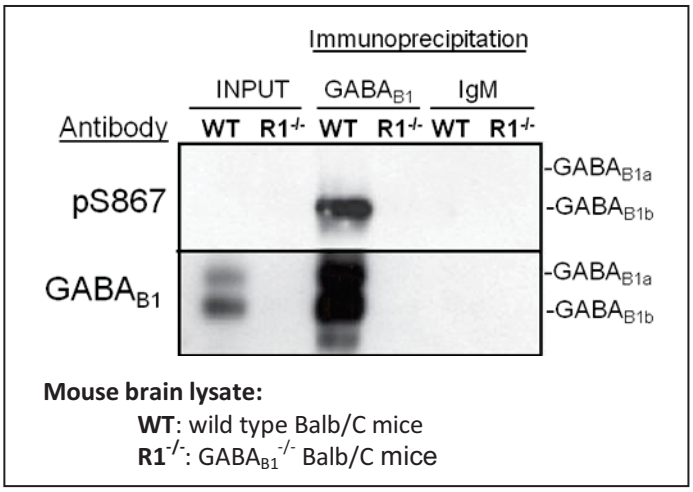

Figure 11. *In vivo* phosphorylation of the GABA$_{B1b}$ subunits isoforms. Brain lysate of wild type Balb/C mice (WT) shows no pS867 phosphorylation signal. After immunoprecipi-tation with a mouse GABA$_{B1}$ antibody the pS867 antibody recognizes the phosphorylated GABA$_{B1b}$ subunit isoforms. The mouse brain lysate from GABA$_{B1}$ knock out type Balb/C mice (R1$^{-/-}$) serves as negative control. And the mouse immunoglobulin (IgM) control guarantees the specificity of the immunoprecipitation.

4.2.5.4 NMDA treatment of cortical cultures increases S867 phosphorylation

CaMKII and glutamate receptors are integral parts of synaptic plasticity that underlie learning and memory. The simplest model for long-term potentiation postulates that CaMKII is activated by Ca^{2+} influx through NMDA (N-methyl-D-aspartate) receptors (Malenka, 1999). This calcium influx induced a potentiation of synaptic efficacy by inducing synaptic receptor insertions (Rongo, 1999, Hayashi, 2000) and increased single-channel conductance of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors (Derkach, 1999). Additionally, translocation of CaMKII to the synapse in hippocampal neurons was shown to be NMDA receptor-induced (Bayer, 2001). Consequently, it seemed essential in the course of the present study to analyze the effect of NMDA receptors on GABA$_{B1}$ S867 phosphorylation. Therefore cultured cortical neurons from wild type Balb/C mouse embryos (DIV 14) were treated with NMDA. During the recovery time, S867 should then be phosphorylated if the activation of NMDA receptors stimulates the phosphorylation by CAMKII. Immunoprecipitation of GABA$_{B1}$ subunits showed the following result; a recovery time of 7 minutes shows a S867 phosphorylation signal (Figure 12, second lane), whereas the untreated cell cultures show no weak basal phosphorylation of S867 (Figure 12, first lane). In the standard NMDA treatment protocol, although it has a longer reaction time of 30 minutes (3 minutes of NMDA application and 27 minutes of recovery time after application), no pS867 phosphorylation signal could be observed in several experiments (not shown). This is not surprising, the decrease of the S867 phosphorylation signal after 30 minutes, compared to 10 minutes, is probably due to the advanced natural dephosphorylation processes, reducing the number of phosphorylated S867. NMDA receptor activation under increased neuronal activity augments phosphorylation of GABA$_{B1}$ at S867.

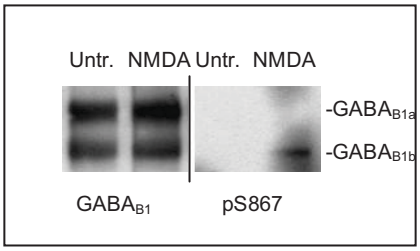

Figure 12. NMDA receptor activation increases S867 phosphorlyation in cortical cell cultures. Cortical cell cultures (Div 14) were treated with NMDA. The loading amount was normalized using a GABA$_{B1}$ antibody (left panel).The first culture is NMDA untreated (untr.). The second culture was treated with NMDA and had a recovery time of 7 minutes (NMDA).

4.2.5.5 Preliminary immunohistochemical results with the pS867 antibody

The pS867 antibody served a crucial role in the discovery of the phosphorylation site S867. It helped to unveil some of the S867 phosphorylating kinases in the *in vitro* approach. Additionally, the pS867 antibody is essential for analyzing the distribution of the phosphorylated S867 in different brain regions. In the immunohistochemical experiments, coronal cryosections of mouse brains from WT mice and $R1^{-/-}$ mice were prepared as described in the paper of Kuramoto et al. (Kuramoto, 2007), and stained with the pS867 antibody. The secondary antibody was a donkey anti-rabbit/Cy2-conjugated (Jackson Immunoresearch). The counterstaining was done with DAPI (4',6-Diamidin-2'-phenylindoldihydrochlorid). The sections were mounted with Fluorsafe (Calbiochem) and visualized with a Leica DMI6000 fluorescence microscope. The primary antibody was used in a dilution of 1:100. Unfortunately, the pS867 antibody does not show specific staining for the pS867 in the $GABA_{B1}$ subunit, since strong background staining in hippocampus and cortex was present in WT and as well $R1^{-/-}$ mice (Figure 13). To solve that problem, further conditions were tested. These conditions that could lead to an appropriate staining include increased CaMKII activity in combination with higher antibody dilutions or less stringent binding conditions.

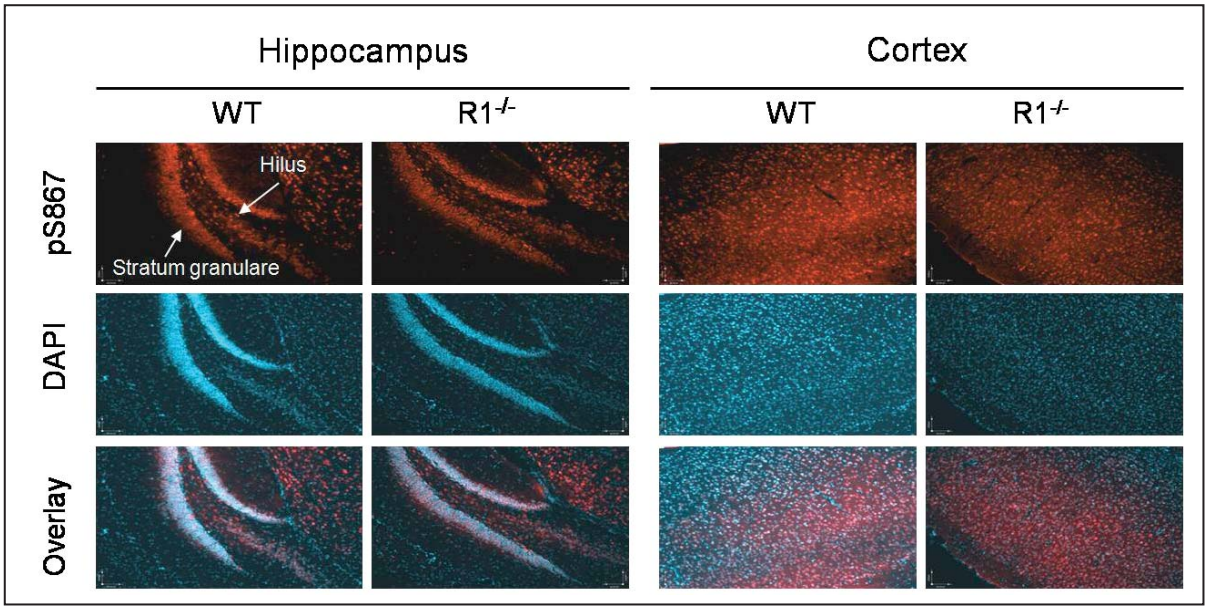

Figure 13. Immunohistochemistry on coronal cryosections with the pS867 antibody. Cryosections were made from wild type Balb/C mice (WT) and $GABA_{B1}$ knock out Balb/C mice ($R1^{-/-}$). Left panels show hippocampal sections and right panels cortical sections. The top level shows the pS867 antibody staining. In the second level, the cell nuclei were marked by DAPI counterstaining. In the last lane both stainings are presented as a merge picture. No difference in pS867 staining could be observed between WT and $R1^{-/-}$ mice, showing unspecific binding of the p867 antibody in this experiment.

4.2.6 The phosphorylation of S867 is physiological relevant

To examine if NMDA receptor mediated S867 phosphorylation regulates physiological functions of native $GABA_B$ receptors in neuronal cells, whole-cell patch clamp recordings were done from cultivated hippocampal neurons treated with NMDA. The $GABA_B$ receptor function at the neurons was monitored by recording of baclofen evoked current responses of G-protein coupled inwardly rectifying K^+ channels. In the first set of experiments CaMKII activity in WT mouse neurons was blocked by perfusion of neurons with intracellular solution containing specific CaMKII inhibitor, KN-93. In the second set hippocampal neurons isolated from $R1^{-/-}$ mouse were transfected with the non-phosphorylable mutant from of $GABA_{B1b}$: $GABA_{B1b}$ S867A.

Dissociated hippocampal neurons (DIV 16-20) were perfused with a solution containing 145 mM:NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Hepes and 25 mM glucose , pH 7.3, 320 mosm. $GABA_B$ receptors were activated by fast application of baclofen (100µM, for 5 seconds). The responses were recorded by patch-clamp recording from neurons dialyzed with intracellular solution (ICS) containing 102 mM K-gluconate, 32.5 mM KCl, 10 mM HEPES, 0.1 mM EGTA (0.1), 4 mM Mg-ATP, 0.5 mM Tris-GTP and 10 mM Tris-phosphocreatine pH 7.2, 289 mosm. First, K^+ currents evoked by baclofen were recorded from neurons voltage clamped at -50 mV before NMDA treatment. The stable response was achieved within 15 min of whole cell recording. Then the holding potential was decreased to -70 mV to prevent activation of voltage dependent Ca^{2+} conductance and NMDA (30µM) was applied for one minute together with the NMDA receptor co-activator glycine (10µM). Mg^{2+} was omitted from the NMDA containing extracellular solution to avoid the Mg^{2+} blocks of the NMDA receptors (middle graph in Figure 14 a-c) shows blocked NMDA receptor dependent glutamatergic currents). Thirty minutes after NMDA application, the baclofen responses was recorded at Vm = -50 mV. In the whole cell patch-clamp recordings of cultured WT mouse hippocampal neurons in absence of KN-93, a marked decline in baclofen-induced K^+ current following NMDA application can be observed (Figure 14 a), upper part). Indeed, the amplitude decreases from 84 pA to 19 pA following NMDA treatment. Treating the hippocampal neurons with KN-93, shows in the K^+ current traces no drastic differences before and after the NMDA application; 69 pA and 36 pA before and after the treatment respectively (Figure 14 a), lower part). To quantify the effect of NMDA treatment on $GABA_B$ receptor function we have analyzed the maximal amplitudes of baclofen evoked responses before- and after NMDA treatment-. Our results showed that the amplitudes of after NMDA treatment responses were inhibited to 19.1 ± 14.4% (n = 5) of the responses obtained before before NMDA treatment (Figure 14. c), first bar graph, - KN-93). It indicates that NMDA receptor activation can effectively regulate the $GABA_B$ function. The inhibitory effect of NMDA receptors was at least partially mediated by CamKII activity as the inclusion of KN-93 into ICS relieved

NMDA-induced reduction of baclofen responses by nearly 50% (mean K^+ amplitude after NMDA response was to 65.5 ± 20.2%, n = 5) (Figure 14.c), second bar graph).

In the next series of experiments we testedthe hypothesis that mechanism underlying the NMDA induced inhibition of $GABA_B$ responses involves CaMKII-mediated phosphorylation of S867, in absence of KN-93. Neurons isolated from $R1^{-/-}$ mouse were transfected with WT $GABA_{B1b}$ (GB1b) or S867A substituted $GABA_{B1b}$ constructs (GB1bS867A) together with $GABA_{B2}$. The GB1b construct has before NMDA treatment K^+ amplitudes of 51 pA and after NMDA treatment amplitudes of 11 pA (Figure 14. b), upper part). Exchanging GB1b by the GB1bS867A construct shows that the before NMDA treatment amplitude was 57 pA before and 32 pA for the after NMDA treatment amplitude (Figure 14. b) lower part). The WT $GABA_{B1b}$ construct shows 80% (SD 11%) reduction of the K^+ response after NMDA application compared to the response before NMDA treatment before the NMDA application. The K^+ current amplitude after NMDA treatment is around 11 pA roughly the same value as the NMDA treated WT mouse neurons. Although NMDA was applied, the S867A $GABA_{B1b}$ substitution rescues the K^+ response to around 64% (SD 16%) of the baclofen elicited K^+ response before NMDA application. The measured K^+ amplitude after NMDA is around 32 pA.
The results obtained from the transfected $R1^{-/-}$ mouse neurons show a similar response to NMDA than the one observed with WT mouse neurons. Indeed, the K^+ current amplitude decreases in the WT mouse neurons and WT $GABA_{B1b}$ construct transfected $R1^{-/-}$ mouse neurons. Conversely, the decrease in the K^+ response was almost prevented in WT mouse neurons in appearance of KN-93 and S867A $GABA_{B1b}$ construct transfected $R1^{-/-}$ mouse neurons.

Both set of experiments confirmed that NMDA receptor mediated activation of CaMKII phosphorylates the $GABA_{B1b}$ isoform subunit at S867, leading to a big decrease of $GABA_B$ receptor induced K^+ response, probably trough the suggested internalization of the $GABA_B$ receptors trough NMDA receptor activation leads to a drastic increase in intracellular Ca^{2+} concentration triggering CaMKII activation. The CaMKII mediated phosphorylation of the $GABA_{B1b}$ subunit isoforms at S867 leads to a strong decrease in postsynaptic inhibitory K^+ current induced by baclofen. The measured decrease indicates a loss of the of the $GABA_B$ receptor response. An internalization of surface $GABA_B$ receptors could straightforwardly explain the observed reduction in the evoked K^+ currents. Additionally, a phosphorylation mimetic S867D $GABA_{B1b}$ construct will be generated. The transfection of that construct should show a reduced K^+ response after the NMDA treatment. Further explanations, on how the S867 phosphorylation could decrease $GABA_B$ receptors response, will be clarified in the following final discussion chapter.

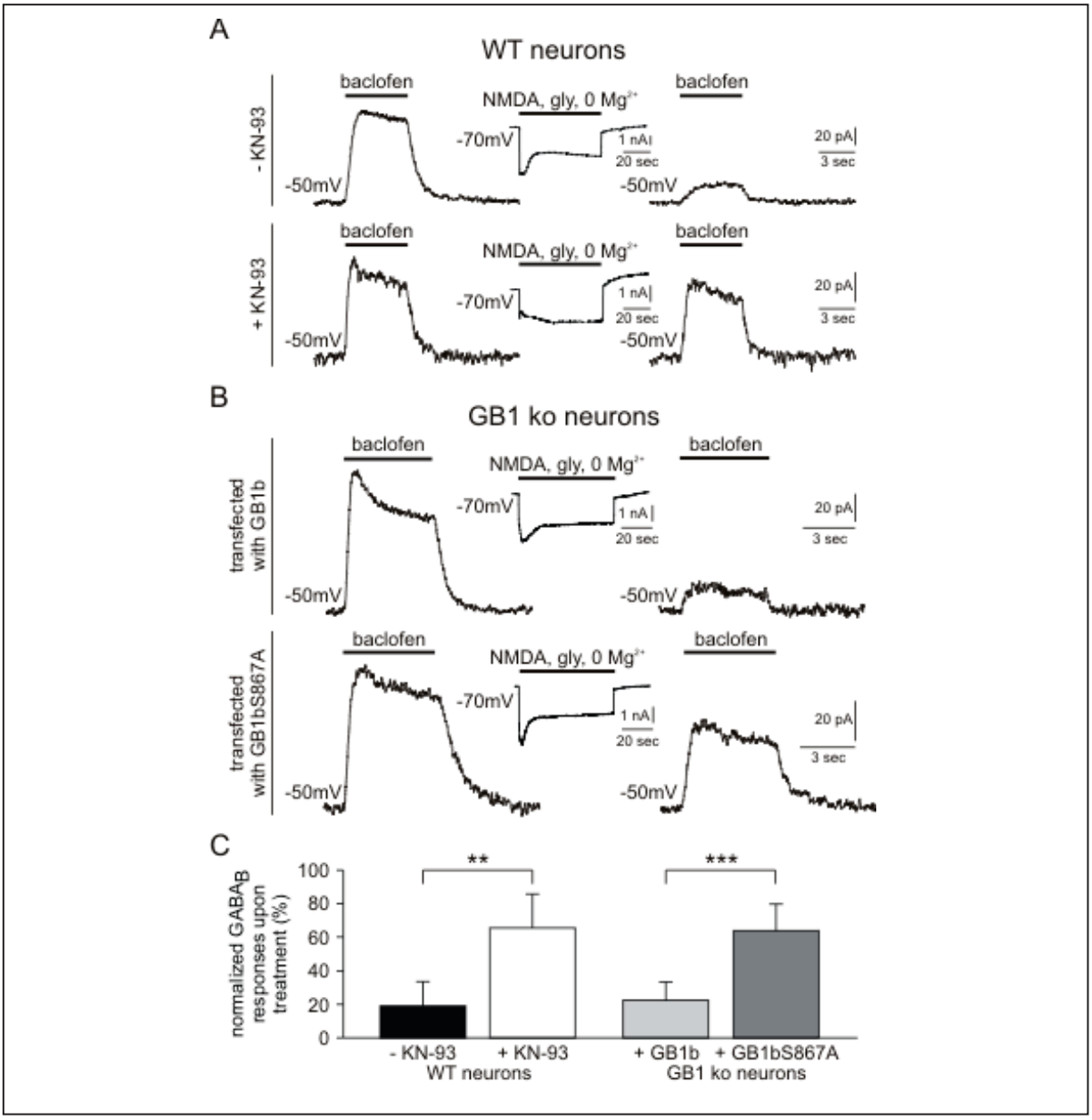

Figure 14. In cultivated hippocampal neurons (DIV 16-20), NMDA reduces the inhibition of K^+ channel activation induced by baclofen. A) Baclofen evoked K^+ response after NMDA application (middle graph shows the blocking of NMDA receptor evoked glutamatergic currents: holding potential was set to -70 mV, NMDA (30µM) was applied for one minute together with the NMDA receptor co-activator glycine (10µM), to avoid the Mg^{2+} blocks of the NMDA receptors, no Mg^{2+} was in the NMDA containing extracellular solution). The whole cell patch-clamp recordings of cultured WT mouse hippocampal neurons in absence of KN-93 show a marked decline in baclofen-induced K^+ current following NMDA application. Indeed, the amplitude decreases from 84 pA to 19 pA following NMDA treatment. The current traces from WT mouse hippocampal neurons pretreated with KN-93 show no strong variation before and after the NMDA application; 69 pA and 36 pA before and after the treatment respectively. B) Dissociated cultured hippocampal neurons from $R1^{-/-}$ mouse (DIV 16-20) cells were transfected in absence of KN-93 with a WT $GABA_{B1b}$ (GB1b) construct; before NMDA the K^+ amplitude was 51 pA and 11 pA after the treatment. Changing the WT GB1b construct by the S867A $GABA_{B1b}$ (GB1bS867A) substitution construct shows that the amplitude was 57 pA before and 32 pA after NMDA treatment. C) The effect of NMDA treatment on $GABA_B$ receptor function has been quantified by normalizing the K^+ amplitude after the treatment to the one before. In WT mouse neurons NMDA application in absence of KN-93 (first bar graph) results in a current of 19% (SD 14%). KN-93 application (second bar graph) rescues the measured K^+ amplitude to 60% (SD 20%). In $R1^{-/-}$ mouse neurons transfected with a WT GB1b construct (third bar graph) the results show a reduction in the K^+ response after the NMDA treatment to 20% (SD 11%). The transfection of the GB1bS867A (fourth bar graph) inhibits the NMDA mediated decrease and shows K^+ currents of 64% (SD 16%) of before NMDA treatment values.

V. Final discussion

The identification of a CaMKII phosphorylation site on a major neuronal receptor is of a special significance. All brain functions need, for an optimal functioning, an adapted balance of excitation and inhibition. The $GABA_B$ receptor was the last major brain receptor in the CNS to be cloned (Kaupmann, 1997). The metabotropic $GABA_B$ receptor binds GABA, the main inhibitory neurotransmitter in the mammalian nervous system. For a long-time, it was generally assumed that the $GABA_B$ receptors system included several pharmacologically distinct receptor subtypes, like the related mGluR family. However, only two receptor subtypes were identified; $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ (Kaupmann, 1998a). It is unclear how this small receptor heterogeneity ensures the differing electrophysiological and pharmacological *in vivo* responses observed for native $GABA_B$ receptors. As previously discussed, the differing subcellular localization of the two receptor subtypes is believed to be the reason for the differing behavior of both subtypes (Vigot, 2006, Guetg, 2009).

The inhibitory effects of $GABA_B$ receptor activation on the brain are typical for a GPCR, slow and prolonged. The presynaptic activation of these receptors lead to the modulation of adenylate cyclase activity or to the blocking of voltage-dependent Ca^{2+} channels which inhibit neurotransmitters release. On the other hand, postsynaptic receptors activation leads to a modulation in the gating of Kir3-type K^+ channels.

Surprisingly, $GABA_B$ receptor regulating mechanisms remain unidentified to a great extent. However, it is thought that receptor-associated proteins and direct posttranslational modifications influence the receptor function. Phosphorylation by protein kinases is known as one of the most important post-translational modifications (Hunter, 1995). The importance of protein kinases in regulating cellular activities is already reflected by the large number of protein kinases related genes present in eukaryotic genomes; protein kinases are one of the largest superfamilies accounting for 1.7% of the human genome (Manning, 2002). Protein kinases were found to be enriched in the brain, where they play a critical role in the nervous systems such as memory formation and learning processes. Protein kinases also influence the $GABA_B$ receptors. For instance, PKA phosphorylates the $GABA_B$ receptor at the site S892 causing an elongation of the inhibitory action of $GABA_B$ receptors (Couve, 2002). This observation was first contradictory to the prevailing dogma being that phosphorylation reduces the GPCRs activity either by increasing receptor desensitization or by removing receptors from the cell surface. S892 phosphorylation by PKA strengthens the presence of $GABA_B$ receptors at cell surface, thereby enhancing the coupling of $GABA_B$ receptors to its effector. S783, another $GABA_B$ receptor phosphorylation site, was later described (Kuramoto, 2007). AMPK phosphorylates S783 thereby stabilizing the activating effects of the receptors on K^+ currents; the K^+ current rundown is finally reduced. AMPK phosphorylation at $GABA_B$ receptors is thought to be a novel neuroprotective

mechanism, which, under conditions of metabolic stress or ischemia, increases $GABA_B$ receptor function. This consequently reduces excitotoxicity and promotes neuronal survival which diminishes the harms of a stroke incident. Both S892 and S783 sites are localized on the carboxyl-terminal of the $GABA_{B2}$ subunit. For a long time, the $GABA_{B2}$ subunit seemed to be the most interesting target for phosphorylation site search on the $GABA_B$ receptor; the $GABA_{B2}$ subunit has the longer carboxyl-terminal and the $GABA_{B2}$ subunit is, due to the G-protein interaction, already localized near second messenger–dependent kinases. Indications for possible phosphorylation sites on the $GABA_{B1}$ subunit existed, but were demonstrated only at the *in vitro* stage, e.g. the S917 was shown on GST-fusion protein containing the $GABA_{B1}$ carboxyl-terminal, but not *in vivo*, and no physiological relevance could be attributed (Kuramoto, 2007).

In my thesis I identified the first phosphorylation site localized on native $GABA_{B1}$ subunits, serine 867 (S867). S867 is juxtamembrane positioned on the $GABA_{B1}$ carboxyl-terminal. It gets phosphorylated by CaMKII, mostly at the $GABA_{B1b}$ subunit isoform. CaMKII is implicated in a wide variety of neuronal functions, e.g. memory formation, receptor functioning, structural modification of the cytoskeleton, axonal transport, and gene expression (Hanson, 1992, Lisman, 1994, Yoshimura, 2000, Yoshimura, 2002). $GABA_B$ receptor activity increases the phosphorylation of threonine 286 on α CaMKII (Xu, 2008). Nevertheless, a CaMKII phosphorylation site on the $GABA_B$ receptor was never shown. The present findings finally confirm that CaMKII is regulating, via $GABA_{B1}$-S867 phosphorylation, $GABA_B$ receptor response.

CaMKII is highly abundant in neurons, especially enriched at synaptic endings (Kelly, 1984) and has a central role in the regulation of glutamatergic synapses. Its implication in glutamatergic transmission has emerged largely from the study of LTP, an activity dependent strengthening of synapses that is thought to underlie some forms of learning and memory. At many excitatory synapses, LTP is triggered by Ca^{2+} entry into the postsynaptic cell. Several lines of evidence indicate that CaMKII detects this Ca^{2+} elevation and initiates the biochemical cascade that potentiates synaptic transmission by recruiting AMPA and NMDA receptors to the postsynaptic membrane (Malenka, 1989b, Malinow, 1989, Bayer, 2001, Lisman, 2002). CaMKII might not only induce LTP but might also be responsible for the persistence of LTP, therefore contributing to memory formation. The strongest evidence for this idea comes from the observation that CaMKII remains activated for at least one hour after LTP induction and autophosphorylation of threonine 286 is crucial for its persistent activation; a mutation that eliminates phosphorylation of this site blocks LTP (Lisman, 2002).

The molecular mechanism underlying the phosphorylation of S867 by CaMKII points in the same direction as synaptic plasticity. Indeed, regulation of synaptic transmission depends on the

availability of surface neurotransmitter receptors and surface $GABA_B$ receptor levels were shown to be decreased in presence of glutamate. The alanine substitution of S867 (S867A) that prevents S867 phosphorylation renders $GABA_B$ receptors refractory to NMDA-mediated internalization. KN-93 application or the use of the S867A mutant abolished, in hippocampal dissociated neurons, the reduction of the $GABA_B$ receptor elicited K^+ currents after the NMDA treatment. Interestingly, the receptors ability to evoke inhibitory K^+ response was preserved. This result also indicates that S867 phosphorylation leads to an internalization of the $GABA_B$ receptors. The glutamate induced reduction of surface $GABA_B$ receptors was postulated to be mediated by a dynamin-dependent clathrin pathway (Vargas, 2008). To confirm that the NMDA mediated internalization works via the same pathway, the internalization should be abolished under hypertonic concentrations of sucrose (Grampp, 2007) or exposure to low K^+ concentrations (Bayer, 2001) conditions inhibiting clathrin-dependent internalizations. Clean and specific inhibition of the dynamin-dependent clathrin pathway in hippocampal cells was made possible by the use of dynasore. Dynasore has a potent *in vitro* inhibitory effect on the GTPase activity of dynamin1, dynamin2 and Drp1but not on other small GTPases (Macia, 2006). The effects of the dynasore administration can be studied using the immunofluorescence-based internalization assay. Additionally, cell surface ELISA and biotinylation assay, with cultured neuronal cells, should be also considered. Optionally, it can be argued that S867 phosphorylation does not contribute to $GABA_B$ receptors internalization but only reduce their recycling to the membrane surface. However, this discrepancy can be solved by transfecting inactive Rab protein mutant in Rab protein deficient cells (Glodowski, 2007). Rab proteins are guanosine triphosphateases that mediate the recycling and trafficking of endocytosed vesicles to the cell surface. The inactive Rab mutant should not reduce the number of surface $GABA_B$ receptors if the phosphorylation is involved in receptor internalization rather than receptor recycling.

The S867 phosphorylation was predominantly found at the native $GABA_{B1b}$ subunit which is, along with CaMKII, concentrated in the same postsynaptic compartments (Kennedy, 1983). The two $GABA_B$ receptor subtypes $GABA_{B(1a,2)}$ and $GABA_{B1(b,2)}$ produce different responses at axonal and dendritic effectors when pharmacologically activated by a saturating concentration of baclofen (Perez-Garci, 2006, Shaban, 2006, Vigot, 2006, Ulrich, 2007). The diverse pharmacological reaction of the two subtypes was explained by the distinct distribution profile of the $GABA_{B1a}$ and $GABA_{B1b}$ subunits to axonal and dendritic compartments respectively (Vigot, 2006, Guetg, 2009). Their expressions are also under separate transcriptional control which influence the ratio of receptor subtypes in individual neurons (Steiger, 2004). S867 phosphorylation represents a selective and specific modification between the two subunits. Primary, the $GABA_{B1b}$ subunits are controlled by the S867 phosphorylation effect. Transient high NMDA release leads to S867 $GABA_{B1b}$ subunit phosphorylation consequently reducing the inhibitory $GABA_B$ response in dendritic spines. This decrease in inhibition

contributes to an increased synaptic transmission efficacy. For this reason, one can anticipate a contribution of S867 phosphorylation to LTP and long-term depression (LTD) formation. LTD, the opposing process of LTP, requires also an NMDAR dependent rise in the postsynaptic Ca^{2+} concentration, which preferentially activates a protein-phosphatase cascade that includes calcineurin and phosphatase 1 (Carroll, 2001). The phosphatases could potentially dephosphorylate the pool of S867 phosphorylated $GABA_B$ receptors. The internalization of the $GABA_B$ would then be reduced consequently leading to an increased inhibitory transmission in the CNS. One can then expect that the dephosphorylation of S867 could probably contribute to LTD like phenomenon; the inhibitory fence of the gabaergic system would be reset and even strengthened.

Further effects on the $GABA_B$ receptor system through the S867 phosphorylation by CaMKII are imaginable. CaMKII phosphorylation was shown to regulate mGluR1 trafficking to different dendritic compartments (Kielland, 2009) and to influence the availability of Homer3 for its targets in the postsynaptic densities (Mizutani, 2008). Similarly, $GABA_{B1}$ subunit phosphorylation could also potentially leads to a conformational change of the subunit which would increase the binding to scaffolding proteins such as β-arrestins, as shown for other GPCRs, e.g. anaphylatoxin receptor (Braun, 2003).

The NMDA receptor mediated Ca^{2+} influx can activate, in addition to CaMKII, the kinase PKA (Moyano, 2004). Since PKA was shown *in vitro* to phosphorylate S867*;* further attention should be paid to a possible *in vivo* phosphorylation of S867 by PKA. This can be done by repeating the NMDA treatment experiments but in the presence of the cAMP–PKA pathway activator or inhibitor; e.g. forskolin or the PKA inhibitor $peptide_{14\text{-}22}$ or Walsh peptide (Moreno-Delgado, 2009). PKA also mediates crucial physiological functions in the brain, especially in processes like LTP (Anwyl, 2009). $GABA_B$ receptors are known to influence PKA activity by decreasing cAMP production, via their restrictive action on adenylate cyclase. PKA phosphorylates large arrays of proteins involved in mood disorders; e.g. Rap-1 (a member of the Ras family and is highly expressed in the CNS) (Dwivedi, 2008). Additionally they are many significant intersections between $GABA_B$ receptors and PKA activity. Indeed, via PKA dependent pathways, presynaptic $GABA_B$ receptors decrease the release of GABA from autoreceptors. This mechanism has an important role in the regulation of cholinergic transmission in the nucleus basalis of Meynert (Kubota, 2003), where a decrease in acetylcholine production is a key component in Alzheimer disease etiology (Freund, 2009). PKA and $GABA_B$ receptors are also essential in the sleep-wake behavior of mammals (Datta, 2007). Application of a PKA activator to Sprague-Dawley rats induces REM sleep, whereas baclofen application has the opposite effect (Datta, 2007).

Concluding, the phosphorylation site S867 can be seen as major part of a feedback mechanism. The active $GABA_B$ receptors decrease the activity of CaMKII and PKA. The physiological S867 phosphorylation by CaMKII and potentially by PKA, antangonizes the decrease in the kinases activity. S867 phosphorylation reduces the inhibitory activity of $GABA_B$ receptors. This feedback mechanism would ensure a fine tunning of the inhibitory transmission in the CNS that could otherwise not be guaranteed. S867 is also part of the cross-talk between the glutamatergic and gabaergic nervous system. The NMDA-dependent phosphorylation of S867 by CaMKII internalizes the whole $GABA_B$ receptor. Consequently, the inhibitory response of the gabaergic system is locally decreased, whereas the excitatory circuit is enhanced; further recruitment of AMPA and NMDA receptor is then facilitated, phenomenon also observed during LTP. $GABA_B$ receptor subtype specificity of S867 phosphorylation could be seen as an additional explanation for the different $GABA_{B(1a,2)}$ and $GABA_{B(1b,2)}$ receptors localization. In conclusion, S867 phosphorylation plays an active part in the orchestration of synaptic plasticity and clearly manifests many possibilities for further scientific findings on the inhibitory effect of $GABA_B$ receptors on the CNS. Especialy the generation of a $GABA_{B1}$ S867A mice model could be an important landmark in the study of the role of S867 phosphorylation on the CNS. On such a mice model the regulatory effect of the S867 phosphorylation on the inhibitory gabaergic is able to get studied under different and the most physiological ways. The development of new research avenues on that subject enables the development of new innovative approches for the treatment of neurological and psychiatric diseases.

VI. References

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VII. Appendix

A. Curriculum Vitae

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PEER-REVIEWED PUBLICATIONS

- Gassmann M., Haller C., Stoll Y., **Abdel Aziz S.**, Biermann B., Mosbacher J., Kaupmann K., Bettler B .
  The RXR-type endoplasmic reticulum-retention/retrieval signal of $GABA_{B1}$ requires distant spacing from the membrane to function.
  Mol. Pharmacol.2005Jul;68(1):137-44
- Biermann B., Bradaia A., Ivankova-Susankova K., **Abdel Aziz S**., Besseyrias V., Kapfhammer J.P., Gassmann M., Bettler B.
  The Sushi domains of $GABA_B$ receptors function as axonal targeting signals.
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- **Abdel Aziz S*.**, Guetg N. *, Gassmann M., Paul Jenoe, Suzett e Moes, Rostislav Turecek, Emilio Casanova and Bettler B.
  NMDA receptor-dependent $GABA_B$ internalization via CaMKII phosphorylation of serine 867 in $GABA_{B1}$
  Proc Natl Acad Sci USA 2010 Aug 3; 107 (31): 13924-9

B. Acknowledgements

I am deeply obliged to Prof. Dr. Bernhard Bettler for giving me the unique opportunity to do my Ph.D. in his laboratory and entrusting me with an exciting project. It was a great experience to benefit from the excellent insights in the scientific and daily life.

Special thanks also to Dr. Martin Gassmann, it was a great honour to work and learn under his supervision. I benefited from his patience and from sharing his critical and supportive thinking in terms of research.

I thank my collaborators Dr. Nicole Guetg, Jan Tchorz, Dr. Rotislav Turecek and Dr. Klara Ivankova-Susankova for assisting me to develop further this project.

I would also thank Dr. Paul Jenoe and his group for allowing me to use their HPLC machine and the precious support and feedback I received during the analysis of my results and during the writing of my thesis.

Thanks to Prof. Dr. Markus A. Ruegg and Prof. Dr. Martin Spiess for accepting to be members of my thesis committee.

Thanks also to Prof. Dr. Mohammed Akaaboune for the fruitful and interesting discussions.
Thanks to Prof. Dr. Markus Wymann, Prof. Dr. Brian Hemmings and Prof. Dr. Peter Parker for their helpful comments on my project.

Great “thank you” to the former/ present lab mates from the Bettler, Brenner and Ruegg laboratories. It was a great privilege to work together with you and to enjoy your colleagueship. I appreciate your invaluable generosity.

Explicit thanks to Dr. Audree Pinard for her indefatigueable, critical inputs to my thesis.

Thanks to all the people on the floor and in the whole Bio- and Pharmazentrum, that helped me address the different questions during my Ph.D.

Last but foremost I want to thank my family, my great parents and my beloved brothers, for their infinite love and support. I bow in front of you and pay you all my love and respect.
And of course all my friends (in and outside the laboratories) for their help, support and love. All my love and respect to you too.