Maturation of GABAergic signaling during brainstem development

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List of abbreviation

aa	amino acid
ad	"till end volume"
app.	approximately
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
C	Celsius
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CNS	central nervous system
C-terminus	carboxy-terminus
DEPC	diethylpyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
et al.	and others
EtBr	ethidiumbromide
EtOH	ethanol
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GABA	γ-amino butyric acid
GIPs	GPCR interacting proteins
GPCR	G protein coupled receptors
HEPES	*N*-hydroxyethylpiperazine-*N´*-2-ethanosulphonic acid
HRP	horseradish peroxidase
HSP70	heat shock protein 70
IgG	immunoglobulin G
IP	immunoprecipitation
kb	kilo base

kbp	kilo basepair
kDa	kilo dalton
M	molar
mA	milli ampere
MAPK	Mitogen-Activated Protein Kinase
MeCP2	methyl-CpG-binding protein 2
mM	milli molar
mRNA	messenger RNA
n	number in study or group
nM	nano molar
NMDA	*N*-methyl-d-aspartate
NP-40	nonidet P-40
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyfluoride
RNase	ribonuclease
rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcript polymerase chain reaction
SDS-PAGE	sodiumdodecylsulfate -polyacrylamide gel
sec	second
TBE	tris borate EDTA buffer
TBS	tris-buffered saline
TEMED	tetramethylendiamine
Tris	tris-(hydroxymethyl)-aminomethan
Tween 20	polyoxyethylene sorbitane monolaurate
U	Unit, enzyme activity
v/v	volume per volume
Vol.	volumes
w/v	weight per weight
X-gal	5-bromo-4-chloro-3-indoyl-D-galactopyranoside

1 INTRODUCTION

1.1 G protein coupled receptors (GPCRs)

The nervous system process a multitude of signals from the environment and within its cells. The ability of an organism to function normally depends on the capacity of nervous system cells to communicate effectively with each other and their environment. Specialized proteins called receptors, which are very specific to the signals they receive, regulate the signaling pathways. In terms of their structural and functional features, transmembrane receptors can be categorized as either ionotropic (transmitter-gated ion channels) or metabotropic receptors. To the metabotropic receptors belong the: G protein coupled receptors, Guanylyl cyclase receptors and Tyrosine Kinases receptors.

G protein coupled receptors (GPCRs) form one of the largest superfamilies of cell-surface receptors whose primary function is to transduce extracellular stimuli into intracellular signals. The chemical diversity of the GPCRs ligants is exceptional and includes endogenous ligants such as hormones and neurotransmitters and neuromodulators such as biogenic amines, amino acids, peptides, glycoproteins, prostanoids, phospholipids, nucleosides and nucleotides, ions (Gether 2000; Howard, McAllister et al. 2001; Lee, George et al. 2001) and exogenious ligants, such as odors, pheromones, light, or tastes (Firestein 2000). Despite the remarkable structural diversity of their activating ligands, all GPCRs are predicted to share a common molecular architecture consisting of a single protein chain (300-1000 amino acids) that crosses the membrane seven times (seven-transmembrane G protein Coupled Receptors, 7-TM GPCRs) (Ulloa-Aguirre, Stanislaus et al. 1999). The seven transmembrane α-helical segments (TM I-VII), usually consisting of 20 to 27 amino acids each, are connected by three intracellular (i1, i2 and i3) and three extracellular (e1, e2 and e3) loops (Baldwin 1993), with the amino terminus located on the extracellular side and the carboxy terminus on the intracellular side (Wess 1997). The N-terminal segment is the site of glycosylation and ligand binding, the C-terminal segment allows palmitoylation and phosphorylation as prerequisites for

desensitization and internalization (Liebmann and Bohmer 2000). The intracellular loops transmit the signal from the receptor to G protein.

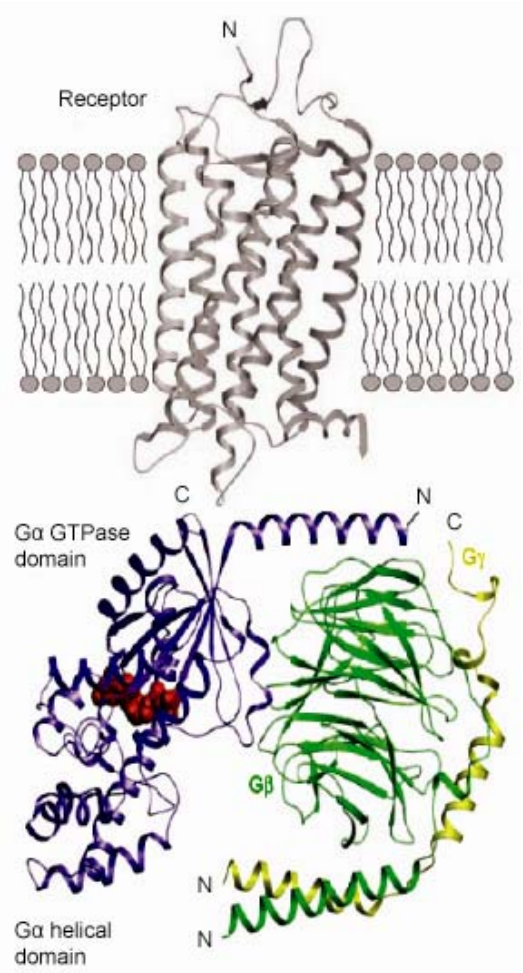


Figure 1.1. GPCR and G protein complex. Ribbon model of bovine rhodopsin, showing the seven transmembrane spanning α-helices (gray), which has been cross linked to several sites on the G protein. The N-terminal and the C-terminal ends of the Gα (blau), Gβ (green) and Gγ (yellow) subunits are marked with the letters N and C respectively. The GDP molecule is buried between the GTPase and helical domain of Gα. Adapted and modified from (Lambright, Sondek et al. 1996).

The structure of the GPCRs has been confirmed by analysis of the crystal structure of Rhodopsin (Baldwin 1993; Palczewski, Kumasaka et al. 2000) that has been extensively used as template for homology-based modelling of GPCRs (see Figure 1.1) (Orry and Wallace 2000).

1.1.1 Evolution & Structural Classification of GPCRs

GPCRs are the oldest known signal transducers present in the genome of plants (Plakidou-Dymock, Dymock et al. 1998), yeast (Dohlman, Thorner et al. 1991) and slime mold (*Dictyostelium discoideum*) (Devreotes 1994), as well as in protozoa and the earliest diploblastic metazoan (Vernier, Cardinaud et al. 1995; New and Wong

1998). In vertebrates, the GPCR family contains 1000–2000 members (>1 % of the genome) including >1000 coding for odorant and pheromone receptors (Bockaert and Pin 1999). In a recent analysis of the GPCRs in the human genome, more than 800 GPCRs were listed (Fredriksson, Hoglund et al. 2003). Of this total, 701 were in the rhodopsin family (type A) (Fredriksson, Lagerstrom et al. 2003).
A study similar to that of Fredriksson et al. (Fredriksson, Hoglund et al. 2003), showed that the repertoire of GPCRs for endogenous ligands consist of 367 receptors in human and 392 in mice [endoGPCR refers to GPCRs for endogenous (non-olfactory) ligands]. In view of the known existence of alternatively spliced variants and editing isoforms of GPCRs, it is likely that the true number of GPCRs will never be known and is much higher than estimated. (Kroeze, Sheffler et al. 2003).

All GPCR members have seven transmembrane domains but, on the base of shared sequence motifs, they are grouped into six classes: A, B, C, D, E and the frizzled/smoothened family (Horn, Weare et al. 1998).
Family A is by far the largest and the most studied. It includes biogenic amine receptors (adrenergic, serotonin, dopamine, muscarinic, histamine), adenosine, cannabionoid, melanocortin, olfactory receptors, melatonin receptors plus others.
Family B receptors include approximately 20 different receptors for a variety of peptide hormones and neuropeptides, such as calcitonin and glucagon.
Family C receptors are characterized by an exceptionally long amino terminus (500-600 amino acids). The receptors include the Metabotropic glutamate and γ-aminobutyric acid (GABA) receptors, the calcium receptors, the vomeronasal, mammalian pheromone receptors, and the recently identified putative taste receptors (Gether 2000; Sadee, Hoeg et al. 2001).
Yeast pheromone receptors make up two minor unrelated subfamilies, family D and family E. In *Dictyostlium Discoideum* four different cAMP receptors constitute yet another minor, but unique, subfamily of GPCRs (Family F).

1.2 GPCR signaling

GPCRs have been named based on their ability to recruit and regulate the activity of intracellular Heterotrimeric G proteins (Gether 2000). When a ligand such as a

hormone, neurotransmitter, or glycoprotein interacts with a heptahelical receptor on the surface of the cell, the ligand either stabilizes or induces a conformation in the receptor that activates a heterotrimeric G protein (composed of α, β, and γ-subunits) on the inner membrane surface of the cell. In the inactive heterotrimeric state, GDP is bound to the Gα-subunit. Upon activation, GDP is released, GTP binds to Gα, and subsequently Gα-GTP dissociates from Gβγ and from the receptor (see Figure 1.1) Both the Gα-GTP and the Gβγ dimer activate a number of enzyme activities (effectors) or ionic channels which regulate the intracellular concentrations of secondary messengers such as cAMP, cGMP, diacylglycerol (DAG), IP3, arachidonic acid, sodium, potassium or calcium cations (Gilman 1987; Sprang 1997). Different GPCR subtypes couple to different Gα subfamilies ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$), thus exerting different downstream effects on a cell (Hamm 1998; Cabrera-Vera, Vanhauwe et al. 2003; Kurose 2003).

The mechanism by which GPCRs transduce extracellular messages into intracellular cellular responses was initially envisioned as a simple model. Recently, this view has been extended in order to explain the complexity of the GPCR signaling. Thus, it has become more important to investigate further the role of the G protein variability, the GPCRs diversity, and the ligants heterogeneity, for the receptor modulation. Furthermore, GPCRs interact not only with G proteins but also with a variety of other proteins. The later interaction influences the signal variability too.

1.2.1 GPCR signaling specificity through G protein variability

There are at least 21 different α, 5 different β, and 13 different γ subunits in human (Downes and Gautam 1999; Venter, Adams et al. 2001) to which GPCRs can be coupled (Hermans 2003; Wong 2003). The influence of the different G protein subtypes in the mechanisms of signaling has been demonstrated, based on the β2-adrenoceptor receptors. Seifert et al. (Seifert, Wenzel-Seifert et al. 1998) proposed that the structural differences between two different splice variants of $G\alpha_s$-protein might influence the binding of β2-adrenoceptor to $G\alpha_s$-protein.

Another layer of complexity arises from the ability of each G protein to activate multiple downstream effectors. Both Gα and Gβγ subunits contribute to the modulation, in a synergistic or antagonistic fashion, of either the same or unrelated

effectors, resulting in dual intracellular signaling. An example is the simultaneous $G\alpha_{i/o}$-mediated inhibition of adenylate cyclase via the Gα subunit and stimulation of phospholipase Cβ via the Gβγ subunit (Exton 1996). In Addition, single GPCRs are able to activate simultaneous multiple G protein pools. Dual coupling to $G\alpha_s$ and $G\alpha_{q/11}$ (Jin, Wang et al. 2001) or to $G\alpha_{i/o}$ and $G\alpha_{q/11}$ (Offermanns, Heiler et al. 1994) has now been reported for many GPCRs. In some cases, a single receptor has been found to simultaneously activate members of three or even four unrelated classes of G protein ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12}$) (Laugwitz, Allgeier et al. 1996).

1.2.2 GPCR signaling specificity through the complexity of the GPCRs.

The existence of receptor subtypes represents the second level of specificity in GPCR signal transduction. Through a variety of mechanisms, genes encoding GPCRs have duplicated and spread throughout eukaryotic genomes. Thus, at least 5 closely related human genes encode muscarinic cholinergic receptors, 5 encode dopamine receptors, and at least 15 encode serotonin receptors (Felder, Graul et al. 1999). A number of GPCR genes exist as a single exon but many GPCR genes are multiexonic. This indicates the existence of splice variants with distinct functions, as has been demonstrated for the prostaglandin EP3 receptor subtype. Alternative splicing of EP3 yields at least 4 isoforms that differ in their C-terminus and couple to different G proteins and second messengers (Namba, Sugimoto et al. 1993). In addition Pindon et al. (Pindon, van Hecke et al. 2002) demonstrated that the human 5-HT4a receptor binds to $G\alpha_s$-protein, whereas it's C-terminal splice variant 5-HT4b receptor binds to both $G\alpha_i$- and $G\alpha_s$-proteins, when expressed in HEK293 cells. Many more splice variants can be expected that have yet to be studied; for a review, see (Gudermann, Kalkbrenner et al. 1996; Sadee, Hoeg et al. 2001).

1.2.3 GPCR signaling specificity through ligands heterogeneity

Ligants interact with different extracellular and/or transmembrane domains of GPCRs, in order to convey their messages to the interior of the cell. For each of the ligands, exist distinct binding sites in the GPCRs, either embedded within the pocket formed by the 7-TM bundle within the membrane (biogenic amines), or at pockets

formed by the extracellular loops (peptides). For the metabotropic glutamate and GABA receptors, the ligand-binding sites are contained within the large extracellular domain characterizing family C receptors (Coughlin 1994).

The binding modes for agonists acting at GPCRs are almost as diverse as the nature of their ligands. Even various agonists acting at the same receptor may not necessarily share an overlapping binding site (Schwartz and Rosenkilde 1996). Thus, ligand-dependant stimulation can activate coupling to different G protein subtypes. In case of β2-adrenergic receptor, the antagonist ICI-118-551, which behaves as an inverse agonist for coupling to $G\alpha_s$, act as an agonist for β2-adrenergic receptor coupling to $G\alpha_i$ (Gong, Sun et al. 2002). An example for ligand-dependant stimulation has been showed from Heubach et al. (Heubach, Ravens et al. 2004), where binding of β2-adrenoceptor to either $G\alpha_i$ or $G\alpha_s$ depends on whether the receptor has been activated by epinephrine or norepinephrine; or in the example of Houston et al. where structurally distinct ligands modulate differently ($G\alpha_i$ or $G\alpha_o$ coupling) the cannabimimeric CB_1 receptor (Houston and Howlett 1998). Lastly, the idea that a given receptor can be coupled to two different pathways depending on the duration of activation (and therefore potential desensitization) has also been reported in the case of mGluRs (Pin 1998).

Despite all this ligand-binding dependant GPCR signaling experiments, they appear cases of spontaneous basal signaling activity in the absence of agonists (also referred to as constitutive activity) (Lefkowitz, Cotecchia et al. 1993). Constitutive activity of wild-type β2 adrenergic (Chidiac, Hebert et al. 1994), serotonin (Barker, Westphal et al. 1994), bradykinin (Leeb-Lundberg, Mathis et al. 1994), d-opioid (Costa and Herz 1989) , and muscarinic (Jakubik, Bacakova et al. 1995) receptors has been reported. It has also been observed that discrete mutations are able to dramatically increase this constitutive agonist-independent receptor activity (Kjelsberg, Cotecchia et al. 1992; Samama, Cotecchia et al. 1993). The majority of the constitutively activating mutations were initially identified after mutational substitutions in the C-Terminal part of the third intracellular loop of the adrenergic receptors (Kjelsberg, Cotecchia et al. 1992; Samama, Cotecchia et al. 1993), but currently activating mutations have been identified in almost any receptor domain in an increasing number of receptors [representative examples in (Porter, Hwa et al. 1996; Burstein, Spalding et al. 1998; Hjorth, Orskov et al. 1998)].

1.2.4 GPCR signaling specificity in a cell- or tissue-dependent manner

The signal transduction also depends on the cell or tissue. Studies on the pleiotropic hormone bradykinin have shown that it is capable of activating different G proteins and multiple signaling pathways (Table 1) according to the cell or tissue that they are expressed.

Table 1.1. Typical bradykinin signaling pathways: multiple coupling and cell specificity

Cell/Tissue	Effector	Mechanism	Ref.
A431 cells, guinea pig ileum, others	PLCb (stimulation)	via $G\alpha_{q/11}$	(Tilly, van Paridon et al. 1987)
fibroblasts, rat myometrial cells	PLA2 (stimulation)	partially via $G\alpha_i$	(Tropea, Munoz et al. 1992)
A431 cells	AC (stimulation)	via $G\alpha_s$	(Liebmann, Graness et al. 1996)
airway smooth muscle cells	AC (stimulation)	via $G\alpha_{q/11}$, PKC, MAPK, PLA2, PGE2	(Pyne, Tolan et al. 1997)
Rat uterus, GPI	AC (inhibiton)	via $G\alpha_i$	(Liebmann, Graness et al. 1995)

1.2.5 GPCR signaling specificity through GIPs

Recent studies have shown that GPCR signaling exhibits greater diversity than previously appreciated. It is now evident that signaling specificity of a GPCR is not only dependent of the nature of the heterotrimeric G proteins, ligands, and GPCR subtypes, but also on the nature of the GPCR interacting proteins (GIP) (Hall and Lefkowitz 2002; Bockaert, Marin et al. 2003; Kroeze, Sheffler et al. 2003). The nature of these GIPs is different, depending on the cell in which the receptor is expressed (Bockaert, Fagni et al. 2004). There are several types of GIPs. Some are transmembrane proteins such as another GPCR (homodimerization and heterodimerization), ionic channels, ionotropic receptors, single transmembrane proteins and soluble proteins. GIPs are implicated in (a) targeting of GPCRs to specific cellular compartment, (b) association with other signaling or structural

proteins, and (c) the fine-tuning of their signal transduction, including desensitization and resensitization.

1.2.5.1 Specificity arising within membrane microdomains

Experimental evidence indicates that GPCRs, G proteins, and effectors are not randomly distributed in the plasma membrane. Indeed, it has been suggested that GPCR signaling mainly occurs within specialized microdomains, implying that the efficiency and specificity of signal transduction are dictated by the stoichiometry of transducer elements within spatially discrete membrane regions (Neubig 1994; Ostrom, Post et al. 2000; Ostrom 2002). Thus the same receptor may regulate a Ca^{2+} channel through one G protein at a nerve terminal and regulate PLCβ at a distal dendrite through another G protein. One of the most studied forms of membrane microdomain are regions of high-density cholesterol, gangliosides, and sphingolipids, referred to as caveolae or lipid rafts (Galbiati, Razani et al. 2001).

1.2.5.2 Association with other signaling or structural proteins

In addition to G proteins, GPCRs are known to interact with many other proteins, some of which may also have signaling functions (Heuss and Gerber 2000). Receptor associated proteins include arrestins, protein kinases and phosphatases, PDZ-domain binding proteins (Bockaert and Pin 1999) and various modifying enzymes, for example those introducing palmitoyl residues into the C-terminus.

a. Interaction with other GPCR (homodimerization and heterodimerization of GPCR)

Most GPCRs are predicted to form homodimers or heterodimers (Chabre et al. 2003; Liang et al. 2003), an idea first proposed by Rodbell (1992). The molecular mechanisms of dimer formation seem to differ considerably among the receptors.
The importance of the heterodimerization has been demonstrated (Kaupmann, Malitschek et al. 1998; White, Wise et al. 1998; Jones, Borowsky et al. 1998; Kuner, Kohr et al. 1999), based on the $GABA_B$ receptors. The functional $GABA_B$ receptor is a heterodimers, composed of the $GABA_BR1$ (GB1) and $GABA_BR2$ (GB2) subunits (Jones, Borowsky et al. 1998; Kuner, Kohr et al. 1999). Formation of the GB1-GB2

dimer within the endoplasmatic reticulum (ER) is needed for the trafficking to the membrane (GB1 is retained within the ER via a RXR (R) motif within its C-terminus).

Another example which shows the importance of the GPCR dimerization has been also demonstrated based on the β2-adrenergic receptor; a peptide corresponding to the sixth TM of the β2-adrenergic receptor inhibits both receptor dimerization and activation (Hebert, Moffett et al. 1996), suggesting that GPCR dimerization may be important for G protein activation.

b. Interaction with ionic channels and ionotropic receptors

An example which shows the fine-tuning of GPCRs through their interaction with ionotropic receptors is the interaction between the dopamine D5 receptor and the ionotropic $GABA_A$ receptors. Both receptors are localized in dentritic shafts and the cell soma/axon hillock area. The C-terminus of D5 (but not D1) receptors interacts with the second intracellular (i2) loop of $GABA_A$ receptor γ2 subunit. This leads to a mutually inhibitory interaction between D5 and $GABA_A$ receptors (Liu, Wan et al. 2000).

c. Interaction with single transmembrane proteins and soluble proteins

A final source of GPCR signaling diversity arises from data suggesting that GPCRs transmit "G protein-independent" signals and that coupling to certain non-G protein effectors exhibits features consistent with agonist-specific trafficking.

The intracellular domains of several GPCRs have been shown to bind to proteins that might function as alternative GPCR signal transducers, among which include GEFs for small G proteins, nonreceptor tyrosine kinases, and several proteins that function as adaptors or scaffolds (Miller and Lefkowitz 2001; Maudsley, Martin et al. 2005). For example the C-termini of $GABA_BR1$ and $GABA_BR2$ receptors, which contribute to their heterodimerization, are engaged in an interaction with the leucine zipper domain of cAMP-responsive element binding protein 2 (CREB2, also called ATF_4) and ATF_x (Nehring, Horikawa et al. 2000; White, McIllhinney et al. 2000; Vernon, Meyer et al. 2001). CREB2 and $GABA_B$ receptors are colocalized in soma and dendrites of cultured hippocampal neurons. $GABA_B$ receptors activate CREB2 translocation to the nucleus and gene transcription in heterologous systems (White, McIllhinney et al. 2000).

d. Desensitization and internalization as a modifier of signal quality

Desensitization has been show to play a role not only in limiting signal duration and intensity, but also in the determination of signal quality. Desensitization is initiated by receptor phosphorylation. Daaka et al. (Daaka, Luttrell et al. 1997) showed that this receptor phosphorylation modifies the coupling specificity of the β2-adrenergic receptor. Activation of β2-adrenoceptor causes primarily an increase of the level of PKA by Gs-mediated signaling pathway. The elevation of PKA phosphorylates the β2-adrenoceptor, which mediated its coupling to $G\alpha_i$-protein and initiates a new set of signaling events.

1.3 The heterotrimeric G proteins

Heterotrimeric G proteins are part of the GTPase superfamily that also includes small GTP-binding proteins and many factors involved in protein synthesis (s. chapter 1.4). "G protein" usually refers to the membrane-associated heterotrimeric G proteins, sometimes referred to as the "large" G proteins. These proteins are activated by G protein coupled receptors and are composed of three subunits: alpha (α), beta (β) and gamma (γ).

When a ligand binds to a G protein coupled receptor (GPCR) on the exterior surface of a cell, it induces a conformational change in the GPCR and that activates the bound heterotrimeric G protein. The G protein then releases the GDP from the Gα subunit, and binds a GTP. This exchange triggers the dissociation of the Gα subunit, the Gβγ dimer, and the receptor. Both Gα-GTP and Gβγ can then activate different signaling cascades and effector proteins, while the receptor is able to activate the next G protein (s. chapter 1.4). The Gα subunit will eventually hydrolyze the attached GTP to GDP, allowing it to reassociate with Gβγ and starting a new cycle.

1.3.1 Structure and diversity of G proteins

Heterotrimeric G proteins consist of α, β and γ subunits (encoded by distinct genes) each of which has multiple isoforms. In addition, there are many splice variants for some of these isoforms, which together can make up hundreds of combinations of G

proteins. The specific combination of α, β and γ subunits affects not only which receptor it can bind to, but also which downstream target is affected, providing the means to target specific physiological processes in response to specific external stimuli (Yan, Kalyanaraman et al. 1996; Scott, Huang et al. 2001).

Table 1.2. Classification of Gα-subtypes and their effectors (Burstein, Spalding et al. 1998)

Family	Subtyp	Effector
$G\alpha_s$	$G\alpha_{s\,(S)}$	↑ AC
	$G\alpha_{s\,(L)}$	↑ GTPase of tubulin
		↑ src
	$G\alpha_{olf}$	↑ AC
$G\alpha_i$	$G\alpha_{i1}$	↓ AC
	$G\alpha_{i2}$	Rap 1GAP
	$G\alpha_{i3}$	GRIN 1 and 2
	$G\alpha_{oA}$	↑ GTPase of tubulin
	$G\alpha_{oB}$	↑ src
	$G\alpha_z$	Ca^{2+} and K^+ channels
	$G\alpha_{t1}$	↑ cGMP-PDE
	$G\alpha_{t2}$	
	$G\alpha_g$	Unknown
$G\alpha_q$	$G\alpha_q$	↑ PLC*βs*
	$G\alpha_{11}$	↑ Bruton´s tyrosine kinase ($G\alpha_q$)
	$G\alpha_{14}$	
	$G\alpha_{15}$ or $_{16}$	
$G\alpha_{12}$	$G\alpha_{12}$	↑ NHE-1
		↑ PLD
	$G\alpha_{13}$	↑ p115RhoGEF
		↑ iNOS

PDE, Phosphodiesterase E; iNOS, inducible nitric oxide synthetase; NHE, Na^+/H^+ exchanger; PLD, phospholipase D; GEF, guanine nucleotide exchange factor; $G\alpha_{s\,(S)}$, $G\alpha_s$ short ; $G\alpha_{s\,(L)}$, $G\alpha_s$ long.

Crystallographic studies of G proteins have shown that the alpha subunit of heterotrimeric G proteins consists of two domains; a GTPase domain and an alpha-helical domain (see Figure 1.1). The GTPase domain consists of five helices surrounding a six-stranded beta sheet with five strands running parallel and one strand antiparallel to the others. The GTPase domain hydrolyzes GTP and contains site for binding to the Gβγ dimmer, heptahelical receptors and downstream effector proteins (Clapham and Neer 1997). The helical domain is composed of six α-helices. This

domain is responsible for increasing the affinity of Gα for guanine nucleotides (Remmers, Engel et al. 1999), for increasing the GTP hydrolysis activity of the protein (Markby, Onrust et al. 1993) and it may also play a significant role in coupling specific G proteins to specific effectors.

The structure of the beta subunit is a "beta propeller" protein, with seven distinct beta sheet domains arranged like blades on a propeller and an α-helical conformation at the N-terminus which forms a coiled coil that is essential for the interaction with the γ subunit (Fong, Amatruda et al. 1987). The gamma subunit is composed of two α-helices connected by a loop, where the N-terminal helix interacts with the N-terminus of the beta subunit and the C-terminal helix (Sondek, Bohm et al. 1996).

G proteins are named after their α subunits. On a basis of sequence identity, at least 21 α subunits encoded by 16 genes, with several alternatively spliced isoforms, have been identified and classified into four subfamilies: (i) $G\alpha_s$ and (ii) $G\alpha_{i/o}$, which stimulate and inhibit respectively adenylate cyclase, (iii) $G\alpha_{q/11}$ which stimulate phospholipase C, and (iv) the less characterized $G\alpha_{12/13}$ subfamily that activates the Na^+/H^+ exchanger pathway (Hamm 1998). Members of this family range in size from 39-52 kDa and share between 35 % and 95 % sequence identity (Downes and Gautam 1999). The $G\alpha_i$ class was named for the ability of some of its members to inhibit adenylyl cyclase and includes α_{i-1}, α_{i-2}, α_{i-3}, which are products of different genes. There is no evidence for alternatively spliced products being encoded by these genes; $G\alpha_o$ (for o ”other” because its function was unclear) is a predominantly neural α subunit, which is also a product of a separate gene that can be spliced to generate at least two polypeptides, α_{o1}, α_{o2}; the rod transducin α subunits α_{t1} and α_{t2}, which transduce visual signals in conjunction with rhodopsin in the retina. $G\alpha_t$ triggers the breakdown of cyclic GMP; $G\alpha_z$ and $G\alpha_{gust}$ (gustducin). All the members of this class, except α_z, can be modified by pertussis toxin.

The $G\alpha_s$ class was first recognized by its ability to activate adenylyl cyclase and includes the $G\alpha_s$ and $G\alpha_{olf}$ (an α subunit from olfactory neuroepithelium which couples to olfactory receptors).

The $G\alpha_q$ class includes $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$. Members of this class activate phospholipase C (see chapter 1.4.1.1) (Strathmann and Simon 1990).

The fourth class includes α_{12} and α_{13}, important for regulating the cytoskeleton, cell junctions, and other processes related to movements

Gβγ is a tightly complexed dimer that dissociates only under denaturing conditions. It can act as a signaling molecule itself, by activating other second messengers or by gating ion channels directly (Ikeda and Dunlap 1999). Both Gβ and Gγ subunits also consist of multiple proteins. There are currently five Gβ and 12 Gγ subunits (Clapham and Neer 1997). The five β subtypes identified so far in mammals have a molecular mass close to 35-36 kDa, and are encoded by 5 genes having a 50-83 % identity between them. The two Gβ subclasses based on amino acid homology are the β1–β4 and β5. The γ subunit is a relatively diverse group of much smaller proteins of 8-10 kDa each. These proteins all undergo posttranslational modifications, leading to further diversity within this subunit family. The 12 mammalian γ subunit types are encoded by 12 genes: γ1 to 12 (Gautam, Downes et al. 1998). The existence of 12 different γ subunits, five different β and at least 20 α subunits and many splice variants indicates that there are many possibilities for different combinations. But, in fact, not all the possible pairs can be formed. For example by transient expression in COS cells, β1 is able to interact with γ1 and γ2, but the very similar β2 molecule is able to form a dimer only with γ2 (Pronin and Gautam 1992). In addition, some subunits are very cell-specific, for example, the α subunits on specialized sensory systems, including olfactory, visual and lingual tissues ($G\alpha_{olf}$/Olfactory neuroepithelium, $G\alpha_{t1}$/rod photoreceptors; $G\alpha_{t2}$/cone photoreceptors and $G\alpha_{gust}$/taste buds) with highly restricted distribution patterns, or the $G\alpha_o$ predominantly expressed in brain and the $G\alpha_{15}$ in hematopoietic cells. The β5, γ3 and β4, are mainly expressed in the nervous system.

1.3.1.1 The complexity of the stimulatory G ($G\alpha_s$) protein

In contrast to the members of the $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ subfamilies, the $G\alpha_s$ gene is located in a rather complex genomic locus (GNAS). Gnas is a complex imprinted gene (genes whose expression is determined by the parent that contributed them) whose major gene products are generated by at least four alternative promoters and first exons that splice onto a common exon (exon 2) (Weinstein, Yu et al. 2001).
There has been a continuous stream of new data, as well as some conflicting reports based on the localisation, function and the number of the alternative splice variants of the Gnas gene products. The human $G\alpha_s$ gene (GNAS1), is located on human

chromosome 20q13.2 while its mouse ortholog (*Gnas*) is located in a distal portion of chromosome 2 (Blatt, Eversole-Cire et al. 1988; Holmes, Williamson et al. 2003).

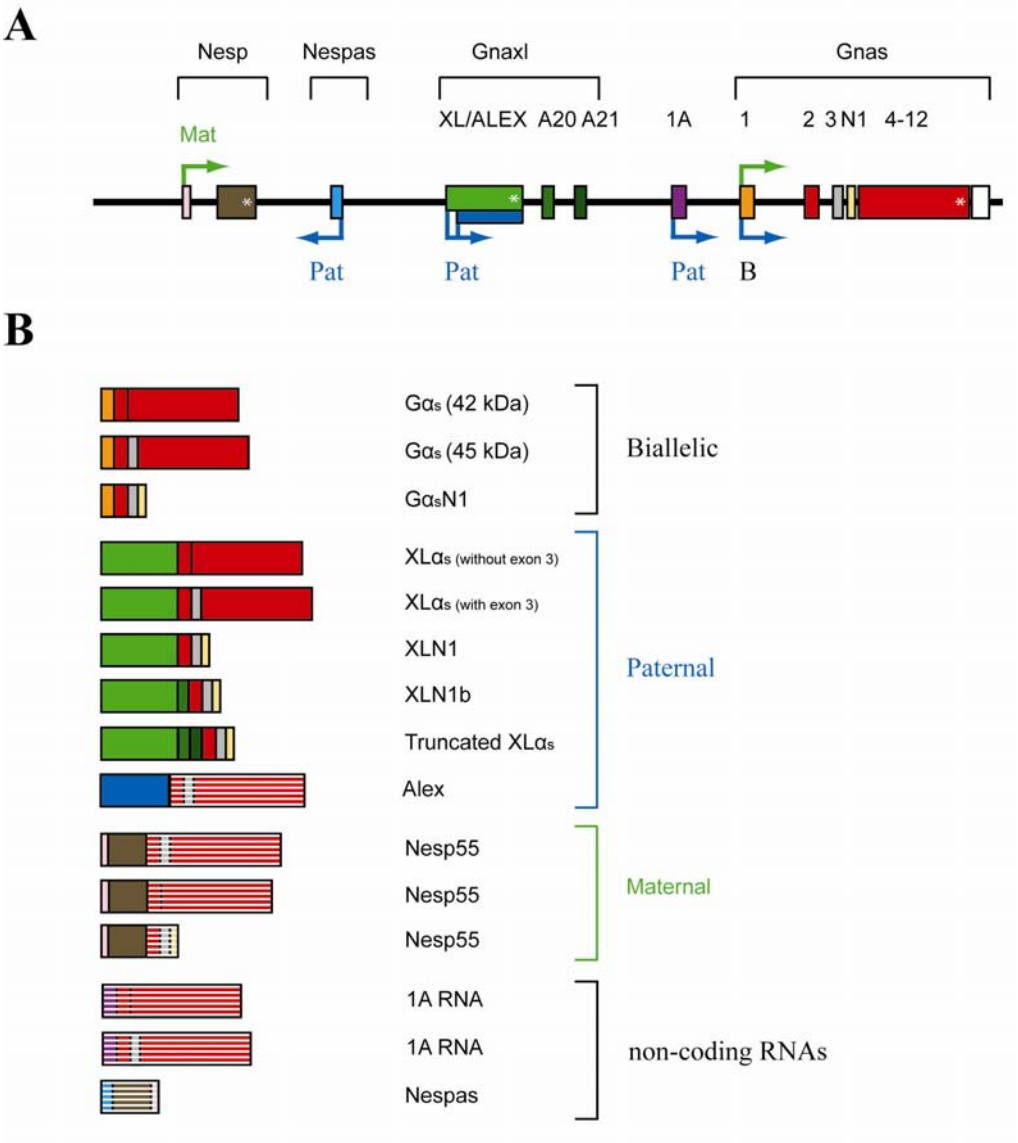


Figure 1.2. Scheme of the imprinted Gnas locus of the mouse. Promoters and the direction of transcription are shown by the arrows; Mat (maternal), Pat (paternal) and B (biallelic). The Gnas promoter (encoding $G\alpha_s$) is biallelically expressed. The neural-specific form is marked as GαsN1. Gnasxl encodes an N-terminal variant of $G\alpha_s$ (XLas) and, truncated proteins (XLN1, XLN1b, truncated XLαs). The Nesp55 protein is encoded by a single upstream Nesp exon. Nespas and exon 1A transcripts produce non-coding RNAs. Asterisks indicate stop codons (A) Intron-exon organization. Neither exons (coding regions, colored; noncoding regions, striped) nor introns (interbox distances) are in scale; however, their relative positions reflect their positions in the genome.
(B) Transcripts and their translation products (Figure modified from reference Plagge et. al 2004, Holmes et. al. 2003, Weinstein et al. 2001 and Abramowitz et.al. 2004.)

As originally described (Kozasa, Itoh et al. 1988), the Gnas locus encodes the α subunit of the stimulatory guanine nucleotide-binding protein $G\alpha_s$, as well as NESP55, a chromogranin-like neuroendocrine secretory protein (Hayward, Moran et al. 1998), and XLas, a large $G\alpha_s$ variant (Kehlenbach, Matthey et al. 1994; Hayward, Moran et al. 1998). In addition, the GNAS locus yields two, paternally derived, noncoding RNAs: the A/B transcript (also called 1A) (Liu, Yu et al. 2000), and the antisence (AS) transcript (called Nespas in mice) (Wroe, Kelsey et al. 2000).

The canonical Gnas consists of 13 exons in human and 12 exons in mouse and it encodes the α subunit of the $G\alpha_s$. $G\alpha_s$ is an essential intermediate in growth, differentiation and homeostatic pathways and is biallelically expressed in most tissues (Hayward, Moran et al. 1998) (see Figure 1.2).

$G\alpha_s$ exist as two splice variants, GαsL (394 aa) and GαsS (379 aa) (Seifert, Wenzel-Seifert et al. 1998; Abramowitz, Grenet et al. 2004) depending on the inclusion or not of the 15 amino acids exon 3. The 15 amino acid insert in the GsαL is localized between the Ras-like domain and the α-helical domain (see Figure 1.1), a position in which the guanine nucleotide-binding site is embedded. In fact it has been show that GαsL releases GDP twice as fast as GαsS. This structural difference, between the two $G\alpha_s$ splice variants (GαsL and GαsS), has important consequences for the functional properties of a GPCR. For example β2-adrenoreceptor fused to GαsL exhibits higher apparent constitutive (agonist-independent) activity than the β2AR fused to GαsS (Seifert, Wenzel-Seifert et al. 1998; Seifert 2001; Wenzel-Seifert, Kelley et al. 2001).

The distribution of long (GαsL) and short (GαsS) variants of the α subunit of the stimulatory G protein ($G\alpha_s$) change under a wide range of metabolic conditions, such as cellular differentiation, ontogenetic development and ageing (Novotny and Svoboda 1998). These distributional differences suggest that these alternative $G\alpha_s$ isoforms have distinct roles in signaling (Jones, Masters et al. 1990).

GαsL and GαsS proteins have been reported to migrate in polyacrylamide gels with apparent values of 52 kDa and 45 kDa by some authors (Jones and Reed 1987), or 45 kDa and 42 kDa by others (Milligan, Mitchell et al. 1990; McFarlane-Anderson, Bailly et al. 1992), depending on the materials and experimental conditions used (Novotny and Svoboda 1998).

Another neural-specific splice variant is the GsαN1. Exon N1 is an alternative terminal exon between exons 3 and 4 (see Figure 1.2). Splicing to exon N1 leads to the generation of a truncated transcript (GαsN1) which does not encode full-length $G\alpha_s$ (Liu, Yu et al. 2000), there importance, if any, is unknown (Plagge, Gordon et al. 2004).

A large variant of $G\alpha_s$, termed XLαs (for extra large), is derived from GNAS through the use of an alternative promoter and first exon that splices onto the common exon 2. XLas is expressed only from the paternal allele (Hayward, Moran et al. 1998; Hayward and Bonthron 2000) and have a molecular mass of 78 kDa (Klemke, Pasolli et al. 2000; Pasolli, Klemke et al. 2000). The C-terminal domain of XLαs is identical to $G\alpha_s$ and is encoded by exons 2-13 (41 kDa) of the $G\alpha_s$ gene, and, hence, contains most of the functional domains of $G\alpha_s$ including receptor and effector binding sites (Kehlenbach, Matthey et al. 1994). The long aminoterminal extension (37 kDa) is encoded by XLαs exon of the $G\alpha_s$ gene. While RT-PCR experiments suggest that XLαs is widely distributed, Northern analysis, immunoblotting, and *in situ* hybridization experiments demonstrate that XLαs expression is limited to neural and endocrine tissue (Kehlenbach, Matthey et al. 1994; Pasolli, Klemke et al. 2000; Pasolli and Huttner 2001). XLas shares many, but not all, functional properties of $G\alpha_s$. It forms a heterotrimer with βγ subunits; it binds GTP and undergoes a conformational change upon GTP binding; it activates, when in the GTP state, adenylyl cyclase (Klemke, Pasolli et al. 2000; Bastepe, Gunes et al. 2002) and mediate receptor-stimulated cAMP production (Klemke, Pasolli et al. 2000; Bastepe, Gunes et al. 2002). However, several different methods failed to demonstrate efficient coupling of XLαs to different G protein coupled receptors (Klemke, Pasolli et al. 2000). Recent findings indicate that XLαs, at least in vitro, is capable of functionally coupling to receptors that normally act via $G\alpha_s$ (Bastepe, Gunes et al. 2002). Thus, the biological role(s) of XLαs within the cell remains obscure. Some findings show that XLαs (or possibly XLN1) appears to play a primary role as a negative regulator of sympathetic nervous system activity or that the alternative truncated Gnasxl product XLN1 is a dominant-negative inhibitor of $G\alpha_s$ signaling. (Xie, Plagge et al. 2006). In addition XLαs and $G\alpha_s$ exert antagonistic functions (Plagge, Gordon et al. 2004).

The XLαs promoter generates also at least three additional transcripts. The neural-specific truncated form of XLαs (XLN1) produced by splicing to exon N1 (Pasolli, Klemke et al. 2000). It remains unknown whether the resultant truncated protein has any biological function or it can act as a dominant negative inhibitor of $G\alpha_s$ or XLαs signaling (Xie, Plagge et al. 2006).

In addition two small exons of 91 and 67 bp in length (referred to as A20 and A21) downstream of the XLαs exon, express two extra truncated forms of XLαs: XLN1a and XLN1b (Weinstein, Yu et al. 2001). While most XLαs transcripts do not contain exons A20 and A21, a small proportion of XLαs transcripts have A20 alone (Pasolli, Klemke et al. 2000) or both A20 and A21 (Hayward and Bonthron 2000) (see Figure 1.2). A role for these transcripts, if any, remains to be determined (Weinstein, Yu et al. 2001).

Another GNAS protein product is the neuroendocrine secretory protein 55 (Nesp55), a chromogranin-like polypeptide (Hayward, Moran et al. 1998). Nesp55 is expressed specifically in endocrine cells and the nervous system, from the maternal GNAS allele (Hayward, Moran et al. 1998; Bastepe, Frohlich et al. 2005). Nesp55 has a predicted size of approximately 27-29 kDa, which due to addition of keratin sulfate glycosaminoglycan chains, appears on immunoblots with a molecular mass of 55 kDa. The entire coding sequence for NESP55 is contained within the upstream exon, and therefore $G\alpha_s$ exons 2-13 are within the 3'-untranslated region of the Nesp55 transcripts (Hayward, Moran et al. 1998). Nesp imprinting is established later in development, either by the action of paternal antisense Nespas transcripts or other mechanisms (Liu, Chen et al. 2005). The function of Nesp55 is not well understood, but it is regarded as a marker for the constitutive secretory pathway (Chen, Perrin et al. 2005).

In addition, there are paternally expressed, non-coding antisense transcripts, Nespas (Wroe, Kelsey et al. 2000), that start approximately 2 kb upstream of the Gnasxl initiation site (Abramowitz, Grenet et al. 2004) and overlap the *Nesp* protein coding exon. *Nespas* function as a switch to enhance *Gnasxl* expression and to repress *Gnas* expression on the paternal allele (Williamson, Turner et al. 2006).

Further analysis of the locus has revealed the presence of additional transcripts, which lack coding potential. An alternative first exon (variously called exon 1A or exon A/B) had been identified upstream of exon 1. This exon generates ubiquitously

expressed transcripts, expressed only from the paternal allele, that are presumed to be untranslated (Liu, Yu et al. 2000; Weinstein, Chen et al. 2002).

It is possible that the transcript level of exon 1A is important in modulating that of *Gnas*. Recent research shows that the 1 A region can suppress $G\alpha_s$ expression from the paternal allele in a tissue-specific manner (Liu, Chen et al. 2005).

Additional complexity arises because the XLαs-coding transcript contains a second overlapping and conserved ORF (open reading frame), which encodes a completely unrelated and paternally expressed protein termed Alex (Freson, Jaeken et al. 2003; Plagge, Gordon et al. 2004). Alex has the ability to interact with XLαs, and inhibits its adenylyl cyclase-stimulating function (Freson, Jaeken et al. 2003; Plagge, Gordon et al. 2004).

1.4 G protein pathways

G protein coupled receptors produce their physiological effects, as the name indicates, via interaction with G proteins (Heterotrimeric and small G proteins). The complexity of GPCR signaling raise through the G protein-dependent and G protein-independent signaling (see chapter 1.2.5) but also through cross-talk between G protein coupled receptors and their intracellular second messenger pathways. Cross-talk between different pathways may occur at the level of receptors, G proteins, effectors or second messengers and may serve to fine-tune cell signaling.

G proteins are so-named because of their ability to bind the guanine nucleotides, guanosine triphosphate (GTP) and guanosine diphosphate (GDP). There are two families of G proteins.

The large family of 'small' G proteins or small GTPases, which lack βγ subunits, includes the Ras family (signal transduction), the Rho/Rac family (cytoskeleton), the Rab and Sar1/Arf families (vescicle trafficking), and the Ran family (nuclear import/export) (Takai, Kaibuchi et al. 1994).

The second G protein family is the membrane-associated heterotrimeric G proteins or 'large' G proteins. These proteins are activated by G protein coupled receptors and are made up of alpha (α), beta (β) and gamma (γ) subunits.

1.4.1 Second messenger signaling

The heterotrimeric G protein activation produces cellular responses through a variety of second messenger cascades (second messenger pathways). Four major types of G proteins, $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12}$, are involved in transduction of signals produced by neurotransmitter binding, and for each one of them exists multiple subtypes.
In G-protein-mediated pathways (Gilman 1987), the hormone, neurotransmitter or drugs (first messenger) binding induces conformational change of the receptor, which induces dissociation of trimeric G protein-complex (non-active) into the free (active) Gα and Gβγ subunits (Bourne, Sanders et al. 1991). Both Gα and Gβγ activate directly (Matsuda, Lee et al. 1992; Lu, Lee et al. 1999; Akam, Challiss et al. 2001; Dascal 2001) or indirectly a great number of enzyme activities (effectors) or ionic channels.

The βγ subunits released from the G-protein-receptor interaction can direct regulate several voltage-dependent Ca^{2+} channels and the G-protein-activated K^+ channels (GIRK). The best established example of this type of mechanism in brain is the coupling of many types of receptors, via subtypes of $G\alpha_o$ and $G\alpha_i$ in many types of neurons, to the activation of an inward rectifying K^+ channel and to the inhibition of a voltage-dependent Ca^{2+} channel, actions that hyperpolarize cells.
The activation of the Gα and Gβγ subunits leads to the activation or inhibition of a variety of second messengers such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), diacylglycerol (DAG), inositol triphosphate (IP3), arachidonic acid, in addition to changes in intracellular calcium levels as well as opening and closing of a variety of ion channels (Kozasa 2001; Marinissen and Gutkind 2001; Offermanns 2003). Which particular second messenger pathway is activated depends on the receptor G protein coupling and is receptor subtype specific. According to the type of the second messenger, there are five main second messenger pathways:

- cAMP-mediated signaling
- IP3/DAG-mediated signaling and
- Ca^{2+}-mediated signaling
- arachidonic acid-mediated signaling
- NO-cGMP-mediated signaling

1.4.1.1 cAMP-mediated signaling

Cyclic adenosine monophosphate (cAMP) is the first characterized second messenger (Walsh, Perkins et al. 1968; Sutherland 1972). In response to receptor activation, $G\alpha_s$ and $G\alpha_{i/o}$ proteins regulate the stimulation or inhibition of the enzyme adenylyl cyclase respectively, which then synthesise then the second messenger cAMP from cytosolic ATP (Reithmann, Gierschik et al. 1990). The main effector for cAMP remains the cyclic AMP-dependent protein kinase (PKA) but recent reports show that not all effects of cAMP are mediated by PKA (Dremier, Pohl et al. 1997). Other targets of cAMP are the cyclic-nucleotide-gated ion channels and the GTP-exchange protein EPAC which specifically activate the monomeric G protein Rap (de Rooij, Zwartkruis et al. 1998; Kawasaki, Springett et al. 1998). Rap links the $G\alpha_s$ signals to activation of mitogen-activated protein kinase (MAPK) signaling modules. PKA remains as the primary effector of cAMP. Increased level of cAMP activates the PKA which phosphorylates then specific serine or threonine residues on multiple target proteins and activate them. Some of the molecules regulated by PKA are: transcription factors, chromosomal proteins, receptors, ion channels and enzymes.

1.4.1.2 Other second messenger signaling pathways

- **IP3/DAG-mediated signaling**

The activation of phospholipase C (PLC) is mediated predominantly by $G\alpha_q$ proteins in response to receptor activation, although recent reports have show that $G\alpha_i$ and $G\alpha_o$ may also be involved in some cell types (Murthy, Zhou et al. 2004). PLC hydrolyses a lipid phosphatidylinositol-4,5-bisphosphate (PIP2) in the plasma membrane, producing inositol trisphosphate (IP3) and diacylglycerol (DAG) (see figure 1.3). IP3 and DAG are the second messengers for many growth factors, hormones, and neurotransmitters and they can also act directly on ion channels (Perraud, Fleig et al. 2001; Tesfai, Brereton et al. 2001).

- **Ca^{2+}-mediated signaling**

Calcium is involved in the regulation of almost all kinds of pathways. Activation of neurotransmitter receptors can alter the flux of extracellular Ca^{2+} into neurons or can regulate release of Ca^{2+} from intracellular stores.

- **Arachidonic acid-mediated signaling**

Arachidonic acid (AA) is involved in synaptic transmission, induction of a variety of signal transduction pathways and stimulation of neuronal damage (Katsuki and Okuda 1995). There are at least three phospholipases which generate free arachidonic acid (AA), through calcium or G-protein-dependent mechanisms (Burch 1989): PLA_2, PLC, and PLD. Recent studies have shown that all of them may be activated by neurotransmitters (Balsinde, Winstead et al. 2002).

- **NO/cGMP-mediated signaling**

The NO/cGMP signaling cascade is of importance in the nervous and cardiovascular systems, where it controls modulation of synaptic transmission, and smooth muscle relaxation. The gas nitric oxide (NO) acts as neurotransmitter and has also a neuromodulatory role (Garthwaite and Boulton 1995). It acts mainly through the activation of a soluble guanylate cyclase enzyme, which when activated; produce the second messenger cyclic guanosine monophosphate (cGMP). Studies have shown that NO can also affect directly ion channel activity (Bolotina, Najibi et al. 1994).

1.4.2 Non second messenger signaling cascades/ MAPK

There are at least three distinct MAP kinase signal transduction pathways in mammalian cells, each named after the particular MAPK associated with it: ERK, JNK, and p38. MAPK (Mitogen-Activated Protein Kinase) signaling pathways modulate many cellular events including: regulation of embryonic development, cell movement and apoptosis, as well as cell and neuronal differentiation.

1.4.3 Cross-talk between GPCR pathways

The term cross-talks refers to the phenomenon that signal components can be shared between different signal pathways and activate multiple responses in the cell. It is known that signaling pathways do not operate in isolation but may regulate and be regulated by one another and so form high complex networks.

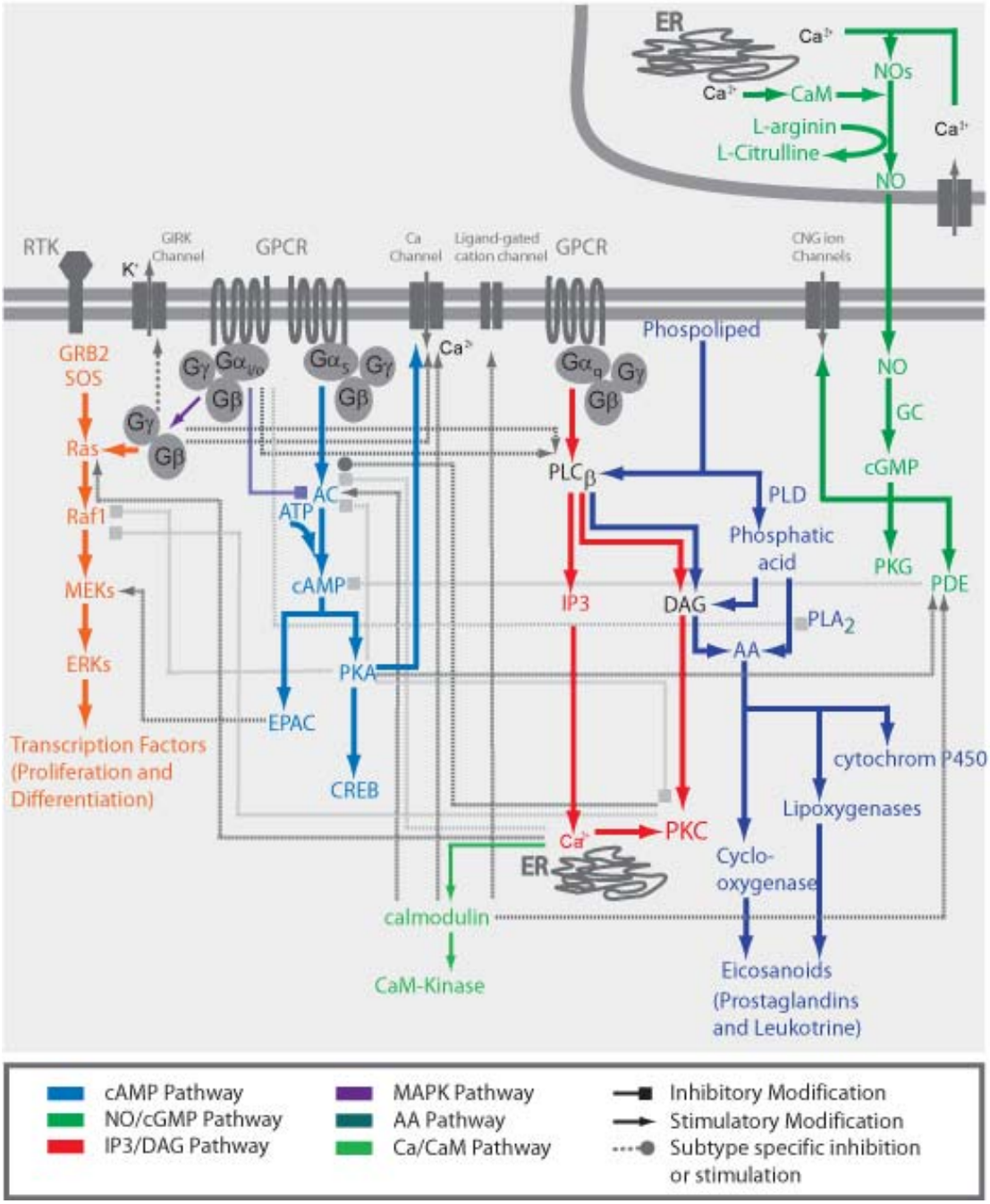


Figure 1.3. G protein-mediated signaling system. This schematic diagram shows the basic pathways and their interactions. This graph is only a first-level representation without many details. The cAMP (blue), IP3/DAG (red), Ca/CaM (green), Arachidonic Acid (dark blue), NO/cGMP (dark green), MAPK (orange) pathways are shown together with their interconnections (dark gray for stimulation and gray for inhibition). The possible routes are explained in the text. AC, adenylyl cyclase; PLC, phospholipase C; cAMP, cyclic adenosine monophosphate; IP3, inositol-1,4,5 tris-phoshate; DAG, diacylglycerol; PKC, C kinase; PKA, protein kinase A; ER, endoplasmatic reticulum; cGMP, cyclic guanosine monophosphate; NO, nitric oxide; CaM, calmodulin; PDE, phosphodiesterase; MAPK, Mitogen-Activated Protein Kinase.

For example cAMP can act through PKA to produce short-term effects on channel functions, and through Rap and MAPK to regulate gene expression and produce long-term effects (Bos, de Rooij et al. 2001); or the βγ from the heterotrimeric Gi proteins couples indirectly to the small GTPase Ras and activates the MAPK pathway and through direct interaction regulates the PLCβ (IP3/DAG Pathway), K^+ channels, adenylyl cyclase (cAMP pathway) and phosphatidylinositol 3-kinase (Neves, Ram et al. 2002). Figure 1.3 shows the basic pathways and their interactions. Although the map appears quite complex, this is only a first-level representation, where multiple isoforms of the proteins and many more details are not shown.

There are many additional factors which influence the type of the interconnection. Thus, investigations have shown that the subtype of the proteins (e.g. PKC, AC, PLC, Gβ, Gγ) which take part in the pathways play also an important role. There are about 10 protein kinase C (PKC), 13 Phospholipase C (PLC) and nine known adenylate cyclase (AC) isoforms and each of them could lead to a different path (Watts and Neve 2005). For example, in the case of AC; CaM can activate AC1 and AC8; PKC activates AC2 and inhibits AC4 (Nasman, Kukkonen et al. 2002; Sunahara and Taussig 2002). Other factors that influence the cross-talks are the type of the ligand, the type of the tissue, the age, the cell type and many more. In addition there are also differences based on which model system was used for the pathway research. For example in transfected COS and HeLa cell lines the 5HT 1A receptor interacts 100 times more potently through the $G\alpha_i$ pathway (AC inhibition) than through the $G\alpha_q$ (PLC regulation). In other cell lines, the 5HT 1A receptor interacts equal through the $G\alpha_i$- and $G\alpha_q$- pathways and in intact cells interact also through the arachidonic acid pathway (Raymond, Mukhin et al. 1999).

Another example is that the hippocampal corticotropin-releasing factor (CRF) receptors from BALB/c mice interact with $G\alpha_{q/11}$, but in C57BL/6N mice the CRF receptors are also coupled to $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_i$ (Blank, Nijholt et al. 2003).

Thus, the individual signaling mechanisms which are most frequently studied in isolation can show all kind of possible connections, but only the in vivo interactions from certain region and organisms, could give a canonical connection map.

1.5 GABA and its receptor system

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate central nervous system, first described in the mammalian brain over 50 years ago (Awapara, Landua et al. 1950; Roberts and Frankel 1950).
GABA regulates, in the immature brain, brain morphogenesis, such as changes in cell proliferation, cell migration, axonal growth, synapse formation, steroid-mediated sexual differentiation and cell death (Belhage, Hansen et al. 1998; Kardos 1999; Varju, Katarova et al. 2001; Ben-Ari 2002; Owens and Kriegstein 2002).
There are two major classes of GABA receptors: metabotropic $GABA_B$ receptors, which produce slow prolonged inhibitory signals and ionotropic $GABA_A$ (including $GABA_C$) receptors, which produce fast synaptic inhibition (Chebib and Johnston 1999; Bormann 2000).

The ionotropic γ-Aminobutyric acid type A ($GABA_A$) receptor is a chloride-selective ion channel. Activation of postsynaptic $GABA_A$ receptor leads to an influx of chloride ions into the postsynaptic cytoplasm and the resulting hyperpolarization of the postsynaptic membrane inhibits the postsynaptic neuron. In contrast to the inhibitory action of GABA on mature neurons, $GABA_A$ receptors exert in immature neurons excitatory actions (Ganguly, Schinder et al. 2001; Hubner, Stein et al. 2001). $GABA_A$ receptors are composed of five subunits in a heteropentameric manner. The most common formation contains two α, one or two β, and one or two γ subunits, with potential substitution of ε or δ for the γ subunit (Benke, Fritschy et al. 1994; Baumann, Baur et al. 2001; Klausberger, Sarto et al. 2001). There are at least 16 different $GABA_AR$ subunits grouped together based on their sequence homology, α (1-6), β (1-3), γ (1-3), δ, ε, θ, and π (Mehta and Ticku 1999) with a protein size of approximately 55 kDa. Specific $GABA_AR$ subunit composition has been associated with certain phenotypes such as anxiety, aggression and learning (Delaney and Sah 1999; Rudolph, Crestani et al. 1999; Collinson, Kuenzi et al. 2002).
$GABA_A$ receptors are targets of a variety of pharmacologically and clinically important drugs, such as benzodiazepines, barbiturates (Ito, Suzuki et al. 1996), ethanol (Celentano, Gibbs et al. 1988), neurosteroids (Harrison and Simmonds 1984), insecticides, and some general anesthetics (Sieghart 2000; Fritschy and Brunig 2003). The pharmacological and biophysical properties of the $GABA_A$ receptors are

primarily determined by their subunit composition (Hevers and Luddens 1998; Mohler, Benke et al. 2001; Luscher and Keller 2004; Rudolph and Mohler 2004). Thus, slight variations in $GABA_A$ receptor subunits composition could contribute to differential behaviour.

The recent identified type of the GABA receptors is the $GABA_C$. Although $GABA_A$ and $GABA_C$ receptors are both ionotropic receptors and linked to chloride channels, the $GABA_C$ receptors differ from the $GABA_A$ and $GABA_B$ receptors in their pharmacological and biophysical properties.
They are insensitive to $GABA_A$-antagonist bicuculline and $GABA_B$ agonists and antagonist (baclofen and saclofen) (Drew, Johnston et al. 1984), as well as to known $GABA_A$ modulators like benzodiazepines, barbiturates, and neurosteroids (Bormann and Feigenspan 1995). They lack prominent desensitisation, and they activate and deactivate more slowly than $GABA_A$ receptors.
$GABA_C$ receptors are pentamers (Amin and Weiss 1996), composed of GABA ρ subunits (Qian et al., 1997a; Enz et al., 1995, 1996) and they are expressed in many brain regions, with prominent distributions on retinal neurons (Sivilotti and Nistri 1991; Boue-Grabot, Roudbaraki et al. 1998; Wegelius, Pasternack et al. 1998; Enz and Cutting 1999).

1.6 The $GABA_B$ receptors

The metabotropic $GABA_B$ receptors were first pharmacologically distinguished by Hill and Bowery in 1981 as a bicuculline-insensitive, baclofen (β-chlorophenyl GABA)-sensitive GABA receptor widely expressed in the mammalian central nervous system (Hill and Bowery 1981). However the $GABA_B$ receptor was cloned many years later by Bettler and colleagues (Kaupmann, Huggel et al. 1997), a decade after the $GABA_A$ receptor (Schofield, Darlison et al. 1987). Since then many agonist and antagonist have been developed for the $GABA_B$. Phaclofen, saclofen and 2-hydroxysaclofen were the first described antagonists (Kerr, Ong et al. 1987; Kerr, Ong et al. 1988), followed by a big variety of antagonists CGP46381, CGP54626, CGP 52432, CGP35348, CGP52432 (Froestl, Bettler et al. 1999).

$GABA_B$ exists as heterodimer with the subunits $GABA_BR1$ and $GABA_BR2$ (Jones, Borowsky et al. 1998; Kaupmann, Malitschek et al. 1998; White, Wise et al. 1998; Martin, Russek et al. 1999) which are formed due to alternative splicing (Kuner, Kohr et al. 1999). Both subunits consist of seven transmembrane spanning proteins.

Heterodimer $GABA_B$ receptors are present at both presynaptic and postsynaptic sites (Chebib and Johnston 1999; Couve, Moss et al. 2000) where they mediate slow synaptic inhibition in the central nervous system. They couple to heterotrimeric G proteins ($G\alpha_i$ and $G\alpha_o$) (Asano, Ui et al. 1985; Hill 1985; Morishita, Kato et al. 1990; Knott, Maguire et al. 1993) and activate second messenger pathways and cause inwardly rectifying K^+ channels to open and voltage-dependent Ca^{2+} channels to close (Bettler, Kaupmann et al. 1998; Bowery, Bettler et al. 2002; Calver, Davies et al. 2002; Bettler, Kaupmann et al. 2004). This receptors function as heterodimers. They are composed of a $GABA_{B1}$ and a $GABA_{B2}$ subunits, which are both required for normal receptor functioning (Bettler, Kaupmann et al. 2004).

$GABA_B$ receptors have been implicated in synaptic inhibition, hippocampal long-term potentiation, short-wave sleep, muscle relaxation, and antinociception (Bettler, Kaupmann et al. 1998; Kaupmann, Malitschek et al. 1998; Bowery and Enna 2000; Cryan and Kaupmann 2005).

1.6.1 Molecular structure of $GABA_B$ receptors

$GABA_B$ receptors belong to the class C subfamily of G protein coupled receptors (Galvez and Pin 2003) together with the vomeronasal (Ryba and Tirindelli 1997), metabotropic glutamate (mGluRs) (Masu, Tanabe et al. 1991), extracellular calcium-sensing (CaSR) (Brown, Gamba et al. 1993), and putative taste receptors (Hoon, Adler et al. 1999).

All the members of this subfamily share low sequence similarity and they are composed of seven transmembrane domains with the intracellular loops being responsible for coupling to heterotrimeric G proteins and they are constitutive dimmers.

The distinctive characteristic of this group is the unusually large extracellular domain (ECD), also known as the “venus flytrap domain”, that is nearly equal in size to the remaining portion of the protein and it dictates the ligand binding specificity (Galvez,

Duthey et al. 2001). In contrast to the other class C GPCRs, the $GABA_B$ receptor is the only one which is a heterodimer composed of two distinct subunits (Marshall, Jones et al. 1999; Mohler and Fritschy 1999; Ng, Clark et al. 1999; Margeta-Mitrovic, Jan et al. 2000; Dean, Higgs et al. 2001; Bowery, Bettler et al. 2002; Calver, Davies et al. 2002; Bettler, Kaupmann et al. 2004; Couve, Restituito et al. 2004) and where the coexpression of both subunits is required for a fully functional $GABA_B$ receptor (Jones, Borowsky et al. 1998; Kaupmann, Schuler et al. 1998; White, Wise et al. 1998; Kuner, Kohr et al. 1999).

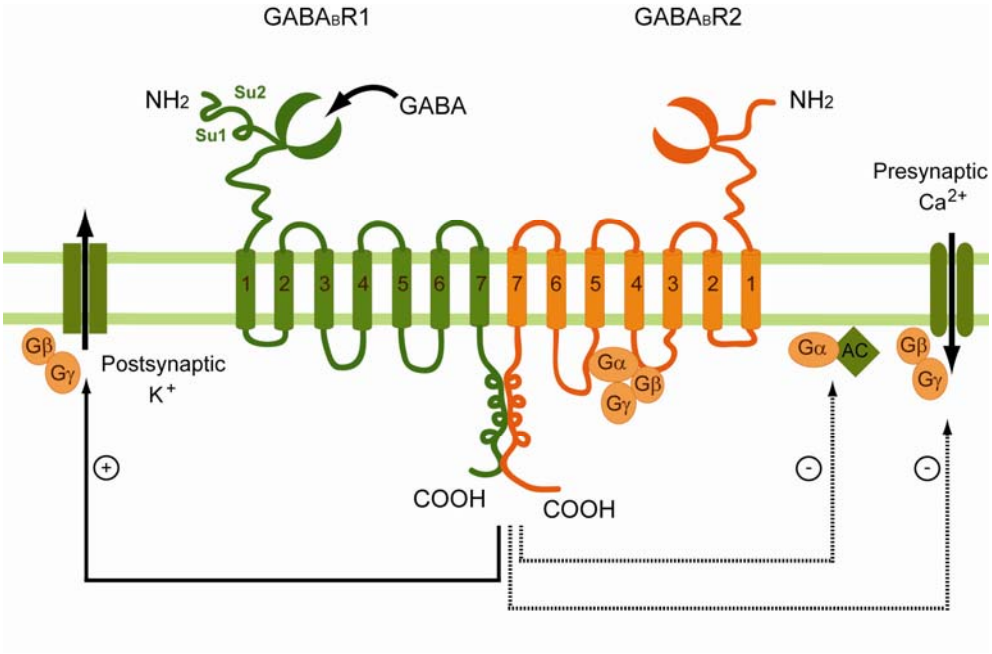


Figure 1.4. Schematic representation of $GABA_B$ receptor system indicating the dimeric nature of the structure. The $GABA_{B1a}$ subunit contains two Sushi repeats (Su1 and Su2) and interact via its C-terminal coiled coil domain with the coiled coil domain of the $GABA_{B2}$ subunit. The N-terminal domain of the $GABA_{B1}$ subunit form two lobes which are able to trap the agonist (Venus Flytrap) (Galvez, Parmentier et al. 1999). Activation of the βγ subunits leads to activation of postsynaptic K^+ currents, mediated by inwardly rectifying potassium channels, and to inhibition of presynaptic Ca^{2+} currents mediated by inactivation of voltage gated calcium channels. Activation of $G\alpha_{i/o}$ subunit results in a negative coupling to adenylate cyclase. $GABA_BR1$, the isoforms $GABA_BR1a$; $GABA_BR2$; α, β, and γ, $G_{i/o}$ protein subunits; AC, adenylate cyclase. Modified from Bettler et al., 1998.

Although tested $GABA_BR1a$ and $GABA_BR1b$ were found to be associated with $GABA_BR2$ and the majority of the $GABA_B$ receptors appear to be heteromers (Benke, Honer et al. 1999; Franek, Pagano et al. 1999).
However, there is evidence that $GABA_BR1a$ and $GABA_BR1b$ alone are able to couple to K^+ channels or adenylyl cyclase, although at low efficiency (Kaupmann, Huggel et al. 1997; Kaupmann, Schuler et al. 1998) and that $GABA_BR2$ expressed in HEK 293 cells can inhibit forskolin-stimulated cAMP production in the presence of GABA (Kuner, Kohr et al. 1999; Urwyler, Mosbacher et al. 2001). Thus, $GABA_B$ receptors with monomeric or diverse, yet unknown, heteromeric structures may occur *in vivo* (Benke, Honer et al. 1999). CGP7930 is described as a positive allosteric regulator of the $GABA_B$ receptor.

Both subunits, $GABA_BR1$ (Kaupmann, Huggel et al. 1997) and $GABA_BR2$ (Jones, Borowsky et al. 1998; Kaupmann, Malitschek et al. 1998; White, Wise et al. 1998; Kuner, Kohr et al. 1999; Martin, Russek et al. 1999; Ng, Clark et al. 1999) of the γ-Aminobutyric acid type B ($GABA_B$) receptor consist of three main regions: an extracellular domain (ECD, N-terminal extracellular domain); a heptahelical domain composed of seven transmembrane α-helices (TMD) and loops and the intracellular C-terminal domain (ICD, C-terminal domain) (Kaupmann, Huggel et al. 1997). The $GABA_{B2}$ protein has 54 % similarity and 35 % homology to $GABA_{B1}$ (Jones, Borowsky et al. 1998; White, Wise et al. 1998; Kuner, Kohr et al. 1999; Martin, Russek et al. 1999; Ng, Clark et al. 1999).
Whereas $GABA_BR1$ contain the ligand binding site, $GABA_BR2$ contains all the molecular determinants for G protein coupling. $GABA_BR2$ is also necessary for the $GABA_BR1$ transportation to the plasma membrane (White, Wise et al. 1998). Through the interaction between $GABA_BR1$ and $GABA_BR2$ at the C-terminal coiled-coil domain $GABA_BR2$ masks the endoplasmic reticulum (ER) retention signal of the $GABA_BR1$ intracellular tail (Margeta-Mitrovic, Jan et al. 2000; Pagano, Rovelli et al. 2001; Grunewald, Schupp et al. 2002; Bettler, Kaupmann et al. 2004; Couve, Restituito et al. 2004) and helps the $GABA_BR1$ subunit to reach the surface associated with the $GABA_BR2$ and be functional (Margeta-Mitrovic, Jan et al. 2000; Calver, Robbins et al. 2001; Pagano, Rovelli et al. 2001).

Different studies have confirmed the function of the three main regions of the $GABA_{B1}$ and $GABA_{B2}$ subunits.

The $GABA_{B1}$-ECD (extracellular domain) is the only determinant for ligand binding (Malitschek et al., 1999), while the ECD of $GABA_{B2}$ does not bind to any known ligands (Kniazeff, Galvez et al. 2002) but it is required for full functionality of the native receptor. The closure of the $GABA_{B1}$-ECD lobes after ligand binding is sufficient to keep the receptor in its active state (Kunishima, Shimada et al. 2000; Kniazeff, Saintot et al. 2004). It has been demonstrated that the ECD of $GABA_{B2}$ acts allosterically to increase agonist affinity for $GABA_{B1}$ by stabilizing the active state of the receptor (Liu, Maurel et al. 2004) and prevents an inhibitory interaction between the ECD and TMD of $GABA_{B1}$ (Galvez, Duthey et al. 2001; Duthey, Caudron et al. 2002; Liu, Maurel et al. 2004; Uezono, Kanaide et al. 2005).

The $GABA_B$-TMDs (transmembrane domains) are essential for maximum efficiency of G protein coupling but only the $GABA_{B2}$-TMD is absolutely required (Galvez, Duthey et al. 2001; Margeta-Mitrovic, Jan et al. 2001). Specifically, the $GABA_{B1}$ intracellular loops are not necessary for specific G protein coupling of $GABA_B$ receptors but among the four $GABA_{B2}$ intracellular segments, all three loops were essential for GIRK channel coupling (Calver, Robbins et al. 2001; Margeta-Mitrovic, Jan et al. 2001; Margeta-Mitrovic, Jan et al. 2001; Robbins, Calver et al. 2001; Duthey, Caudron et al. 2002; Havlickova, Prezeau et al. 2002). $GABA_{B1}$ intracellular loops might be involved in signaling pathways not involving G proteins or in the regulation of subcellular $GABA_B$ receptor localization, for example through binding with the transcription factors CREB2 and ATFx or other proteins.

The C-terminus domains of $GABA_{B1}$ and $GABA_{B2}$ subunits are important but not a requirement for proper function (Calver, Robbins et al. 2001; Pagano, Rovelli et al. 2001; Grunewald, Schupp et al. 2002), although there may be differences between Gα and Gβγ activated signaling cascades (Margeta-Mitrovic, Jan et al. 2001).

1.6.2 $GABA_B$ receptor subtypes and their function

Many splice variants of the $GABA_BR1$ and $GABA_BR2$ have been described (Kaupmann, Huggel et al. 1997; Jones, Borowsky et al. 1998; Kaupmann, Malitschek et al. 1998; White, Wise et al. 1998; Pfaff, Malitschek et al. 1999) and various studies predicted pharmacologically distinct $GABA_B$ receptor subtypes (Enna 2001).

Initially, two major isoforms of $GABA_BR1$, $GABA_BR1a$ and $GABA_BR1b$, have been identified. Since then further splice variants have been observed: the $GABA_BR1c$ (Isomoto, Kaibara et al. 1998; Pfaff, Malitschek et al. 1999), $GABA_BR1d$ (Poorkhalkali, Juneblad et al. 2000), $GABA_BR1e$ (Schwarz, Barry et al. 2000), $GABA_BR1f$ (Wei, Jia et al. 2001), and $GABA_BR1g$ (Wei, Jia et al. 2001). Beside variants 1a and 1b, for the rest $GABA_B$ (c-g) splice variants could not be demonstrated the existence of a protein *in vivo* or they do not occur across different species (Bowery, Bettler et al. 2002; Bettler, Kaupmann et al. 2004). For example $GABA_BR1c$ appears to act as a functional subunit but is expressed only in humans. Splice variants have also been described for $GABA_BR2$. The two transcripts $GABA_{B2b}$ and $GABA_{B2c}$ have been proposed to arise from alternative splicing of the $GABA_BR2$ message (Calver, Medhurst et al. 2000; Clark, Mezey et al. 2000). Although no difference in expression or function has been detected for the reported $GABA_BR2b$ or $GABA_BR2c$ variants (Martin, Russek et al. 2001). $GABA_{B2b}$ and $GABA_{B2c}$ transcripts are likely to represent artefacts arising during cDNA synthesis and/or PCR amplification. Therefore, at the present time, there are no confirmed $GABA_{B2}$ splice variants (Bettler, Kaupmann et al. 2004; Steiger, Bandyopadhyay et al. 2004).

The predominant $GABA_BR1$ receptor subtypes are $GABA_BR1a$ and $GABA_BR1b$, both of which combine with the $GABA_{B2}$ subunit to form heterodimers at mainly different subcellular locations (Benke, Honer et al. 1999; Steiger, Bandyopadhyay et al. 2004). $GABA_BR1a$ and $GABA_BR1b$ are expressed in the brain by the presence of alternative transcription sites in the $GABA_{B1}$ gene and differential promoter usage (Kaupmann, Huggel et al. 1997; Steiger, Bandyopadhyay et al. 2004).

The human $GABA_BR1$ gene on chromosome 6p21.3 (Grifa, Totaro et al. 1998) is in the genetic locus for neurobehavioral disorders that include schizophrenia, juvenile myoclonic epilepsy and dyslexia (Cryan and Kaupmann 2005). The human $GABA_BR2$ gene on chromosome 9q22.1 is a candidate gene for hereditary sensory neuropathy type 1 (Martin, Russek et al. 1999; Ng, Clark et al. 1999; Martin, Russek et al. 2001).

Information about the molecular structure of the $GABA_B$ receptor is only available from human and rat. $GABA_{B1a}$ is a protein of 960 amino acids (120 kDa) and $GABA_{B1b}$ is a protein of 844 amino acids (100 kDa) (Kaupmann, Huggel et al. 1997).

They share the C-terminal amino acids and they differ solely in their N terminus, where the first 147 residues of $GABA_BR1a$ are replaced by a sequence of 18 different amino acids in $GABA_BR1b$ (Kaupmann, Huggel et al. 1997; Martin, Russek et al. 2001). The N-terminal sequence of the $GABA_BR1a$ protein contains two sushi domains (Hawrot, Xiao et al. 1998). These domains are involved in protein-protein interactions used also for cell-cell adhesion (Bettler, Kaupmann et al. 2004). It is suggested that the $GABA_{B1a}$ sushi-repeats interact with the extracellular matrix protein fibulin (Blein, Ginham et al. 2004). The pharmacological profile of the $GABA_{B1a}$ and $GABA_{B1b}$ splice variants is similar (Kaupmann, Huggel et al. 1997). To date no convincing pharmacological differences have been described to any of the functional $GABA_B$ splice variants. Although studies by Ng and colleagues reported that gabapentin acts as an agonist to $GABA_BR1a$ but not to $GABA_BR1b$ (Ng, Bertrand et al. 2001), additional experiments in brain slice preparations (Lanneau, Green et al. 2001) or *in vivo* could not replicate this findings (Jensen, Mosbacher et al. 2002).

Although the pharmacological profile of the two isoforms seems to be similar, the subcellular distribution and their physiological behaviour is different. Studies with rat and human cerebellum and spinal cord show that $GABA_BR1a$ isoform is localized preferentially at presynaptic sites and the $GABA_BR1b$ appears to be preferentially located postsynaptically (Kaupmann, Schuler et al. 1998; Billinton, Upton et al. 1999; Bischoff, Leonhard et al. 1999; Ritter, Zschuntsch et al. 2004). In different areas of the brain however, $GABA_{B1b}$ protein is located at presynaptic terminals and the $GABA_{B1a}$ at the postsynaptic sites (Benke et al., 1999; Princivalle et al., 2001).

In addition recent studies show that the two $GABA_{B1}$ isoforms have also distinct functions in synaptic physiology and behaviour (Perez-Garci, Gassmann et al. 2006); the $GABA_{B1a}$ mainly assembles presynaptic heteroreceptors, while $GABA_{B1b}$ receptor mainly mediates postsynaptic inhibition (Huang 2006).

1.6.3 $GABA_B$ receptor-mediated signaling

Downstream effects of $GABA_B$ receptor activation are mediated by G proteins (Hill, Bowery et al. 1984; Asano, Ui et al. 1985) and predominantly through coupling to $G\alpha_i$ and $G\alpha_o$ types of G proteins (Asano and Ogasawara 1986; Morishita, Kato et al. 1990; Menon-Johansson, Berrow et al. 1993; Greif, Sodickson et al. 2000), although G

protein independent effects have been also described (Harrison 1990). Through heterotrimeric G proteins the presynaptic $GABA_BRs$ inhibit neurotransmitter release by down-regulating high-voltage activated Ca^{2+} channels (Mintz and Bean 1993; Kerr and Ong 1995), whereas postsynaptic $GABA_BRs$ activate inwardly rectifying K^+ channels (GIRK) (Andrade, Malenka et al. 1986; Misgeld, Bijak et al. 1995) and inhibit adenylyl cyclase (Nishikawa, Hirouchi et al. 1997).

A. Coupling to K^+ channels

Postsynaptic $GABA_B$ receptors activate inwardly rectifying K^+ channels (GIRK or Kir3), via the βγ subunits of the $G\alpha_{i/o}$ G proteins (Newberry and Nicoll 1984; Gahwiler and Brown 1985), which induce a slow inhibitory postsynaptic current (IPSC) (Luscher, Jan et al. 1997; Schuler, Luscher et al. 2001). This baclofen stimulated IPSC can be inhibited by the Kir3 channel blocker, barium (Jarolimek, Bijak et al. 1994). There is evidence that $GABA_B$ receptors could act also through voltage-gated K^+ channels (Blaxter, Carlen et al. 1986; Saint, Thomas et al. 1990) and Ca^{2+}-activated K^+ channels (SK channels) (Blaxter, Carlen et al. 1986; Gerber and Gahwiler 1994). In addition, $GABA_B$ receptors also activate presynaptic Ba^{2+}-sensitive K^+ channels (Thompson and Gahwiler 1992) which are composed of different subunits than the postsynaptic channels (Luscher, Jan et al. 1997).

B. Coupling to Ca^{2+} channels

The presynaptic $GABA_B$ receptors are subdivided in autoreceptors and heteroreceptors. The autoreceptors control the GABA release and the heteroreceptors inhibit all other neurotransmitter release (Calver, Davies et al. 2002; Bettler, Kaupmann et al. 2004), including glutamate (Yamada, Saitow et al. 1999; Hirono, Yoshioka et al. 2001), monoamines and many neuropeptides (Bowery, Hill et al. 1980; Bonanno and Raiteri 1993; Morton, Manuel et al. 2001).

Presynaptic $GABA_B$ receptors inhibit neurotransmitter release by blocking Ca^{2+} influx via high-voltage activated Ca^{2+} channels (Mintz and Bean 1993; Poncer, McKinney et al. 1997) through Gβγ subunits of a pertussis toxin-sensitive G proteins (Dascal 2001; Kajikawa, Saitoh et al. 2001; Zamponi 2001) or through a direct modulation of synaptic vesicle priming (Sakaba and Neher 2003). The presynaptic $GABA_B$ receptor

effect is associated with high-voltage activated Ca^{2+} channels (Wu and Saggau 1997) of N type (Cav2.2) and P/Q type (Cav2.1) (Menon-Johansson, Berrow et al. 1993; Mintz and Bean 1993; Amico, Marchetti et al. 1995; Herlitze, Garcia et al. 1996; Poncer, McKinney et al. 1997; Takahashi, Kajikawa et al. 1998; Ikeda and Dunlap 1999). Both types of Ca^{2+} channels are expressed in presynaptic terminals and were shown to trigger neurotransmitter release (Wu and Saggau 1997).

In addition, inhibition (Maguire, Maple et al. 1989; Amico, Marchetti et al. 1995) or facilitation (Shen and Slaughter 1999) of L-type Ca^{2+} channels by $GABA_B$ receptors are also described. This effect was shown to be indirect and to depend on protein kinase C (PKC) activity. $GABA_B$ receptors also inhibit or disinhibit T-type Ca^{2+} channels (Scott and Dolphin 1986; Crunelli and Leresche 1991; Matsushima, Tegner et al. 1993). A postsynaptic inhibition of Ca^{2+} channels of N- and P/Q-type by $GABA_B$ receptors (Harayama, Shibuya et al. 1998) and G protein-independent $GABA_B$ effects on neurotransmitter release (Harrison 1990) were also postulated.

C. Coupling to adenylyl cyclase

Besides modulation of ion channels through Gβγ, pre- and postsynaptic $GABA_B$ receptors activate and inhibit adenylyl cyclase (Hashimoto and Kuriyama 1997; Olianas and Onali 1999; Bowery, Bettler et al. 2002; Calver, Davies et al. 2002) via the $G\alpha_{i/o}$ and Gβγ subunits (Bowery 1993; Bettler, Kaupmann et al. 2004).

The inhibitory effect on adenylyl cyclase (AC) through activation of $GABA_B$ receptors (Wojcik and Neff 1984) is mediated by $G\alpha_i$ and $G\alpha_o$ proteins (Xu and Wojcik 1986; Morishita, Kato et al. 1990; Kaupmann, Huggel et al. 1997). The pertussis toxin-sensitive Gα proteins inhibit AC types I, III, V, and VI (Bettler, Kaupmann et al. 2004) and thus decrease the cAMP levels and the PKA activity, which results in the inhibition of GABA release.

Activation of the $GABA_B$ receptor can also stimulate adenylyl cyclase types II, IV, and VII, in response to $G\alpha_s$-coupled GPCRs activated by hormones (Kelly and Wagner 1999) and neurotransmitters, such as noradrenaline, vasoactive intestinal peptide, adenosine and prostaglandins (Karbon, Duman et al. 1984; Karbon and Enna 1985; Schaad, Schorderet et al. 1989; Simonds 1999; Bowery and Enna 2000).

Early studies in brain slices have suggested that facilitation of cyclic AMP formation occurs indirectly, as a consequence of phospholipase A2 stimulation by $GABA_B$

receptors and may be mediated by arachidonate or some other fatty acid (Duman, Karbon et al. 1986).

In addition, it is proposed that $GABA_B$ receptors enhance adenylyl cyclase II and IV activities by a mechanism involving βγ subunits of $G\alpha_{i/o}$. Adenylyl cyclases II and IV are two AC isoforms which can be stimulated by $G\alpha_{i/o}$ protein βγ subunits (Tang and Gilman 1992) and it is proposed that $GABA_B$ receptors, by interacting with $G\alpha_{i/o}$ proteins, may promote the release of a sufficiently high amount of βγ subunits to stimulate type II/IV adenylyl cyclase activities (Tang and Gilman 1992; Olianas and Onali 1999).

It is shown that in specific layers of rat olfactory bulb, a brain area expressing a high level of type II adenylyl cyclase (Feinstein et al., 1991), other $G\alpha_{i/o}$-coupled receptors, such as the acetylcholine muscarinic and opioid receptors, stimulate basal adenylyl cyclase activity and potentiate the responses of $G\alpha_s$-linked neurotransmitter receptors (Olianas and Onali 1993; Olianas and Onali 1999).

1.6.3.1 Modulation of $GABA_B$ receptor function

A number of studies have reported many factors which can lead to modulation of $GABA_B$ receptors such as cross-talks with other receptors or pathways and interaction with $GABA_BR$ associate proteins (Karbon, Duman et al. 1984; Karbon and Enna 1985; Duman, Karbon et al. 1986; Schaad, Schorderet et al. 1989; Olianas and Onali 1999; Simonds 1999; Bowery and Enna 2000; Kubota, Katsurabayashi et al. 2003; Balasubramanian, Teissere et al. 2004)

Cross-talks between PKA and PKC pathways which affect GABA release have also been reported. It has been suggested that the PKC cascade has no direct effects on GABA release, but it is involved in GABA release via cross-talk with the cAMP/PKA (Kubota, Katsurabayashi et al. 2003).

The complexity in the modulation of $GABA_B$ receptor function rise with a number of $GABA_B$ receptor associated proteins which produce diverse physiological and pharmacological effects. Different studies have demonstrated that both $GABA_{B1}$ and $GABA_{B2}$ subunits associate with other proteins.

One of this proteins is the transcription factor CREB2/ATF4, which interacts directly with the $GABA_BR1$, suggesting a novel G protein independent mechanism of signal

transduction (Nehring, Horikawa et al. 2000; White, McIllhinney et al. 2000; Vernon, Meyer et al. 2001). CREB, ATF4 and USF have been shown to regulate expression of the $GABA_{B1a}$ and $GABA_{B1b}$ receptors (Steiger, Bandyopadhyay et al. 2004). CREB stimulates expression of both $GABA_{B1a}$ and $GABA_{B1b}$, USF inhibits expression of $GABA_{B1b}$, and ATF4 which interact with $GABA_B$R1a and R1b (Ritter, Zschuntsch et al. 2004) and stimulates expression of $GABA_{B1a}$ but inhibits expression of $GABA_{B1b}$ (Steiger, Bandyopadhyay et al. 2004).

Further more, $GABA_B$R1 is associated with the 14-3-3 proteins, which compete with $GABA_{B2}$ for binding to $GABA_{B1}$ *in vivo*, something which may regulate $GABA_B$ receptor heterodimerization (Couve, Kittler et al. 2001). Marlin-1 was proposed to regulate the cellular levels of $GABA_{B2}$ and thereby to affect the number of functional $GABA_B$ receptors (Couve, Restituito et al. 2004).

The fact that protein fibulin-2 *in vitro* binds to the two sushi repeats of the $GABA_{B1a}$, but not to the $GABA_{B1b}$ which lacks the two sushi repeats, provides evidence for the existence of subtype-selective interacting proteins (Blein, Ginham et al. 2004). Another protein which interacts in a subtype specific manner is the transcription factor CHOP, also known as Gadd153, which interacts selectively with $GABA_{B1a}$/$GABA_{B2}$ receptors and results a reduced cell surface expression of the receptor (Sauter, Grampp et al. 2005). A physical interaction between $GABA_A$ and $GABA_B$ receptor subunits was also reported (Balasubramanian, Teissere et al. 2004). Association of $GABA_{B1}$ with the γ2S subunit of $GABA_A$ receptors robustly promotes cell surface expression of $GABA_{B1}$ in the absence of $GABA_{B2}$. The cross-talk between $GABA_A$ and $GABA_B$ subunits regulates $GABA_B$ receptor trafficking (Bettler and Tiao 2006).

Another protein that interact with $GABA_B$R2 receptors is MUPP-1 which may serve as an adaptor protein, linking $GABA_{B2}$ to various signaling molecules (Milligan and White 2001; Bettler, Kaupmann et al. 2004). NEM-sensitive factor ,or NSF, has been shown to interact with the C-terminal end of $GABA_{B2}$ (Bettler, Kaupmann et al. 2004) but also with $GABA_A$ receptors and possibly provides a structural link between the ionotropic and metabotropic GABA receptor systems. Tamalin is yet another scaffolding protein that interacts with $GABA_B$, as well as mGlu receptors (Kitano, Kimura et al. 2002).

1.7 Changes in the distribution of the GABAergic system

GABA and its receptors (ionotropic and metabotropic) show particular ontogenetic distribution in different brain areas. Specific GABA receptors subtypes are highly expressed in the embryonic and/or postnatal brain, whereas others are mainly present in the adult brain. These changes in the distribution and composition of the GABA receptor subunits are crucial for normal development of the brain areas (Lujan, Shigemoto et al. 2005). Extended investigations on the developmental changes of $GABA_A$ receptors demonstrate the influences of GABA receptor subunit alteration. In the adult CNS, GABA is a predominant inhibitory neurotransmitter, whereas in the immature brain serves as an excitatory transmitter (Ben-Ari 2002; Owens and Kriegstein 2002). This switch is associated with a differential expression of $GABA_A$ receptor subunits.

Although consequence and effects of this differential expression of subunits is not fully understood, it shows that the ontogenetic change in a subunit composition regulates the function of the receptor (Takayama and Inoue 2004; Lujan, Shigemoto et al. 2005). The expression of $GABA_B$Rs in the adult brain is described in many papers, but the functional significance of these receptor during postnatal development is largely unknown (Zhang, Elsen et al. 1999).

1.7.1 Distribution of $GABA_A$ receptor variants during brain development

Findings in animal models demonstrate that $GABA_A$R subunit expression varies across cell types, structural regions, and over the course of brain developments (Gambarana, Beattie et al. 1991; Killisch, Dotti et al. 1991; Laurie, Seeburg et al. 1992; Laurie, Wisden et al. 1992; Wisden, Laurie et al. 1992; Fritschy, Paysan et al. 1994; Gutierrez, Khan et al. 1994; Fritschy and Mohler 1995; Gutierrez, Khan et al. 1996; Gutierrez, Khan et al. 1997; Brunig, Scotti et al. 2002; Wei, Zhang et al. 2003; Lopez-Tellez, Vela et al. 2004; Yu, Wang et al. 2006). Altering subunit composition changes receptor kinetics, pharmacology and physiology, which impacts behaviour (Pirker, Schwarzer et al. 2000). So that light variations in $GABA_A$R subunit composition could contribute to both normal behaviour, as well as pathologic conditions (Brussaard, Kits et al. 1997; Gao, Newman et al. 1999; Porter, Zhang et al. 2005).

For example specific $GABA_AR$ subunit compositions have been associated with certain phenotypes such as anxiety, aggression, learning and memory deficits (Delaney and Sah 1999; Rudolph, Crestani et al. 1999; Collinson, Kuenzi et al. 2002; Armstrong, Sheffield et al. 2003; Miczek, Fish et al. 2003; Ishikawa, Mizukami et al. 2004; Porter, Zhang et al. 2005).

The aging-related changes in the expression patterns of the $GABA_A$ receptor subunits lead also to functional differences in $GABA_A$ receptors during ontogeny (MacLennan, Brecha et al. 1991).

Although the subunit distribution of the $GABA_A$ receptors varies from region to region, we can generally say that the immature brain expresses several subunits, including α1, α2, α3, α5, β2, and γ2. Subunits α2, α3, and α5 were found to decrease with age and in contrast, α1, β2, and γ2 levels are maintained or rise with age (Laurie, Wisden et al. 1992; Brooks-Kayal, Jin et al. 1998). α1, β2, and γ2 are the predominant subunits in the adult brain (Fritschy and Mohler 1995; Pirker, Schwarzer et al. 2000) and were found throughout the brain, although differences in their distribution were observed. Subunit α2, α3, α4, α5, α6, γ1, and δ are more confined to certain brain areas.

Generally, the $GABA_AR$ subunit expression is more significant among α subunits than among the other subunits (Bosman, Rosahl et al. 2002), and in the majority of changes involves a developmental decrease in α2 or α3 expression and an increase in α1 expression (Laurie, Seeburg et al. 1992; Fritschy, Paysan et al. 1994; Bosman, Rosahl et al. 2002).

The alpha 1 expression is low at birth, restricted to a few areas, and increases dramatically during the first postnatal weeks. In contrast, the alpha 2 subunit, has a widespread distribution throughout the brain at birth, and disappears from numerous areas soon after the appearance of the alpha 1-subunit.

During a short time window, many individual neurons show a coexistence of both (α1 and α2), which indicates that the alpha 1-subunit gradually replaces receptors containing the alpha 2-subunit (MacLennan, Brecha et al. 1991; Laurie, Wisden et al. 1992; Fritschy, Paysan et al. 1994; Gutierrez, Khan et al. 1996; Lopez-Tellez, Vela et al. 2004).

The β2, β3, and γ2 subunits, which are a major constituent of $GABA_A$ receptors in both immature and adult rat brain, show no significant changes in their expression (Fritschy, Paysan et al. 1994).

In the rat pre-Bötzinger complex (PBC) has been shown an aging-related switch in the expression of α1 and α3 subunits. The α3-subunit is at the age of P0 (postnatal day 0) at relatively high levels and it decreases with development, whereas the α1-subunit, which is relative low at P0, increases with age. The expression of α2-subunit is rather constant throughout the postnatal development.

Although there is no direct evidence, it is postulated that the switch from depolarisation to hyperpolarisation during the early brain development is associated with a switch of $GABA_A$ receptor α subunit composition, which contributes to functional changes in GABA transmission (Liu and Wong-Riley 2006). This excitation-inhibition switch of $GABA_A$ receptor function takes place in the early postnatal period and depends on the animal species, the brain regions and the type of neurons. In hypothalamic and spinal cord neurons the switch from depolarisation to hyperpolarisation happens between P8 and P12 (Gao and van den Pol 2001), in hippocampal neurons between P8 and P12 (Ben-Ari, Cherubini et al. 1989; Michelson and Wong 1991; Ruano, Araujo et al. 2000), and in brainstem during the first postnatal week (Ritter and Zhang 2000; Marchetti, Pagnotta et al. 2002).

The combination of the α subunit age depended alteration with the $GABA_A$ receptor functional switch, means that the α2 (or α3) composition would contribute to the depolarizing GABA transmission in the neonate, and the α1 composition could contribute to the synaptic inhibition in the adult (Fritschy, Paysan et al. 1994).

In addition, it has been reported that $GABA_A$ receptors can depolarize also in adult neurons, such as in the CA1 pyramidal neurons (Isomura, Sugimoto et al. 2003), which expresses predominantly α2 subunit (Laurie, Wisden et al. 1992).

1.7.1.1 Neurodevelopmental disorder (MECP2)

Rett syndrome is an X-linked neurodevelopmental disorder caused by mutations in the gene encoding the transcriptional repressor methyl-CpG-binding protein 2 (MeCP2) (Amir, Van den Veyver et al. 1999).

MeCP2 is expressed widely, but is increasingly expressed during development (Kishi and Macklis 2004). It binds DNA methylated at CpG sites and once bound to DNA it

regulates transcriptional repression (Shahbazian and Zoghbi 2002). Thus, loss of MeCP2 function leads to improper gene expression programs.

Rett syndrome affects almost exclusively girls and is usually fatal for males. After a period of apparently normal development (6-18 months), Rett syndrome patients enter a period of regression, characterized by loss of acquired language, acquired microcephaly, autistic manifestations, loss of purposeful hand skills, stereotypic hand movements, gait ataxia, and breathing abnormalities such as hyperventilation and apnea (Coleman, Brubaker et al. 1988; Amir, Van den Veyver et al. 1999; Hagberg 2002; Zoghbi 2005).

Since the availability of mecp2 mutant mice which provides a very good model system for the research of Rett syndrome (Amir, Van den Veyver et al. 1999), many molecular, anatomical or behavioural studies have been done.

It is known that, Rett syndrome is a disorder of abnormal neuronal maturation (Kaufmann, MacDonald et al. 2000; Kaufmann, Johnston et al. 2005). Studies on the expression of neurotransmitter receptors such as NMDA, AMPA, kainite, metabotropic glutamate receptors and GABA receptors show age related abnormalities in receptor densities in the frontal cortex and basal ganglia (Blue, Naidu et al. 1999; Blue, Naidu et al. 1999); Nevertheless, remains open question as to whether these morphological abnormalities are the cause or consequence of the Rett syndrome phenotype.

In Rett syndrome the balance between excitation and inhibition, that exist in healthy brains, is shifted (Dani, Chang et al. 2005; Moretti, Levenson et al. 2006). Many studies suggest that this synaptic imbalance may cause the Mecp2 observed abnormalities (Dani, Chang et al. 2005; Moretti, Levenson et al. 2006), but it is still unknown which transmitter and receptors are predominantly involved.

1.7.2 Distribution of $GABA_B$ receptor variants during brain development

All three $GABA_B$ receptor proteins, namely R1a, R1b and R2, display a broad distribution, being present in all brain areas. However, the presence of each splice variant differs among brain regions, age, and cellular distribution.

In situ hybridization and immunohistochemical studies have shown an early and strong $GABA_{B1}$ receptor expression in discrete brain regions during embryonic

development (Kim, Li et al. 2003; Lopez-Bendito, Lujan et al. 2003; Panzanelli, Lopez-Bendito et al. 2004).

$GABA_{B1}$ receptor mRNA is intensely expressed by E12 and is detected in hippocampus, cerebral cortex, intermediate and posterior neuroepithelium, and the pontine neuroepithelium (Lopez-Bendito, Shigemoto et al. 2002; Kim, Li et al. 2003).

The most studied $GABA_{B1}$ splice variants, $GABA_{B1a}$ and $GABA_{B1b}$ are differentially regulated during postnatal maturation (Malitschek, Ruegg et al. 1998; Fritschy, Meskenaite et al. 1999; Liang, Hatanaka et al. 2000).

$GABA_BR1a$ is dominant in neonatal brain and increases within the first postnatal days (P0–P5) and then decreases to adult levels, whereas $GABA_BR1b$ levels rise during the second and third postnatal weeks, i.e. during the peak of synaptogenesis, and is predominant in adulthood (Malitschek, Ruegg et al. 1998; Fritschy, Meskenaite et al. 1999; Calver, Medhurst et al. 2000; Martin, Russek et al. 2001).

In contrast to the immature brain, in the adult rat brain the $GABA_{B1b}$ levels are higher than $GABA_{B1a}$ levels in most structures. Exceptions are the olfactory bulb and the striatum where higher levels of $GABA_{B1a}$ than $GABA_{B1b}$ protein are detected (Bettler, Kaupmann et al. 2004).

Also sex dependant differences have been shown in combination with developmental changes of $GABA_BR1$ variants. Specifically, in rat hypothalamus $GABA_BR1a$ was much higher expressed in females at birth whereas in males the higher expression of $GABA_BR1a$ starts after 38 days. $GABA_BR1b$ showed no sex differences along development (Bianchi, Lux-Lantos et al. 2005).

Another developmental mediated difference is the alteration of the $GABA_B$ receptor binding affinity to L-baclofen. Although the binding affinity of the $GABA_B$ receptor to the agonist is the same between $GABA_{B1a}$ and $GABA_{B1b}$ (Kaupmann, Huggel et al. 1997) in early postnatal development the affinity at R1a and R1b is 10-fold lower than in adult brain and gradually increases with aging (Malitschek, Ruegg et al. 1998).

1.8 Aim of this thesis

When starting this study it was well documented that in the Central Nervous System there is a developmental switching in the function of the GABAergic system from early excitation to late mature inhibition. However, little is known about what controls this developmental shift in $GABA_A$ and $GABA_B$ receptors.

The aim of the study was to determine which factors are associated with the GABAergic functional differentiation during postnatal development in mouse brainstem. The following main questions were set:

- In order to specify what kind of modifications could influence the $GABA_B$ receptor function in brainstem during brain maturation, we were interested in understanding the following issues:
  - Is there any developmental change in the expression of the $GABA_BR2$ subunit?
  - How does the G protein subtyp expression differ in animals according to their developmental stage?
  - How does the two factors mentioned above influence the interaction between G proteins and $GABA_B$ receptors in adult and newborn mice?
- By which mechanism(s) does the age-related irregulation between excitation and inhibition in Mecp2 knockout mice (Rett syndrome) is associated with the $GABA_A$ receptors?

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

Equipment	Manufacturer
Electroporator	Bio-Rad, Hercules, California, USA
Electrophoresis chambers for DNA	Biometra, Göttingen, Germany
Elektrophoresis Power supply	EPS601, Amersham Biosciences, Freiburg, Germany
Gel dryer	Model 583, Bio-Rad Laboratories GmbH, München, Germany
Incubators for cell culture	Forma scientific 3250, Water Jacketed Incubator, Marietta, Ohio, USA
Light microscopes	Carl Zeiss, Jena, Germany
Magnetic stirrer with heating block	IKA RCT basic, North Carolina, USA
Minishaker	MS1, IKA, North Carolina, USA
PCR-Cycler	Biometra, Göttingen, Germany
Plate reader	GENios Pro, Tecan Deutschland GmbH, Crailsheim, Germany
Spectrophotometer	GeneQuant pro, Amersham Biosciences, Freiburg, Germany
Speed Vac Concentrator	Beta, Christ, Osterode am Harz, Germany
Thermo-shaker	T5-100, Kisker, Steinfurt, Germany

2.1.2 Chemicals

Name	Manufacturer
AA30 / Rotiphorese gel 30	Roth, Karlsruhe, Germany
Agarose	Merck, Darmstadt, Germany Invitrogen, Groningen, The Netherlands
Aprotinin A 1153	Sigma, Taufkirchen, Germany
Aprotinin Ct. Nr.A 1153	Sigma, Taufkirchen, Germany
BSA	Amersham Pharmacia Biotech, Little Chalfont, UK

Chloroform	Merck, Darmstadt, Germany
Coomassie Brilliant Blue	Merck, Darmstadt, Germany
DEPC-Treated water	Ambion, Huntingdon, Cambridgeshire, UK
(RS) Baclofen	Tocris, Bristol, UK
Dimethylsulfate (DMSF)	Sigma, Taufkirchen, Germany
EGTA	Sigma, Taufkirchen, Germany
EDTA	Roth GmbH & Co., Karlsruhe, Germany
Ethanol	Calbiochem, San Diego, California, USA
D (+) Glucose	Roth, Karlsruhe, Germany
DMSO	Sigma, Taufkirchen, Germany
Glycin	Sigma, Taufkirchen, Germany
Guanosine 5´-diphosphate sodium salt	Sigma, Taufkirchen, Germany
Guanosine 5´-triphosphate sodium salt	Sigma, Taufkirchen, Germany
HEPES	Roth, Karlsruhe, Germany
Jodacetatamide	Sigma, Taufkirchen, Germany
Klenow enzyme	Roche, Mannheim, Germany
LB agar	Invitrogen, Groningen, The Netherlands
Leupeptin L 9783	Sigma, Taufkirchen, Germany
Imidazol	Roth, Karlsruhe, Germany
NP-40	Calbiochem, San Diego, California, USA
Nuclease-free water	Ambion, Huntingdon, Cambridgeshire, UK
Oligo(dT)15	MWG-Biotech AG, Ebersberg, Germany
Phenol/Cloroform/Isoamylalcohol	Amersham Pharmacia Biotech, Little Chalfont, UK
Phenylmethanesulfonyl fluoride (PMSF)	Calbiochem, San Diego, California, USA
Ponceau S	Sigma, Taufkirchen, Germany
Protein A immobilized on Sepharose CL-4B	Sigma, Taufkirchen, Germany
Ribonuclease inhibitor (RiboLock)	MBI Fermentas, St. Leon-Rot, Germany
Sodium Dodecyl Sulphate (SDS) ultra pure	Roth, Karlsruhe, Germany
Sodium Chloride	Roth, Karlsruhe, Germany

T4 DNA ligase, buffer	Roche, Mannheim, Germany
TEMED	Roth, Karlsruhe, Germany
Tris (hydroxymethyl) aminomethane (TRIS)	Roth, Karlsruhe, Germany
TritonX100	Roth, Karlsruhe, Germany
Tween 20	Roth, Karlsruhe, Germany
β-Mercaptoethanol	Sigma, Taufkirchen, Germany

2.1.3 Additional materials

KodakX-O mat AR Film	Kodak, Rochester, USA
35S-GTPγS Cat. Nr. KS 302/0925	Hartmann Analytic GmbH, Braunschweig, Germany
Membrane hybond ECL	Amersham Biosciences, Freiburg, Germany
Whatman GB58 blotting-Paper B002	Heinemann Labotechnik GmbH, Hainichen, Germany

2.1.4 Software

Image processing	Adobe Photoshop CS, Adobe
Microscopy	Leica confocal software, Leica
Sequence analysis/ Virtual cloning	DNAstar, Lasergene Navigator
Text processing	Microsoft Word, Microsoft Corp.
Graphs and tables	Excel, Microsoft Corp. Adobe Illustrator CS, Adobe Prism 4.0, GraphPad Software, Inc. Instat 3 for Mac, GraphPad Software, Inc
Western analysis	Chemi-Smart 5000, Vilber Lourmat Bio-1D, Vilber Lourmat Gel-ProAnalyzer 3.1, Media Cybernetics

2.1.5 Web pages

BLAST	www.ncbi.nln.nih.gov./BLAST
PubMed	www.ncbi.nln.nih.gov

Protein calculation	www.mrc-Imb.cam.ac.uk/rpg/proteincalculator.html.
ClustalW	www2.ebi.ac.uk/clustalw

2.1.6 Molecular markers

2.1.6.1 DNA molecular weight standard

100 bp DNA Ladder	Invitrogen, Groningen, The Netherlands
DNA Ladder N3014S	New England Biolabs, Frankfurt am Main, Germany
1 Kb DNA Ladder	Invitrogen, Groningen, The Netherlands

2.1.6.2 Protein molecular weight standard

The approximate molecular weight of proteins in SDS-polyacrylamide gels was determined according to (Cat. No.: 10748-010) BenchMark Pre-Stained Protein molecular weight marker (6.0-181.8 kDa) from Invitrogen.
Protein molecular weight calculations according to the amino acid sequence were performed using the program at www.mrc-Imb.cam.ac.uk/rpg/proteincalculator.html.

Additional molecular weight markers:

SDS-PAGE standards, Low range (34.3-103 kDa)	BioRad, California, USA
SDS-PAGE standards, High range (48-204 kDa)	BioRad, California, USA
SDS-PAGE standards, Broad range (7.1-209 kDa)	BioRad, California, USA

2.1.7 Kits

Gel extraction kit-QIAEX® II	Qiagen, Hilden, Germany
Omniscript RT kit (Cat Nr. 205111)	Qiagen, Hilden, Germany
ECLTM western blotting detection reagents	Amersham Biosciences, Freiburg, Germany
Membrane blocking agent	Amersham Biosciences, Freiburg, Germany
GTP-Eu	PerkinElmer life sciences, Boston, USA

cAMP Biotrak enzymeimmunoassay (EIA) system	Amersham Biosciences, Freiburg, Germany
Expand long template PCR system	Roche, Manheim, Germany
High fidelity PCR	Roche, Manheim, Germany
MBS mammalian transfection kit	Stratagene, La Jolla, USA
Total protein kit	Sigma, Taufkirchen, Germany

2.1.8 Antibodies

2.1.8.1 Primary antibodies

Antibodies	Raised in	Clonality	Company
Anti-metabotropic Glutamate receptor 2/3	Rabbit	Polyclonal	Chemicon
Anti-Glutamate Receptor 1	Rabbit	Polyclonal	Chemicon
Anti-NMDA NR1 Subunit, splice Variant C1	Mouse	Polyclonal	Biomol
Anti-phospho-NR1 (Ser896)	Rabbit	Polyclonal	Biomol
Anti-phospho-NR1 (Ser897)	Rabbit	Polyclonal	Biomol
Anti-NMDA Receptor 2A&B	Rabbit	Polyclonal	Chemicon
Anti-NMDA Receptor 3B,	Rabbit	Polyclonal	Biomol
Anti-NMDA Receptor 1, (54.1)	Mouse	Polyclonal	upstate
Anti-NMDA Receptor1, (54.2)	Mouse	Polyclonal	SynapticSystems, Göttingen
Anti-NMDA Receptor2A	Rabbit	Polyclonal	Chemicon
anti-synaptophysin (Clone Sy38)	Mouse	Monoclonal	Dako, Denmark
Actin	Rabbit	Monoclonal	Sigma
Hsp70	Goat	Polyclonal	Santa cruz biotechnology, inc.
Glycine Receptor (all Subunits)	Mouse	Polyclonal	Alexis
$GABA_A$ Receptor alpha 1	Goat	Polyclonal	Santa cruz biotechnology, inc.
$GABA_A$ Receptor alpha 2	Goat	Polyclonal	Santa cruz biotechnology, inc.
$GABA_A$ Receptor alpha 3	Goat	Polyclonal	Santa cruz biotechnology, inc.
$GABA_A$ Receptor alpha 5	Goat	Polyclonal	Santa cruz biotechnology, inc.

$GABA_A$ Receptor beta 3	Goat	Polyclonal	Santa cruz biotechnology, inc.
$GABA_A$ Receptor alpha 2	Rabbit	Polyclonal	Abcam
$GABA_A$ Receptor alpha 3	Rabbit	Polyclonal	Abcam
$G\alpha_s$ (K-20) Sc-823	Rabbit	Polyclonal	Santa cruz biotechnology, inc.
$G\alpha_{q/11}$ (C-19) Sc-392	Rabbit	Polyclonal	Santa cruz biotechnology, inc.
$G\alpha_{i/o}$ (K-20) Sc-387	Rabbit	Polyclonal	Santa cruz biotechnology, inc.
$G\alpha_{i-3}$ (C-10) Sc-262	Rabbit	Polyclonal	Santa cruz biotechnology, inc.
$G\alpha_{13}$ (A-20) Sc-410	Rabbit	Polyclonal	Santa cruz biotechnology, inc.
Gβ (M-14) Sc-261	Rabbit	Polyclonal	Santa cruz biotechnology, inc.
$GABA_BR1$ (H-300)	Rabbit	Polyclonal	Santa cruz biotechnology, inc.
Anti-$GABA_BR1$ Receptor	Guinea Pig	Polyclonal	Chemicon
Anti-$GABA_BR2$ Receptor	Guinea Pig	Polyclonal	Chemicon

2.1.8.2 Secondary antibodies

Secondary antibodies specificity	Host	Conjugates	Company
ECL Rabbit IgG,	Donkey	HRP	Amersham Bioscience
ECL Mouse IgG,	Sheep	HRP	Amersham Bioscience
Anti-Goat IgG (H+L)	Donkey	HRP	Dianova
Anti-Mouse IgG	Goat	HRP	Chemicon
Guinea pig	Goat	HRP	Dianova
Guinea Pig IgG (H+L)	Donkey	Cy3 (indocarbocyanin)	Dianova
Rabbit Cy5 IgG (H+L)	Goat	Cy5 (indodicarbocyanin)	Dianova

2.1.8.3 Peptide

$GABA_A$ Receptor alpha 2

$GABA_A$ Receptor alpha 3

$GABA_A$ Receptor alpha 5

$GABA_A$ Receptor alpha 6

$GABA_A$ Receptor beta 3

$G\alpha_s$

2.1.9 Animals

Adult (between 3-4 weeks) and young (between postnatal day 0 and 3; P0-P3) NMRI mice, were used in the experiments. The mice were purchased from the animal center of the University of Gottingen. Animal experiments were carried out in accordance with the guidelines of the Ethics Committee of the University of Göttingen.
New born mice (P0-P3) were rapidly decapitated at the C3-C4 level; The Adult animals were anesthetized with ether and then decapitated. The skulls were opened and the brains were rapidly removed and placed in ice cold artificial cerebrospinal fluid (ACSF). The solution (ACSF) was gassed continuously and saturated with 95 % O_2 and 5 % CO_2. The further procedure depends on the method which is going to follow (see section 2.2.1.1; 2.2.2.1; 2.2.5, 2.2.6.1).

2.1.10 Cell culture

- Eukaryotic cell lines: HEK293 Flp-In (Invitrogen, Karlsruhe, Germany)
- cDNA plasmids: pcIneo-$GABA_B$ R1a and R2 cDNA of rat, kindly provided by Dr. Klemens Kaupmann, Novartis Pharma AG.
- Bacterial Strains: *Escherichia coli* XL1-Blue MRF´ (Stratagene, La Jolla, California, USA)

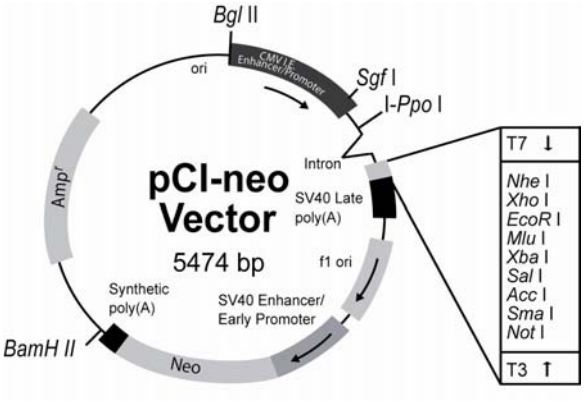


Figure 2.1. pCI-neo vector circle map (Neo= neomycin-phoshotransferase). Amp^r, gene conferring the ampicillin resistance in *E.coli*; f1 ori, origin of replication derived from filamentous phage; ori, origin of replication. Arrows indicate the direction of transcription.

■ Antibiotics:	Ampicillin	Amersham Pharmacia Biotech, Little Chalfont, UK
	Hygromycin B	Invitrogen, Karlsruhe, Germany
	Zeocin	Invitrogen, Karlsruhe, Germany
	Penicillin-Streptomycin	Invitrogen, Karlsruhe, Germany

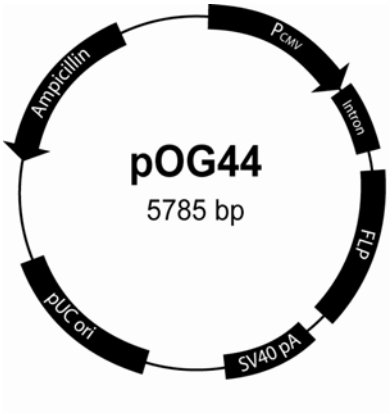


Figure 2.2. pOG44 vector circle map. Ampicillin, Ampicillin resistance gene; pCMV, CMV promoter; FLP, Flp recombinase, synthetic intron, SV40 late poly-adenylation signal, pUC origin. Arrows indicate the direction of transcription.

■ Vectors:	pcDNA5/FRT	Invitrogen, Karlsruhe, Germany
	pCI-neo	Invitrogen, Karlsruhe, Germany
	pOG44	Invitrogen, Karlsruhe, Germany
	pBudCE4.1	Invitrogen, Karlsruhe, Germany

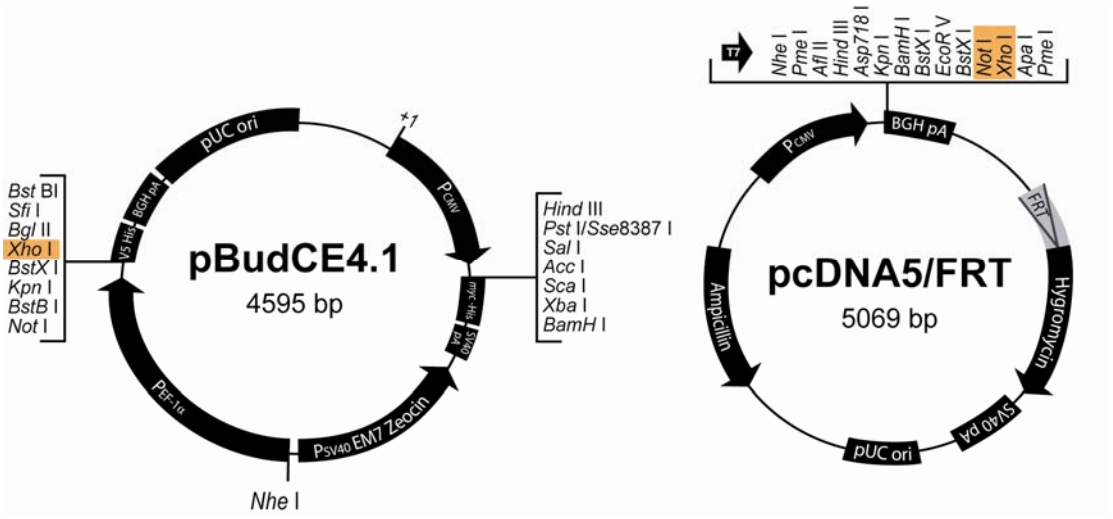


Figure 2.3. Structure of the pBudCE4.1 and pcDNA5/FRT vectors. The restriction sites *Xho* I and *Not* I were used to insert the $GABA_B$ receptor genes (for more details see figure 2.5). Arrows indicate functional direction of genes. Ampicillin, Ampicillin resistance gene; Hygromycin, Hygromycin resistance gene (no ATG); Zeocin, Zeocin resistance gene; P_{CMV}, CMV promoter, *myc* epitope, 6xHis tag; SV40 polyadenylation sequence, EM7 promoter; P_{SV40}, SV40 early promoter; pUC origin; V5 epitop; BGH polyadenylation sequence.

2.1.10.1 Reagents for cell culture

dH2O	Invitrogen, Groningen, The Netherlands
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen, Groningen, The Netherlands
D-PBS	Invitrogen, Groningen, The Netherlands
Trypsin/EDTA	Invitrogen, Groningen, The Netherlands

2.2 Methods

2.2.1 Molecular biology methods

2.2.1.1 Isolation of total RNA from mice brainstem

The typical eukaryotic cell contains ~10-5 µg of total RNA.

- 80-85 % is rRNA (ribosomal) (mostly 28S, 18S and 5S)
- 15-20 % is low MW species tRNA(transfer), snRNA (small nuclear) plus others and
- 1-5 % is mRNA (messenger)

The major concern in handling RNA is the control of ribonuclease activity. For preventing RNase contamination it was essential to use RNase-free gloves, sterile Reagents, RNaseZap (Sigma), pre-packed RNase-free pipette tips and autoclaved glass- and plastic ware.

Total RNA was isolated from NMRI mice brainstem using, TRIzol reagent according to manufacturer's protocol (Invitrogen life technologies) (Chomczynski, P., and Sacchi, N., 1987). Briefly, after decapitation (see section 2.1.9), the brainstem were dissected, weighed, and homogenized in TRIzol reagent at a ratio of 1 ml/100 mg brainstem wet weight.

TRIzol is a monophase solution of phenol and guanidine isothiocyanate which maintains the integrity of the RNA, while disrupting cells and dissolving cell components. An additional isolation step was required because of the high content of proteins and lipids in the brain compared to other tissues. For this reason the samples were centrifuged at 12.000 x *g* for 10 min. at 4 °C. The resulting pellet contains extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains the RNA.

The supernatant was transferred to a new tube and after 5 min incubation at RT, 200 µl chloroform was added, mixed and incubated for 3 min at RT. After centrifugation at 12.000 x *g* for 15 min. at 4 °C, three phases were separated: a low phenol-chlorophorm phase, an interphase, and a colorless upper RNA containing aqueous phase. For the RNA precipitation the aqueous phase was transferred to a new tube and mixed with 500 µl isopropylalcohol. RNA was precipitated for 10 min at RT and pelleted by centrifugation at 12.000 x *g* for 10 min at 4 °C.

The RNA pellet was washed twice with 75 % ethanol solution (diluted with DEPC-treated water), air dried and, dissolved in 50 µl RNase-free water. After 10 min incubation at 37 °C, followed:

- Quantification of the RNA concentration (OD 260 nm).
- Purity determination of the RNA (OD 260/280 nm).
- Examination of the RNA integrity (18S/28S).

I Quantification of RNA concentration (OD 260 nm)

Concentration of the total cell RNA was determined by measuring the OD at 260 nm. An OD_{260} of 1 corresponds to approximately 40 µg of RNA per ml.

II Purity determination of the RNA (OD 260/280 nm)

The ratio A_{260}/A_{280} is an indication of the purity of the RNA. The ratio (for pure RNA) should be in the range of 1.8-2.0. But even if the ratio is less than 1.8, the RNA quality may be acceptable and this can be confirmed by gel electrophoresis.

III Examination of the RNA integrity (18S/28S)

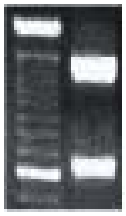


Figure 2.4. Intact RNA. Five µg of total cell RNA were run on a 1.5 % agarose gel. The 18S and 28S ribosomal RNA bands are clearly visible.

The quality of the RNA was checked by loading 5 µg RNA on 1.5 % agarose gel in TBE with 2,5 µg/ml ethidium bromide. The gel was visualised under UV light to evaluate RNA yield.

The 28S rRNA band was approximately twice as intense as the 18S rRNA band. This 2:1 ratio (28S:18S) is a good indication that the RNA is intact. Partially defragged RNA would have a smeared appearance, or would not exhibit a 2:1 ratio.

2.2.1.2 Reverse transcription-Polymerase Chain Reaction (RT-PCR)

The OmniScript kit (Qiagen, Hilden) was used for the reverse transcription of mRNA in a complementary DNA (cDNA).

Reaction mixture for RT-PCR (20 µl):

Component	Volume/reaction	Final concentration
Master mix		
10x Buffer RT	2 µl	1x
dNTP Mix (5 mM each dNTP)	2 µl	0.5 mM each dNTP
Oligo-dT primer (100 pmol/µl)	0.2 µl	1 mM
RNase inhibitor (40 units/µl)	0.25 µl	10 units (per 20 µl reaction)
Omniscript Reverse Transcriptase	1 µl	4 units (per 20 µl reaction)
RNase-free water	Variable	
Template RNA (added at the end)	Variable	10 µg (per 20 µl reaction)

The isolated RNA from 2.2.1.1 was used as a template and the reaction was performed according to the protocol provided by Qiagen.
10 µg of total RNA was used for the PCR reaction with 100 pmol/µl Oligo (dT) 15-Primer (MWG-Biotech AG) in a final volume of 20 µl.
The mix was prepared (on ice) as listed in the table above. The reaction mixture was incubated for 60 min. at 37 °C. The isolated cDNA was used for following PCRs

2.2.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis was performed for separation, identification and purification of DNA. The concentration of agarose was used for separation of the fragments with different lengths.
DNA fragments were separated according to their molecular weight through electrophoresis in horizontal 0.8-1.5 % agarose gels.

Agarose [%]	Fragment length [kb]
0.5	1-30
0.7	0.8-12
1.0	0.5-10
1.2	0.4-3

1.5	0.2-3
2.0	0.01-1

Agarose solutions were prepared by dissolving the desired amount of agarose in TAE buffer, in a microwave oven. Ethidium bromide was added to a final concentration of 1 µg/µl. The samples were loaded onto gels with 0.2 volume loading Buffer. Gels were run horizontally at 60-80 mV in 1xTAE buffer for minimum of 30 min.

The DNA bands were made visible and photographed under UV light. Size of DNA fragments were determined by comparison with DNA ladders.

Stock solution 50xTAE (for 1 l):	242 g	Tris
	57.1 ml	Acetic acid
	100 ml	0.5 M EDTA pH 8.0
		H_2O
Loading buffer:	10 mM	EDTA pH 8.0
	100 mM	Tris pH 8.0
	57 %	glycerol
		bromphenolblue

2.2.1.4 Extraction of the DNA fragments from agarose gels

DNA fragments were separated according to their size by Agarose gel electrophoresis. DNA band was cut from the agarose gel with a clean scalpel. DNA Purification was carried out according to the manual of Qiagen Gel Extraction Kit (Qiagen, Germany).

2.2.2 Biochemical methods

2.2.2.1 Preparation of protein extracts

Aliquots (see section 2.1.9) of different mice brain areas (cortex, hippocampus and brainstem) were weighted, immediately frozen in liquid nitrogen, and stored at -80 °C until homogenization.

The brain samples were homogenized with a glass teflon homogenisator (homgen^{plus}, Schütt) at setting 2000 rpm and 10 strokes, in a buffer (40 w/v) containing 1 mM EDTA, 20 mM HEPES pH 7.4, 0.1 mM PMSF, 2 µg/ml aprotinin and 2 µg/ml leupeptin. The homogenates were placed in ice-cold lysis buffer (1:1) (RIPA with 1 % NP 40) and incubated at 4 °C for 2 h. The supernatant was cleared by centrifugation at 14.000 rmp at 4 °C for 30 min. The protein measurement was determined by Lowry assay as described in section 2.2.2.2. The samples were combined with 3x loading buffer (62,5 mM Tris-HCl, 20 % glycerol, 6 % SDS, 0,01 % bromphenolblue, 10 % β-mercaptoethanol) and boiled at 100 °C for 5 min. Boiling denatures proteins, unfolding them completely. SDS then surrounds the protein with a negative charge and the beta-mercaptoethanol prevents the reformation of disulfide bonds.

2.2.2.2 Determination of protein concentration

The total protein concentration was assessed by the Lowry technique, as modified by Peterson using the total protein kit from Sigma and with bovine serum albumin (BSA) (Sigma) as standard.

Aliquots of samples (5 µl) were diluted in water to a final volume of 50 µl. Each protein concentration determination was carried out in triplicate.

The standard curve was prepared as follows. Bovine serum albumin (BSA) powder was dissolved in distilled water and diluted to a concentration of 400 µg/ml. A series of dilutions (0, 50, 100, 200, 300, 400 µg/ml) were prepared in triplicates with a final volume of 50 µl. Standards and samples were transferred to a microplate (96 well plate, Newton Inc.), 50 µl of Lowry reagent was added to each well and mixed with repeated pipeting. The mixture was then allowed to incubate at RT for 20 min. prior to the addition of 25 µl per well of Folin & Ciocalteu's Phenol reagent. Samples were mixed immediately with repeated pipeting with each addition. Absorbance was measured at a wavelength between 500 and 800 nm. All absorbance determinations were performed using a Microplate Reader (BioRad). Analysis and statistics of the curve were performed using Sigma Plot and Excel software.

2.2.2.3 Sodium Dodecyl Sulfate-Polyacrylamide gel Electrophoresis (SDS-PAGE)

SDS-PAGE is a qualifying and quantifying method for proteins. With this Method proteins can be separated on the basis of mass by electrophoresis in a polyacrylamide gel according to Laemmli *et al.* (1970).

SDS is an anionic detergent which binds to the proteins and confers a negative charge to the polypeptides in proportion to there length.

The negative charges of SDS destroy most of the complex structure of proteins which migrate towards the anode in an electric field.

Finally the polyacrylamide gels with there porous structure separate the proteins according to there relative molecular weight.

Solution	Separation gel (10 %)	Stacking gel (5 %)
AA30	2,5 ml	0,325 ml
4xTris/HCL SDS 8.8	1,875 ml	-
4xTris/HCL SDS 6.8	-	0,787 ml
bidest. H2O	3,125 ml	1,525 ml
Themed	7,5 µl	3,7 µl
APS	40 µl	20 µl

Protein gel electrophoresis was performed using a minigel vertical apparatus under reducing conditions. The vertical gels were poured between two glass plates (10,5x10 cm) sealed with a silicone rubber band giving an internal thickness of 0.6 mm between the two plates. The gels were composed of a separation gel (10 %) that separates the proteins according to their size and a stacking gel (5 %) that insures the simultaneous entry of the proteins into the separating gel. After pouring the separation gel, the gel was overlaid with isopropanol to straighten the gel surface. When polymerisation was completed the isopropanol was removed, the stacking gel was poured above the separation gel and a ten wells comp of 0.6 mm was fixed. After polymerisation of the stacking gel, the comp and the silicone rubber were removed and the gel was assembled in the gel electrophoresis apparatus with the electrophoresis buffer. Air bubbles were removed and samples and ladder were loaded into the wells of the stacking gel using a Hamilton Syringe (Hamilton company).

4xTris/HCl SDS, pH 8.8 (500 ml):	91 gr	Tris-HCl
	2 gr	SDS
4xTris/HCl SDS, pH 6.8 (500 ml):	30,25 gr	Tris-HCl
	2 gr	SDS
10x Electrophoresis buffer (1 l):	30,2 gr	Tris-HCl
	144 gr	glycine
	10 gr	SDS
	Add. H20	

Power supply was attached and the gel electrophoresis was carried out in SDS electrophoresis buffer at 80 Volt until the bromphenolblue dye front had passed stacking gel and afterwards at 150 Volt until the end of the electrophoresis. Electrophoresis was terminated, after the bromphenolblue dye front had passed through the majority of the separating gel. For size markers, BenchMark pre-stained protein molecular weight marker (6.0-181.8 kDa) from Invitrogen, and the low range, high range and broad range prestained standards from BioRad were used.

2.2.2.4 Coomassie blue staining of SDS gels

After electrophoresis, stacking gel was removed and the separating gel was stained at RT for 30 min in a staining solution.

The coomassie solution was then removed and the gel was covered with destain solution under gentle agitation. The destaining solution was changed several times, removing it at each change by aspiration. The gel was completely destained until the protein bands appeared against a clear background. Afterwards the gel was left overnight covered with water. The next day the gel was dried placed onto a sheet of Whatman paper, covered with plastic wrap and dried for 1 h in a gel dryer (Model 583, BioRad) at 80 °C.

Coomassie stain (300 ml):	30 ml	Acetic acid
	75 ml	isopropanol
	195 ml	H_20

	0.2 gr	Coomassie Brilliant Blue R250
Destaining solution (1 l):	100 ml	Acetic acid
	900 ml	H_20

2.2.2.5 Western blot analysis

For the immunological detection of proteins, separated by SDS-PAGE, proteins were transferred onto nitrocellulose membranes (Hybond ECL, Amersham) using a semi-dry technique

A piece of nitrocellulose membrane and 6 pieces of Whatman papers with the same size as the gel were soaked with transfer buffer for 15 min. The blot was assembled from the cathode to the anode as follow: 3 Whatman papers, gel, nitrocellulose membrane, and 3 Whatman papers. The transfer was performed under a current density of 1 mA/cm^2 for 2 h. To verify transfer efficiency the nitrocellulose membranes were reversibly stained with Ponceau S solution.

10x Transfer Buffer (1 l)	30,2 gr	Tris-HCl
	144 gr	Glyzin
	5 gr	SDS
	Add.	H_20
Transfer Buffer (100 ml):	10 ml	10x Transfer Buffer
	20 ml	Methanol
	70 ml	H_2O

2.2.2.6 Staining membranes with Ponceau S

For control and detection of proteins on a membrane after transfer, the membrane was incubated in Ponceau S solution for 2 min at RT. The colored membranes were rinsed with water and the position of proteins and weight standard was marked. For further use, the membranes were washed with PBS-Tween, until they became white again.

Ponceau S solution (1 l):	2 gr	Ponceau S
	50 ml	Acetic acid
	950 ml	H_20

2.2.2.7 Immunodetection of proteins in western blot using horseradisch-peroxidase-conjugated antibodies.

After protein transfer the unspecific binding sites were blocked by incubation in 5 % milk powder (blocking agent Amersham) in PBS/Tween for 2 h at RT. The membranes were then incubated with the primary antibody, diluted to the desired concentration in 5 % milk-PBS/Tween solution overnight at 4 °C under gentle agitation. The primary antibody (monoclonal or polyclonal) recognises a specific epitop in the protein of interest. The membrane was then washed four times with PBS/Tween (5, 15, 30 and 30 min) to remove the unbound primary antibody. The appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was diluted in PBS/Tween, added to the membrane and incubated at RT for 30 min, and washed as above. The choice of the secondary antibody depends on the species of animal in which the primary antibody was raised (host species) and it binds to the Fc region of the primary antibody.

The immunological detection of the proteins was performed using chemiluminescent detection reagents (ECL western blotting detection Reagent, Amersham Bioscience; AceGlow reagents, peqlab biotechnologie GmbH) based on a luminol-HRP-chemiluminescence reaction. The HRP reduces the hydrogen peroxide and the resulting oxygen oxidizes the luminol which emits light. The light was then detected either by photographic film or by a CCD camera which capture a digital image of the western blot.

Densiometric analysis of scanned images: blots were developed using the ECL western blotting detection reagent (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Briefly the membrane was incubated for 1 min at RT with the ECL solutions 1 and 2 (1:1). The membrane was then covered with plastic film and placed in a developing cassette. The membranes were exposed to KodakX films (Kodak) for different time periods and films were developed using the Kodak developer and fixer solutions (GBX Fixer, Kodak; GBX developer, Kodak). The

densiometric quantitation of the scanned images was performed with the Gel-Pro Analyzer Version 3.1 software (Media Cybernetics).
For the densiometric analysis of the digital images from a CCD camera: the detection was performed with the AceGlow Chemilumineszenzsubstrat (peqlab) in a mixture of solution A and B (1:1) by incubation for 1 min. Quantitation of chemiluminescent signals was performed with the image acquisition system Chemi-Smart 5000 and the Bio-1D software (Vilber Lourmat).
Membrane was washed with PBS-Tween, blocked and incubated with a new primary antibody as described above.

10xPBS (1 l) pH7.4:	92,3 gr	NaCl
	2 gr	KCl
	11,5 gr	Na_2HPO_4
	2 gr	$KHPO_4$
PBS/Tween (2 l) pH 7.4:	200 ml	10x PBS
	1800 ml	H_2O
	1 ml	Tween

Membranes were stored after use in plastic bags at 4 °C. To verify the results all experiments were repeated at least four times.
For control staining, membranes were incubated with the same concentration of primary antibody pre-absorbed with blocking peptide. 10 µl of primary antibody and 50 µl of blocking peptide were mixed in 500 µl PBS and incubated at 4 °C overnight. Membranes were than incubated with the antibody-peptide mix, diluted in 5 % milk-PBS/Tween solution overnight at 4 °C under gentle agitation. Membranes were then washed with PBS-Tween, blocked and incubated with the appropriate secondary antibody as described above.

2.2.3 Recombinant DNA techniques

The $GABA_BR1\alpha$ gene fragment was cut out from the pCI-neo vector with *Nhe* I & *Not* I and cloned into the pcDNA5/FRT at the same restriction sites (The new plasmid: pcDNA5/FRT- $GABA_BR1\alpha$, see figure 2.5). The $GABA_BR2$ gene fragment

was cut with *Xho* I and inserted to the pBudCE4.1 at the *Xho* I site (The new plasmid: npBudCE4.1- $GABA_BR2$, see figure 2.5). The new plasmid, pBudCE4.1- $GABA_BR2$, was digested with *Mlu* I and *Xba* I and ligated to the pcDNA5/FRT-$GABA_BR1\alpha$ fragment which was cut with *Pme* I.
The final vector pcDNA5/FRT- $GABA_BR1\alpha$-npBudCE4.1- $GABA_BR2$ was used to transfect the HEK293 cells. All enzymatic reactions (ligation, dephosphorylation, blunt-end and restriction) were used in order to obtain the final vector are summarized in figure 2.5.

2.2.3.1 $GABA_B$ cDNA

The $GABA_B$ receptor cDNAs were derived from Novartis Pharma AG. The $GABA_B$ receptor 2 and $GABA_B$ receptor 1α from rat were used for the cloning.

2.2.3.2 DNA maxi preparation

For the preparation of larger amounts of high copy plasmid DNA Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany) was used. The procedure was carried out according to the manufacturer's instructions. DNA was stored in 1xTE buffer (10 ml 1 M Tris pH 8.0, 2 ml 0.5 M EDTA pH 8.0, Up to 1 lit with H_2O) at 4 °C.

2.2.3.3 DNA sequencing

The sequencing was performed by Fritz Benseler at the Department of Molecular Neurobiology, Max Planck Institute for Experimental Medicine in Göttingen, using the Applied Biosystems 373 DNA sequencer according to Sanger et. al. (1977). Analyses of the sequences were performed using Lasergene software (DNASTAR, Madison, Wisconsin, USA).

2.2.3.4 DNA digestion with restriction enzymes

Restriction digestion is the process of cutting DNA with special enzymes (Restriction Endonucleases). These enzymes recognize and cut, at specific sequences the DNA.

Restriction analyses were performed as a control for the composition of the DNA (analytical digest) or for cloning purpose (preparative digest).

Analytical digest were performed for 2-3 hours at 37 °C in 15 µl reaction volume.

Analytical digest mixture:
1 µl DNA
1.5 µl 10 x buffer (enzyme specific)
0.5 µl restriction enzyme
H_2O up to 15 µl

Preparative digest were performed at 37 °C in 60 µl reaction volume overnight.

Preparative digest mixture:
4 µl DNA
6 µl 10 x buffer (enzyme specific)
2 µl Restriction enzyme (10 U/µl)
H_2O up to 60 µl

DNA fragments obtained from the restriction digestions were directly analyzed by agarose gel electrophoresis.

2.2.3.5 Purification of DNA

DNA fragments were purified by phenol-chloroform extraction or separated on an agarose gel and the fragments of interest were purified using the QIAquick Gel extraction Kit (Qiagen).

- Purification of DNA from agarose gel (see section 2.2.1.4).
- DNA purification (phenol-chloroform extraction) and DNA precipitation

DNA can be purified by phenol-chloroform extraction to remove proteins from DNA solutions. After restriction digestion or blunting the DNA fragments were purified by phenol-chloroform extraction and then precipitated.
The DNA solution was mixed with 0.1xvolume 3M sodium acetate and 1xvolume phenol-chloroform-isoamyl alcohol (25:24:1) followed by centrifugation at 13.000 rpm for 10 min. The top aqueous phase (supernatant) was collected and 1xvolume of isopropanol was added followed by brief mixing and incubation for 5 min on ice. After centrifugation for 15 min at 13.000 rpm the supernatant was removed and the pellet was washed with 70 % ethanol. The pellet was dried in air and then re-suspended in 10 µl dH_2O.

2.2.3.6 Dephosphorylation of DNA fragments

To prevent the self-ligation of a vector in ligation reactions (e.g. when a plasmid was cut with only one enzyme or blunt-ended), 5´ phosphate groups were removed by hydrolysis. The dephosphorylation was performed using alkaline phosphatase (AP) in 10 x AP dephosphorylation buffer (Boehringer Ingelheim, Germany) for 30 min at 37 °C.

The following reaction mixture was used:
60 µl vector DNA (after preparative digestion)
20 µl 10xAP dephosphorylation buffer
2 µl AP (alkaline phosphatase)
120 µl H_2O

2.2.3.7 Blunt-end cloning

Blunt end cloning was performed when no compatible restriction sites were present. After restriction digestion, dephosphorylation and DNA purification from agarose gel slices, blunting was done using Kleenow Polymerase (Boehringer Mannheim GmbH, Germany).

The linearised vector was dissolved in 21.5 µl dH_2O and the following reaction mixture was set up:
21.5 µl DNA
2.5 µl Kleenow Buffer
0.5 µl dNTP 10 mM
0.5 µl Kleenow Polymerase 2 U/µl

The reaction was carried out at 37 °C for 30 min and the DNA fragments were purified by phenol-chloroform extraction and ethanol precipitation. The blunt-ended Vector and fragment were then used for the ligation reaction.

2.2.3.8 Ligation of DNA fragments

DNA ligations were performed by incubating appropriately treated DNA fragments with an appropriately treated vector and T4 DNA ligase in buffer.
The molar ratio of linear vector to insert fragments was ca 1:10. The components in the table below were mixed in a 1.5 ml tube. The mixture was then incubated at 12 °C overnight for ligating reaction. A parallel ligation in the absence of insert DNA was performed as a control.

Composition of the ligating reaction:
1 µl vector DNA
3.5 µl insert DNA
2.5 µl 10x ligase buffer
1 µl T4 ligase (400 U/µl)
17 µl H_2O

The ligation mixture was used then for the transformation of *E. coli* competent cells by electroporation (see section 2.2.3.9).

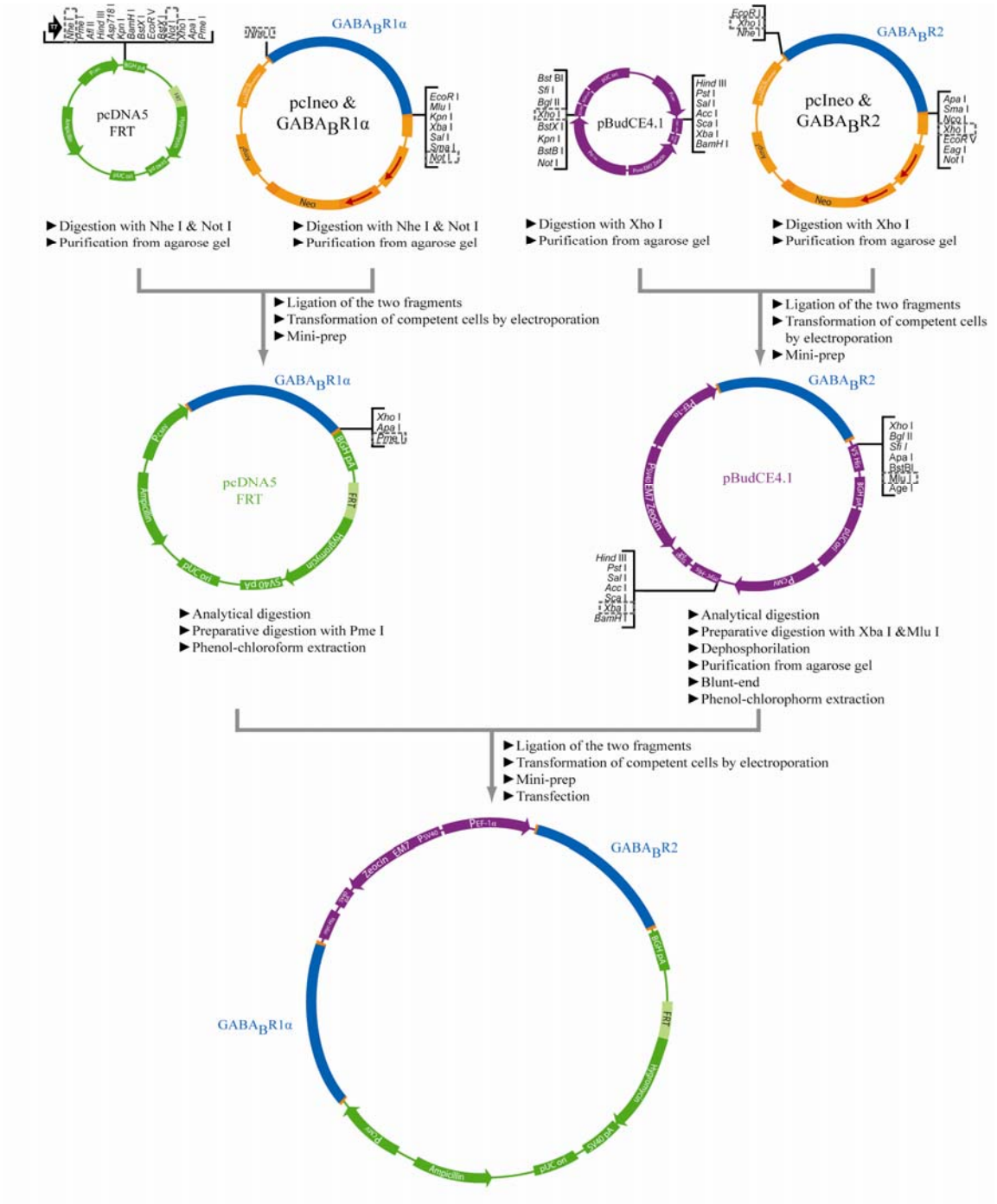


Figure 2.5. The Cloning of the GABA$_B$ R1α and R2 genes. GABA$_B$ R1α was cut out from with *Nh*e I & *Not* I and cloned into the pcDNA5/FRT. The GABA$_B$ R2 gene fragment was cut with *Xho* I and inserted to the pBudCE4.1. The new plasmids, pBudCE4.1- GABA$_B$ R2, was digested with *Mlu* I and *Xba* I and ligated to the pcDNA5/FRT-GABA$_B$ R1α fragment which was cut with *Pme* I. The final vector was used to transfect the HEK293 cells.

2.2.3.9 Transformation of competent cells by electroporation

DNA is introduced into competent *E. coli* cells (*Escherichia coli* XL1-Blue MRF´) by exposing them to a short-high-voltage electrical discharge. 4µl of plasmid DNA (from ligation mixture diluted 1:4 with dH_2O for Mini-prep, or 4 µl plasmid DNA for Maxi-prep) was mixed with 40 µl of electro-competent cells in pre-chilled electroporation cuvette and kept on ice for 1 min. The mixture was pulsed at 2.5 kV in a *E. coli* pulser (BioRad). The transformed cells were then incubated with 1 ml LB medium at 37 °C for 1 h (shaking at 250 rpm).

For Maxi-prep DNA, 4 ml LB medium with the appropriate antibiotic was added to the cell suspension and incubated for further 4 h. 100 µl of the cells were then added to 500 ml LB medium with the appropriate antibiotic and incubated overnight at 37 °C.

For the mini-Prep DNA, the cell suspension was plated onto LB agar plates containing the appropriate antibiotic and grown overnight at 37 °C.

2.2.3.10 DNA mini preparation

White colonies were isolated from plates using sterile toothpicks and suspended in 5 ml of LB medium with the appropriate antibiotic. The colonies were allowed to grow overnight at 37 °C and 400 rpm. The plasmid DNA was isolated from the cells using the QIAprep Spin Miniprep Kit (Qiagen). Briefly, the mini prep DNA was resuspended in 50 µl of EB buffer (10 mM Tris-HCl pH 8.5) at 4 °C. Purified DNA was analysed by restriction digestion.

2.2.3.11 Estimation of DNA concentration

The concentration of nucleic acids was determined using a spectrophotometer (Eppendorf) by measuring the absorption at 260 nm of 10-fold diluted samples in a quartzcuvette. An OD_{260}=1 in a case of 1 cm cuvette corresponds to 50 µg/ml double-stranded DNA, 33 µg/ml single-stranded DNA and 30 µg/ml oligonucleotides (Sambrook et al., 1989).

2.2.4 Culture of HEK293 cells stably transfected with $GABA_BR1\alpha$ and $GABA_BR2$ from rat

2.2.4.1 The Flp-In system

The Flp-In system was used to generate stable expression cell lines. This System creates isogenic cell lines that means, all transfected clones produce equivalent levels of the protein of interest.

The major components of the Flp-In system are:

- A host cell line, Flp-In HEK 293 cell. The host cell line was generated by stable transfection of the pFRT/lacZeo Vector. The vector contains a lacZ-Zeocin gene whose expression is controlled by the SV40 early promoter. The FRT site is located downstream of the ATG initiation codon of the lacZ-Zeocin gene.

- A plasmid containing a FRT site linked to the hygromycin resistance gene, pcDNA5/FRT, into which the gene of interest can be inserted and expressed under the control of the human cytomegalovirus (CMV). The expression vector was constructed as shown in section 2.2.3 and used in this study.

- A Flp recombinase expression Vector, pOG44, for expression of the Flp recombinase under the control of the human CMV promoter.

The pOG44 plasmid and the pcDNA5/FRT vector containing the gene of interest were cotransfected into the Flp-In host cell line.
Upon cotransfection, the Flp recombinase expressed from pOG44 mediates a homologous recombination event between the Recombination Target (FRT) sites (on host cell line genome and on pcDNA5/FRT-constract) so that the pcDNA5/FRT construct is inserted into the host cell genome, at the integrated FRT site.

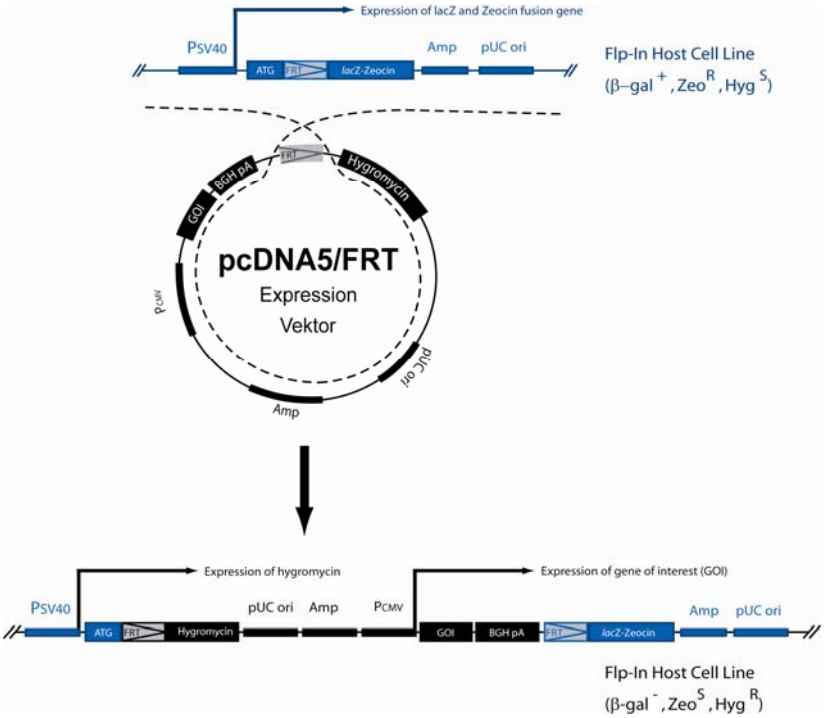


Figure 2.6. Scheme of FLP-In system. (Adapted from the Flp-In system manual, Invitrogen Co.)

Insertion of pcDNA5/FRT into the genome at the FRT site brings the SV40 promoter and the ATG initiation codon (from pFRT/lacZeo) into proximity and frame with the hygromycin resistance gene, and inactivates the lacZ-Zeocin fusion gene. Thus, stable Flp-In expression cell lines can be selected for hygromycin resistance, Zeocin sensitivity, lack of β-galactosidase activity, and expression of the recombinant protein of interest (see figure 3.1).

2.2.4.2 Cell culture and transfection of HEK293 cells

Flp-In HEK293 cells were maintained in Dulbecco Modified Eagle's Medium (DMEM) containing 2 mM L-Glutamine, 100 µg/ml penicillin-streptomycin and 10 % heat inactivated fetal bovine serum (FBS).

Cells were plated one day before transfection onto 60 mm ∅ Petri-dishes to allow attachment of the cells under normal conditions (37 °C, 5 % CO_2, in DMEM medium

described above). Cells were cotransfected with pOG44 and the final vector (pcDNA5/FRT-GABA$_B$R1α-pBudCE4.1-GABA$_B$R2) at a ratio of 1:1 (w/w) by means of calcium phosphate precipitation method (MBS mammalian transfection kit). The Flp-In HEK293 cells were grown to 50-80 % confluence, the medium was changed with a transfection medium (solution 3: Modified Bovine Serum-MBS) and the mixture was drop wise added to the cells.

Transfection mixture for one dish (60 mm):

2.5 µg	pcDNA/FRT-construct
2.5 µg	pOG44
225 µl	H2O
25 µl	CaCl2 2.5 M
250 µl	2xBBS pH 6.95 (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid and buffered saline)

After 3 h incubation (37 °C, 5 % CO2) the transfection medium was discarded. The cells were further incubated overnight, in fresh culture medium (2.5 ml per plate: DMEM, 2 mM L-Glutamine, 100 µg/ml penicillin-streptomycin and 10 % FBS), at 37 °C in a CO_2 incubator.
The cells were further incubated in culture medium with hygromycin till the foci were formed on the petri-dish. The hygromycin resistance Foci were picked up and analysed for β-galactosidase expression.

For purposes of further analysis, cells were frozen. The cells were grown to 80-90 % confluence. The dish was washed with D-PBS and 4 ml culture medium and 2 ml trypsin was applied to detach the cells. Then, cells were transferred to 15 ml Falcon tubes and harvested by centrifugation (1000 rpm for 1 min). The pellet was resuspended in 2 ml of culture medium and 0.5 ml of the resuspended cells were mixed with 0.5 ml freezing medium. The cells were kept in a cryo-freezing box at -80 °C for 48 hours before transferred to the liquid nitrogen (-180 °C).

For X-Gal cell staining, cells were grown onto 16 mm glass cover-slips. For cell lysate preparation, cells were washed with D-PBS, scraped in 1 ml lysis buffer (1h

incubation at 4 °C), centrifuged at 7000 x *g* for 10 min and the supernatant was collected and used for western blotting.

2.2.4.3 X-Gal staining

β-galactosidase hydrolyzes X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) to an insoluble dense blue compound 5-bromo-4-chloro-indigo (Horwitz et al., 1964; Davies and Jacob, 1968). Thus, cells expressing ß-galactosidase are stained in blue and can be easily distinguished from those without *lacZ* expression.

The X-gal staining of the HEK293 cells used in this study was performed in coverslips. Briefly, cells were washed three times with 1X PBS, and then fixed with 3 % formaldehyde for 5 min on ice. After fixation, cells were washed three times with 1X PBS and incubated in X-gal staining solution (1 ml DMF, 40 mg X-Gal, 50 µl 1 M $MgCl_2$, 41 mg potassium hexacyanoferrate III, 53 mg potassium hexacyanoferrate II and in 25 ml PBS) for 1 h at 37 °C.

Stable Flp-In expression cell lines with the $GABA_B$ receptors can be selected for lack of β-galactosidase activity. Insertion of pcDNA5/FRT-construct into the genome at the FRT site (see figure 2.6) results in loss of β-galactosidase activity. Cell lines with inactive β-galactosidase (lac^-) will remain white and not appropriate transfected clones (lac^+) will forme blue colonies (see figure 3.1).

2.2.5 cAMP immunoassay

The cAMP Biotrak Enzymeimmunoassay (EIA) kit from Amersham Biosciences was used for the cyclic AMP measurements. The assay is based on competition between unlabelled cAMP and a fixed quantity of peroxidase-labelled cAMP, for a limited number of binding sites on a cAMP specific antibody (see figure 2.7).
The assay was performed followed the acetylation EIA procedure (protocol 2), which allows cAMP measurements in tissue extracts where higher sensitivity is required.

The acetylation assay, the cAMP Biotrak (EIA) includes:

- 96 well plate coated with donkey anti-rabbit IgG
- Assay buffer: 0.05 M sodium acetate buffer, pH 5.8 containing 0.02 % Bovine Serum Albumin and 0.01 % preservative.
- Standard: cAMP standard 10.24 pmol, for acetylation assays in the range 2–128 fmol/well, lyophilised. On reconstitution this bottle contains 2.56 pmol cAMP/ml.
- Antibody: Rabbit anti-cAMP, lyophilised.
- Peroxidase conjugate: cAMP- Horseradish Peroxidase, lyophilised.
- Wash buffer concentrate: 0.01 M phosphate buffer, pH 7.5 containing 0.05 % Tween™ 20.
- TMB substrate: enzyme substrate containing 3,3',5,5'–Tetramethylbenzidine (TMB)/Hydrogen Peroxide.
- Acetic Anhydride and Triethylamine

Brainstem from neonatal or adult mice was minced and incubated in physiological solution (118 mM NaCl, 3 mM KCl, 1.5 $CaCl_2$, 1 mM $MgCl_2$, 25 $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM glucose, equilibrated with carbogen at 25-27 °C p.H 7.4 in the presence or absence of agonist or antagonist (100 µM baclofen, 50 µM CGP 55845A). Samples were stored at -40 °C.

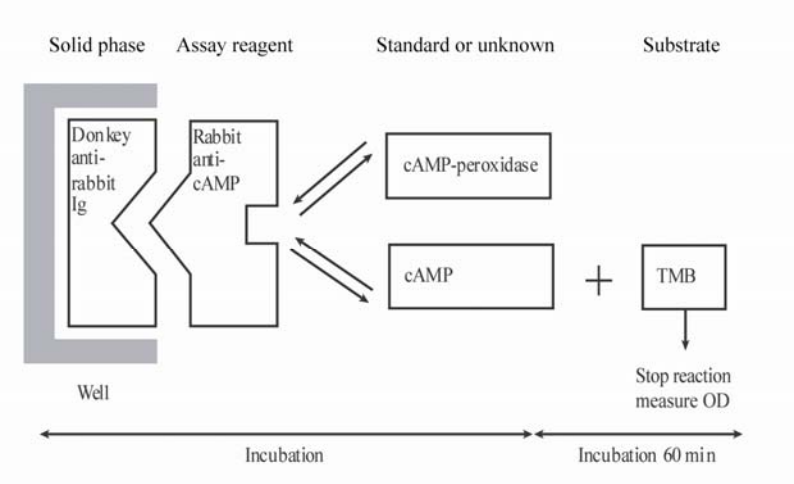


Figure 2.7. Enzyme-immunoassay principle. (Adapted from the cAMP Biotrak EIA System manual, Amersham Biosciences).

The frozen tissues were homogenized in cold 6 % (w/v) Trichloroacetic acid at 2–8 °C (10 % (w/v) homogenate). 200 µl of the homogenates were centrifuged at 2000 *g* for 15 minutes at 4 °C. The supernatant was washed 4 times with 5 volumes of water saturated diethyl ether. The aqueous extract was dried in a dry speedvac (-80 °C) and stored at -40 °C until use.

The rest of the assay was performed, as described in the kit protocol. Briefly the actual amount of cAMP was determined for each sample in comparison to a standard curve of known amounts of cAMP provided in the cAMP kit.

2.2.6 GTP binding assay

2.2.6.1 Immunoprecipitation of [^{35}S] GTPγS-labelled G proteins.

Mice were decapitated and brainstems were rapidly excised at 4 °C (see section 2.1.9). The tissue was homogenized using a glass teflon homogenisator in 20 volumes (w/v) of ice-cold Tris-HCl buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$ and 1 mM EGTA. The homogenate was centrifuged at 4 °C for 30 min at 16000 *g*. The pellet was resuspended in assay buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EGTA and 100 mM NaCl and centrifuged for 30 min at 16000 *g* at 4 °C. The final pellet was resuspended in assay buffer and stored at -80 °C.

The membrane homogenate (10 µg protein/assay) was resuspended in 70 µl of assay buffer with 10 µM guanosine-5´-diphosphate (GDP). Reaction mixtures additionally contained $GABA_BR$ agonist (100 µM baclofen) or $GABA_BR$ agonist (100 µM baclofen) preincubated with $GABA_BR$ antagonist (10 µM CGP 55845A) or assay buffer as contro).

Incubation was performed at 27 °C for 1 h shaking, after adding [^{35}S] GTPγS (1300 Ci/mmol) to a final concentration of 30 nM. The reaction was terminated by adding 140 µl of 50 mM Tris-HCl pH 7.4, containing 20 mM $MgCl_2$, 150 mM NaCl, 0.5 % Nonidet P-40, 200 µg/ml Aprotinin, 100 µM GDP and 100 µM GTP and incubating on ice for 15 min.

The samples were agitated for 1 h at 4 °C with 10 µl of appropriate Gα subunits-directed antibody with 100 µl of 10 % suspension of protein A-Sepharose.

Immunoprecipitates were washed three times, boiled in 0.5 ml of 0.5 % SDS and radioactivity was measured by scintillation spectrometry.

2.2.6.2 Immunoprecipitation of Eu-GTP-labelled G proteins.

The europium-labelled GTP binding assay was performed according to some modifications of the [^{35}S] GTPγS binding assay described previously (see section 2.2.6.1).

The membranes were prepared as described above and the homogenate (10 µg protein/assay) was resuspended in 70 µl of assay buffer with 10 µM GDP. The membrane aliquots contained 100 µM baclofen or 100 µM baclofen preincubated with 10 µM CGP 55845A or assay buffer as control. After a 10 min preincubation at 27 °C on a plate shaker, 100 nM Eu-GTP was added. Incubation was continued for another 50 min, and the reaction was terminated by adding 140 µl of 50 mM Tris-HCl pH 7.4, containing 20 mM $MgCl_2$, 150 mM NaCl, 0.5 % Nonidet P-40, 200 µg/ml Aprotinin, 100 µM GDP and 100 µM GTP and incubating on ice for 10 min.

The samples were agitated for 1 h at 4 °C with 10 µl of appropriate Gα subunits-directed antibody with 100 µl of 10 % suspension of protein A-Sepharose. Immunoprecipitates were washed three times (50 mM Tris-HCl, pH 7.4) and the pellet was boiled in 0.5 ml of 0.5 % SDS at 99 °C for 4 min.

The protein A-Sepharose beads were then pelleted and the supernatant was transferred to a 96 well plate.

The bound Eu-GTP was measured using the factory-set protocol for europium measurements (340 nm excitation/ 615 nm emission) with the GENios Pro plate reader (Tecan Deutschland GmbH).

3 RESULTS

3.1 Cloning and expression of $GABA_BR1a$ and $GABA_BR2$ from rat in HEK 293 cells

The first $GABA_B$ receptor cDNA, $GABA_BR1a$ from rat, was cut out from pcIneo by *NheI* and *NotI* endonuclease and subcloned in pcDNA5. Respectively rat $GABA_BR2$ cDNA was cut out from pcIneo by *XhoI* and subcloned in pBudCE4.1.

The new $GABA_BR1a$ and part of the new $GABA_BR2$ constructs were ligated to a pcDNA5-FRT/$GABA_BR1a$_pBudCE4.1/ $GABA_BR2$ final vector (see figure 2.5).

This final construct contained the two $GABA_B$ receptor subunits which are under the control of the CMV promoter from pcDNA5 (with the $GABA_BR1a$) and EF-1α promoter from pBudCE4.1 (with the $GABA_BR2$). This construct had in addition an FRT (recombination target) site, which in combination with the pOG44-FLP site, insert into the final construct (with the two $GABA_B$ subunits) into the integrated FRT site of the Flp-In HEK293 host cell genome. Thus the Flp-In HEK 293 cells were stably expressing the $GABA_BR1a$ and $GABA_BR2$ subunits. To detect cell lines that express the receptor, i.e cells with the $GABA_BR$ subunit-construct in the proper position (FRT site) of the HEK293 cells, we used X-gal staining. Thus HEK 293 cells stably transfected with $GABA_BR$ subunits, showed lack of β-galactosidase activity and they appeared white (see figure 3.1).

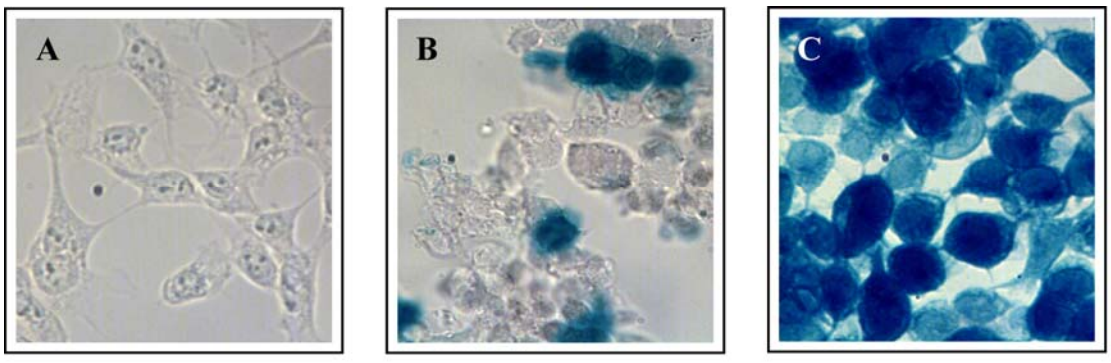


Figure 3.1. X-gal staining of HEK293 cells stably transfected with $GABA_BR1α$ and $GABA_BR2$. Cells expressing β-galactosidase (lac^+) are stained in blue (C), lac^- cells that do not express β-galactosidase remain white. B shows mixed colonies with lac^+ and lac^- cells.

To detect co-expression of $GABA_BR1a$ and $GABA_BR2$, the β-galactosidase negative cell lines where stained with primary antibodies directed against the $GABA_{B1a}$ and $GABA_{B2}$ subunits, and secondary antibodies marked with Cy3 and Cy5. The cells were then analysed by confocal laser scan microscopy, as shown in Figure 3.2.
This immunofluorescence staining showed a proper expression of the $GABA_BR1a$ protein mainly on the cell surface of the transfected HEK293 cells.

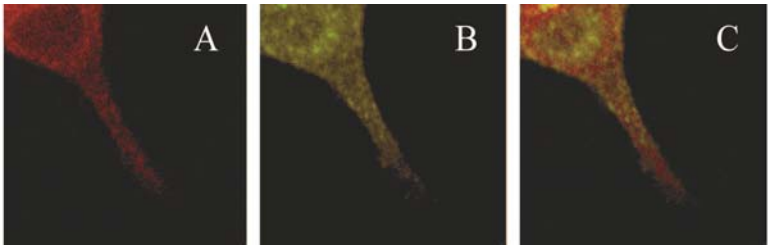


Figure 3.2. Double-labelling of $GABA_BR1a$ and $GABA_BR2$ subunits. HEK293 cells stably transfected with $GABA_B$ R1a (A, red; Cy5) and $GABA_B$ R2 (B, green; Cy3). (C) Overlay of panels A and B shows that $GABA_B$ R1a and R2 are not co-localized.

In contrast, the $GABA_BR2$ showed an irregular distribution; big spots and mainly intracellular. $GABA_BR1a$ and $GABA_BR2$ showed no co-localization (Figure 3.2/C). A 3D picture showed that the $GABA_B$ R2 subunit can not reach the surface.

3.2 Ontogenetic development of the $GABA_B$ receptor in mouse brain

To examine whether $GABA_BR$ shows any age dependant molecular changes, brains from NMRI mice (P0-2 and adult) were analyzed by molecular biological methods. Information about the molecular structure of the $GABA_B$ receptor was untill recentlly only available from human and rat. To identify the mouse $GABA_{B2}$ receptor cDNA sequences we performed a BLAST search of the GenBank expressed sequence tag (EST) database using the amino acid sequence of the rat $GABA_BR2$ as the query. $GABA_BR2$ was our subunit of interest, thus this subunit contains all the molecular determinants for G protein coupling. A mouse $GABA_{B2}$ receptor cDNA was detected (GenBank Accession No. NP_001074610) and in addition a second mouse EST from a new born mouse with a Accession No. XM_143750, predicted as $GABA_BR2$ (http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val=XM_143750.4). An alignment

of the two detected $GABA_BR2$ sequences the NP_001074610 and the predicted XM_143750.4 shows that the latter one has 35 additional amino acids at the end of the fifth transmembrane region in the intracellular part of the receptor. As our main interest was age related differences at the protein levels of $GABA_BR2$ in the G protein coupling regions (heptahelical domains, intracellular loops and intracellular C-terminal domain) this was a promising difference, which had to be further investigated.

To analyze the eventually altered expression of $GABA_BR2$ between P0 and adult animals, total RNA was isolated from brainstems and the resulting $GABA_BR2$ cDNA was analyzed by sequencing. In detail, RNA from brainstems was isolated with trizol reagent and analyzed by spectrophotometry and reverse transcriptase-PCR (RT-PCR).

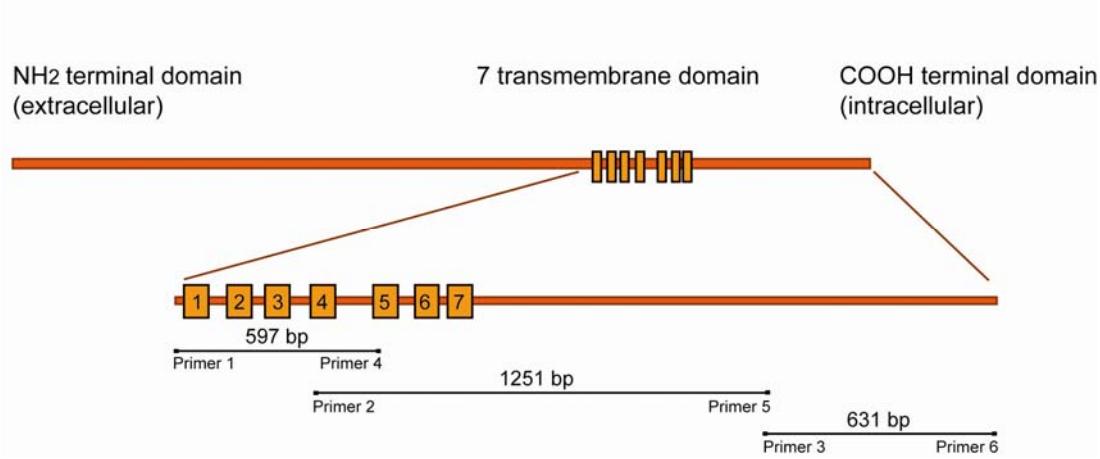


Figure 3.3. Diagram of polymerase chain reaction (PCR) products and relative primer positions on $GABA_BR2$ cDNA. The upper part illustrates the $GABA_BR2$ protein structure; boxes represent the 7 TMD; the line joining them illustrates the NH_2-, COOH-terminal domains and the inter- and extracellular loops. The mid part is an enlargement of the region of interest (7 TMD and COOH-terminal domain) and the last part shows the PCR primer design regions.

Primer 1: TTCGGAAGATCTCGCTTCCACTGT Primer 4: AACAACCGAACAACATGAGGAGCC
Primer 2: TGCTGATCGATCTGTGCATCCTGA Primer 5: CGCCAAGGAGAAGTCATTGCCATT
Primer 3: TCCTTGGCGTCTGCAAACAAAGAG Primer 6: CCATGGGAGAGGTTAAAGTGCACAA

For the reverse transcription of the mRNA to a complementary DNA (cDNA) we used Omnoscript (Qiagen, Hilden) with 10 µg of mRNA as template and the resulting first-strand cDNA was then amplified by PCR. Three primer pairs were designed to amplify three DNA fragments, which screen the receptor $GABA_{B2}$ G-protein coupling regions. The first primer pair 5'-TTCGGAAGATCTCGCTTCCACTGT-3' and 5'-

AACAACCGAACAACATGAGGAGCC-3', amplifies the $GABA_{B2}$ cDNA from the first transmembrane region untill part of the fifth transmembrane region, yielding a fragment of 597 bp after 30 cycles (see figure 3.3). Analog, the second primer pair 5'-TGCTGATCGATCTGTGCATCCTGA-3' and 5'-CGCCAAGGAGAAGTCAT-TGCCATT-3' amplifies the forth transmembrane region till half of the C-terminal region, yielding a fragment of 1251 bp and the third primer pair 5'-TCCTTGGCGTC-TGCAAACAAAGAG-3' and 5'-CCATGGGAGAGGTTAAAGTGCACAA-3' yields a fragment of 631 bp which encodes the second half of the C-terminal region till the end of it (see figure 3.3). After amplification the products (6 µl) were analyzed on a 1.5 % agarose gel (see figure 3.6). PCR products from adult $GABA_BR2$ with the desired size were identified, as shown in figure 3.6. The three bands with the expected size of 597, 1251 and 631 bp are present in the last three bags of the gel.

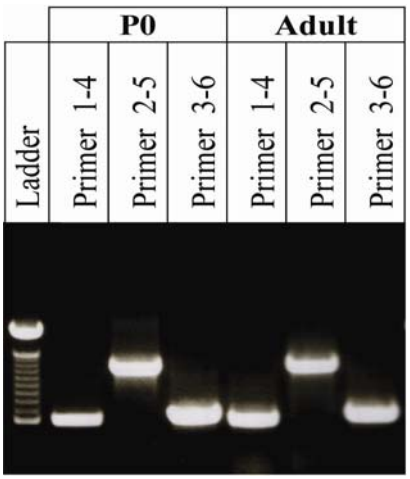


Figure 3.6. Agarose gel showing PCR amplified products obtained using the primer pairs 1-4, 2-5, and 3-6 from newborn and adult mouse brainstem $GABA_BR2$ cDNA. Line 2 to 4 are PCR products from newborn cDNA (597, 1251, and 631 bp respectively); line 5 to 7 are PCR products from adult cDNA (597, 1251, and 631 bp respectively), along with marker DNA (line 1).

In case of the existence of a 35-extra amino acid fragment in the $GABA_BR2$ at the fifth transmembrane region of new born mice, the second PCR product should have a size of 1356 bp, but as shown in figure 3.6., the three PCR fragments from new born have the same size as the adult mouse (597, 1251 and 631 bp). Thus, the predicted $GABA_BR2$ protein with the accession No. XM_143750 is not expressed at any of the investigated ages and there is no difference in the expression of the $GABA_{B2}$ receptors between adult and young mice.

In addition, the isolated three cDNA $GABA_BR2$ fragments from both ages were analyzed by sequencing to examine the existence of potential point mutations or any

other substitute, which can have influence on the expression and eventually the function of $GABA_{B2}$ receptors.

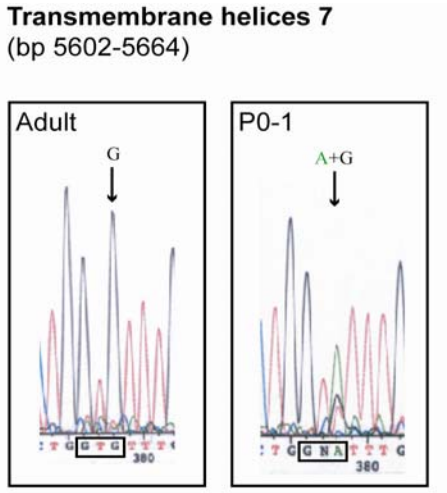


Figure 3.4. Electropherograms showing part of the transmembrane helices 7 sequence in adult and newborn mice. A G to A/G substitution at nucleotide position 5658 is marked with an arrow. The marked codon GTG in adult encodes a valine (V). The mixed A/G signal at the same position in the P0 mouse does not modify the amino acid. Both triplets GTG and GTA encode valine.

For this reason we isolated and sequenced the six PCR fragments. Sequence analysis revealed differences between adult and newborn mouse genome of $GABA_{B2}$ receptor at the positions 5658 and 5790. The electropherograms (see figure 3.4 and 3.5) show at those positions the two mixed signals in the cDNA of the newborn mice.

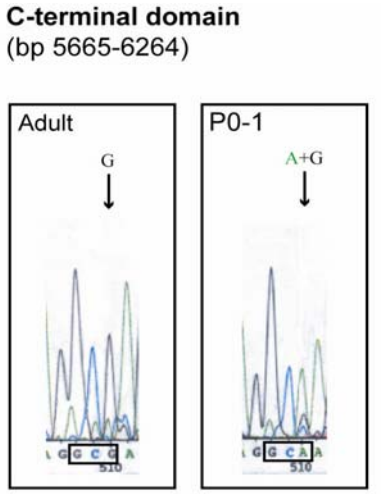


Figure 3.5. Electropherograms showing part of the C-terminal domain sequence in adult and newborn mice. A G to A/G substitution at nucleotide position 5790 is marked with an arrow. The marked codon GCG in adult encodes alanin (A). The mixed A/G signal at the same position in the P0 mouse does not modify the amino acid. Both triplets GCG and GCA encode alanin.

At the position 5658, which is part of the seventh transmembrane region, the cDNA of the younger mice shows a double peak of adenin and guanin. In contrast to the adult cDNA, shows a single G-peak. The same switch takes place at the position 5790.

In both cases the G/A switch to G leads to silent mutations. Both codon GTG and GAA (position 5658) encode the amino acid valine and at the position 5790, GCG and GCA encode the amino acid alanin.

Therefore, it can be concluded that there is no change in the expression of $GABA_B$ receptor 2 in the developing mouse brainsteam.

3.3 Changes in the $GABA_B$ receptor signaling in the developing mouse brainstem

$GABA_B$ receptor functionality and selectivity to diverse G proteins subtypes was studied by baclofen stimulated [^{35}S]-GTPγS (Harrison and Traynor 2003) and GTP-Eu binding assay.

The [^{35}S]-GTPγS-binding assay measures the level of the G protein activation, following agonist occupation of a GPCR, by determining the binding of [^{35}S]-GTPγS to the Gα subunits. In general, the assay is experimentally more feasible for receptors coupled to the abundant $G\alpha_{i/o}$ proteins. Nevertheless, [^{35}S]-GTPγS binding assays are used also with GPCRs that couple to the $G\alpha_s$ and $G\alpha_q$ families of G proteins. For the measurement of the labeled Gα the labeled-Gα subunits are immunoprecipitation with Gα-antibodies which are cross-linked with protein A-Sepharose.

[^{35}S]-GTPγS binding to $GABA_B$ receptor was performed in young and adult mouse brainstem homogenates. To evaluate whether receptor activation could increase GTPγS binding, cells were incubated in the presence of a $GABA_B$ receptor agonist and receptor antagonist. The following immunoprecipitation was then performed with antibodies against $G\alpha_{i/o}$, $G\alpha_{i3}$, $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12}$ and $G\alpha_{13}$ as described under chapter 2.2.6.1. Fused proteins were then isolated on protein A-Sepharose, and bound [^{35}S]-GTPγS was eluted and quantified by scintillation counting.

The experiments were done in duplicate. The figure 3.7 shows the results as the percentage of mean control values. The $G\alpha_{i/o}$ subunit showed increase of the bounded [^{35}S]-GTPγS after treatment with baclofen and a decrease after the addition of an antagonist, compared to the control (inactivated $GABA_B$ receptor). It is known that $GABA_B$ receptors interact with $G\alpha_{i/o}$ proteins, but in addition was observed an increase of $GABA_B$ receptors binding to $G\alpha_{i/o}$ in newborn mouse brainstem. $GABA_B$

receptors binding affinity to $G\alpha_{i3}$, $G\alpha_{12}$ and $G\alpha_{13}$ shows no interactions between the receptor and those Gα proteins.

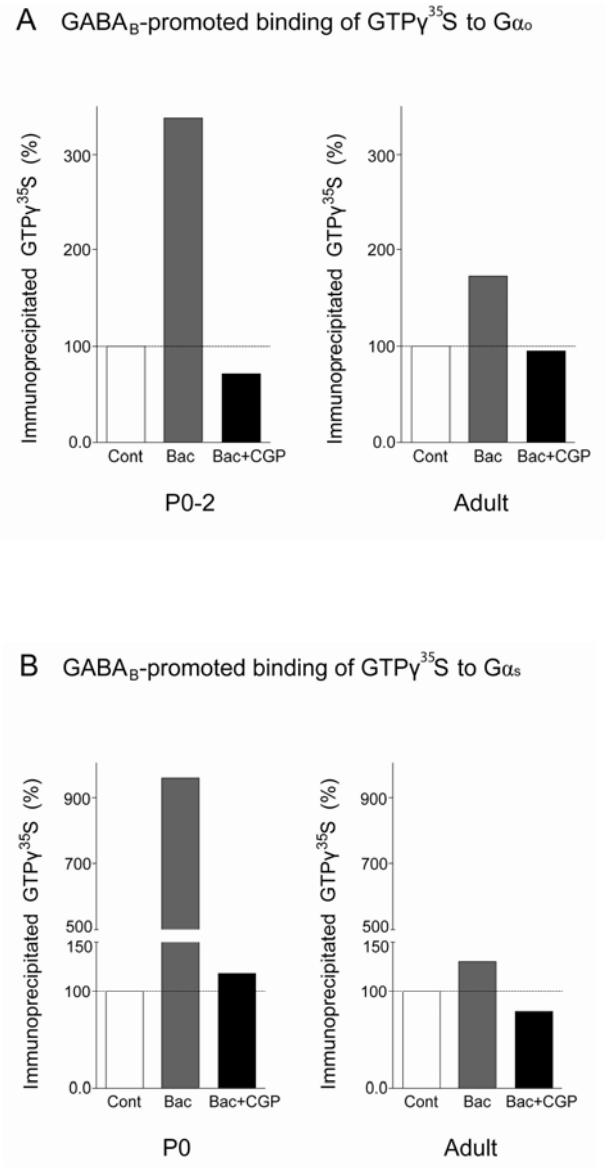


Figure 3.7. The diagram shows $GABA_B$ receptor agonist-stimulated [^{35}S]-GTPγS binding to specific Gα protein subtypes in brainstem.
Membranes prepared from brainstem of P0 and adult mice were incubated with [^{35}S]-GTPγS in presence of buffer, 100 μM baclofen and 10 μM CGP 55845A.
Immunoprecipitation was performed with appropriate antibodies against different Gα subunits and bounded [^{35}S]-GTPγS was quantified by scintillation counting. Results were calculated as the percentage of mean control values and represent two independent experiments.
A) Shows, as expected, an increase of the immunoprecipitated $G\alpha_{i/o}$ at both ages after stimulation with baclofen.
B) Shows an unexpected dramatical increase of the immunoprecipitated $G\alpha_s$ in young animals, which hardly appears at adult mouse brainstem after activation with baclofen.

The figure 3.7.B shows the $GABA_B$ receptors interaction to $G\alpha_s$. In this case an unexpected dramatical increase of the immunoprecipitated $G\alpha_s$ in young animals was observed in contrast to the near negligble shift at adult mouse brainstem after activation with baclofen. As the results showed an unexpected difference of $GABA_B$ receptor interaction to the $G\alpha_s$ protein subtypes between the two ages (P0 and adult), additional experiments were necessary.

Further experiments for the $GABA_B$ receptor functionality and selectivity to diverse G proteins subtypes were done with a non-radioactive, non-hydrolyzable, europium

labeled GTP analogue, the GTP-Eu. The GTP-Eu binding assay yielded similar values to the traditional [^{35}S]-GTPγS assay (Frang, Mukkala et al. 2003; Labrecque, Anastassov et al. 2005) but with less intensity. We developed Eu-GTP-binding assay with immunoprecipitation in order to avoid problems involved in working with radioactivity and to help us to increase the number of experiments.

The assay was optimized as described under 'Material and methods'. The young and adult mouse brainstem homogenates were incubated in the presence of a $GABA_B$ receptor agonist and antagonist together with the europium-labeled GTP.

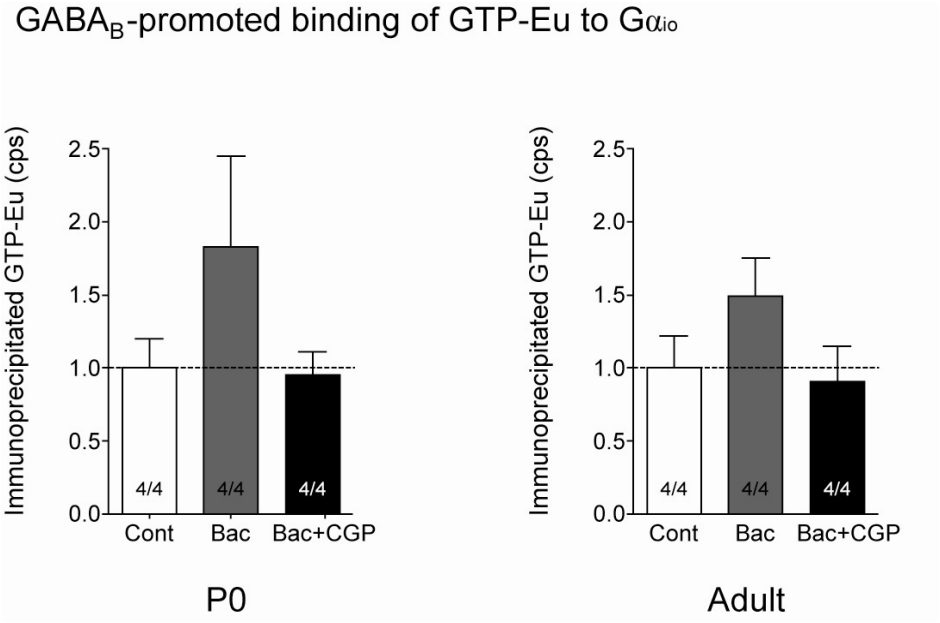


Figure 3.8. The diagram shows $GABA_B$ receptor agonist-stimulated Eu-GTP binding to specific $G\alpha_{i/o}$ protein subtypes in brainstem. Membranes prepared from brainstem of P0 and adult mice were incubated with Eu-GTP in presence of buffer, 100 μM baclofen and 10 μM CGP 55845A. Immunoprecipitation was performed with an antibody against $G\alpha_{i/o}$ subunits and bounded Eu-GTP was quantifie using the autofluorescent properties of Eu (340 nm excitation/615 nm emission) by using the GENios pro plate reader. The experiments were done in fourplicate and the figure shows the means ± standard deviation.

The samples were further incubated with antibodies against $G\alpha_{i/o}$, $G\alpha_{i3}$, $G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{12}$ and $G\alpha_{13}$. The fused proteins were isolated on protein A-Sepharose, and bound Eu-GTP was washed, eluted and quantifie by using the autofluorescent properties of europium (340 nm excitation and 615 nm emission).

The experiments were done in fourplicate. The figures 3.8, 3.9 and 3.10 show the means ± standard deviation.

The G protein subunits alpha i3, alpha 12 and alpha 13 did not bind appreciable levels of Eu-GTP, at both ages (P0 and adult), consistent with a minimal level of GDP/Eu-GTP exchange. In contrast, the subunits alpha i/o (see figure 3.8) and alpha s (see figure 3.9) bound measurable levels at the age of P0.

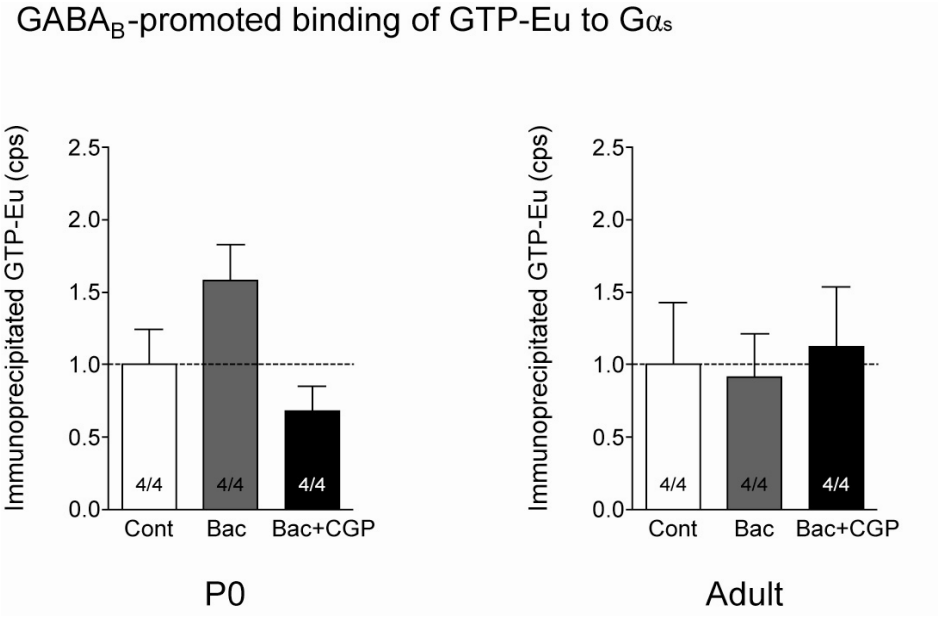


Figure 3.9. The diagram shows $GABA_B$ receptor agonist-stimulated Eu-GTP binding to specific $G\alpha_s$ protein subtypes in brainstem. Membranes prepared from brainstem of P0 and adult mice were incubated with Eu-GTP in presence of buffer, 100 μM baclofen and 10 μM CGP 55845A. Immunoprecipitation was performed with an antibody against $G\alpha_s$ subunits and bounded Eu-GTP was quantifie using the autofluorescent properties of Eu (340 nm excitation/615 nm emission) by using the GENios pro plate reader. The experiments were done in fourplicate and the figure shows the means ± standard deviation.

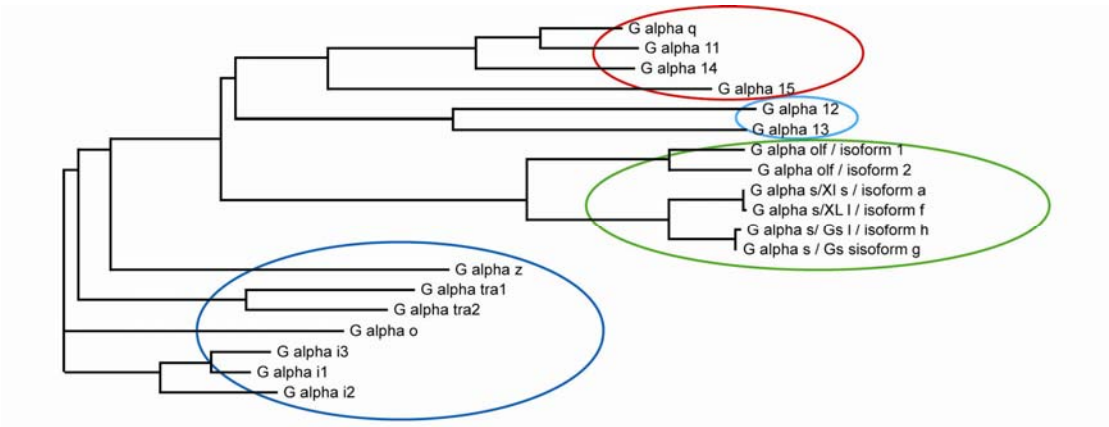
In mature brainstems only the $G\alpha_{i/o}$ shows measurable levels of Eu-GTP, the $G\alpha_s$ binding in adult brainstems was not different compare to control. Binding to $G\alpha_{q/11}$ was weak and not significant.

In summary, the results of the Eu-GTP-binding and [^{35}S]-GTPγS binding assays with immunoprecipitation confirm the coupling of the $GABA_B$ receptor to $G\alpha_{i/o}$ at both ages in brainstem with more robust increases at younger ages. The $G\alpha_{i3}$, $G\alpha_{12}$ and

$G\alpha_{13}$ did not show any interaction to $GABA_B$ receptor. The $G\alpha_{q/11}$ was in both cases not significant (Eu-GTP and [^{35}S]-GTPγS), although there was a weak signal after activation with baclofen. The surprisingly result was the $G\alpha_s$ interaction with the $GABA_B$ receptors only in new born mice, something which disappears in adult animals.

3.4 Maturation of the mouse brain is associated with a differential expression of variants of G protein alpha subunits

To investigate and analyze the expression of the Gα-protein subtypes we had to identify which subtypes exist in mouse. Nineteen Gα protein sequences were collected from Genbank at NCBI by keyword search and sequence similarity using BLAST.



$G\alpha_{s\text{-}f}$	NP 001070975	$G\alpha_o$	NP 034438	$G\alpha_{13}$	NP 034433	$G\alpha_{olf\text{-}1}$	NP 034437
$G\alpha_{s\text{-}a}$	NP 034439	$G\alpha_q$	NP 032165	$G\alpha_{15}$	NP 034434	$G\alpha_{i\text{-}3}$	NP 034436
$G\alpha_{s\text{-}h}$	NP 963910	$G\alpha_{t1}$	NP 032166	$G\alpha_{11}$	NP 034431	$G\alpha_{14}$	NP 032163
$G\alpha_{s\text{-}g}$	NP 001070978	$G\alpha_{12}$	NP 034432	$G\alpha_{i1}$	NP 034435	$G\alpha_{olf\text{-}2}$	NP 796111
$G\alpha_{i\text{-}2}$	NP 032164	$G\alpha_z$	NP 034441	$G\alpha_{t2}$	NP 032167		

Figure 3.10. Phylogenetic tree of the mouse Gα subtypes. The phylogenetic tree was constructed from ClustalW multiple sequence alignment of the amino acid sequences of the Gα family members derived from mouse. The Gα amino acid sequences were collected from the National Center of Biotechnology Information (NCBI) database.
The encircled areas represent the four Gα families: $G\alpha_s$, $G\alpha_{12}$, $G\alpha_q$, $G\alpha_i$. The $G\alpha_s$ major branch has two main sub branches. One of these contains the $G\alpha_s$ isoforms olf-1 and olf-2 and the second consists of the $G\alpha_s$ isoforms g, h and a, f. In addition, it is not clear if all the $G\alpha_s$ mouse subtypes are known.

The sequences were analyzed by multisequence alignments and phylogenetic analysis (see figure 3.10). The phylogenetic tree produced by CLUSTALW organises the proteins by sequence similarity by using the program PHYLIP.

All Gα proteins could be assigned to four known families: $G\alpha_s$, $G\alpha_{12}$, $G\alpha_q$, $G\alpha_i$. The six primary antibodies that were used in this work represent one or two variants from each family group: $G\alpha_s$ antibody for the $G\alpha_s$ family; $G\alpha_{12}$ and $G\alpha_{13}$ for the $G\alpha_{12}$ family; $G\alpha_{q/11}$ for the $G\alpha_q$ group; and $G\alpha_{i\text{-}o}$ and $G\alpha_{i3}$ representatives for the $G\alpha_i$ family.

Multisequence alignments from the Gα family members were generated for checking the binding sites recognised by the antibodies, and for the size calculation of the Gα protein subtype variants. This was especially necessary for the $G\alpha_s$. Thus the GNAS locus encodes many $G\alpha_s$ variants. In our case the $G\alpha_s$ (Santa cruz biotechnology, sc-823) antibody binds the fourth and part of the fifth exon (see figure 3.11). The third exon which is spliced in almost each second Gnas product is not included in the sequence recognized by the $G\alpha_s$ antibody. That means that this antibody is able to bind to almost all known splice variants of the $G\alpha_s$ proteins (see figure 1.2). For example the $G\alpha_s$ short (GenBank Acc. Nr. NP_001070978) and long (GenBank Acc. Nr. NP_963910) isoforms differ in the existence or not of exon 3. The same applies to the GαsXL short (GenBank Acc. Nr. NP_001070975) and long (GenBank Acc. Nr. NP_034439) isoforms, they also differ in exon 3. In addition, the antibody should not disturb the interaction between the $G\alpha_s$ subunit and $GABA_B$ receptors. Like other GPCRs, $GABA_B$ receptors recognize the very COOH terminus of Gα subunits, encoded by exon 12 in mouse and 13 in human, exons 4 and 5 are in the N-terminus.

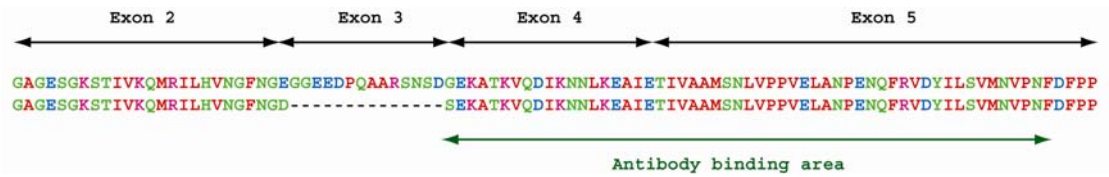


Figure 3.11. Alignment of the $G\alpha_s$ (short and long subtypes). The alignment of protein sequences was perfomed using ClustalW algorithm. Web Site: www.ebi.ac.uk/clustalw. Residues are colored as follows: red, small and hydrophobic amino acids; blue, acidic; magenta, basic; green, hydroxyl and amine amino acids, including Gln and Tyr. The green line shows the area where the $G\alpha_s$ antibody from Santa cruz (sc-823) binds.

3.4.1 Distribution of the $G\alpha_{i/o}$ and $G\alpha_{q/11}$ proteins in adult and P0 mouse brain areas

Western blot analyses were performed in order to examine the developmental changes in the distribution of the Gα subunits in brainstem. Using SDS PAGE and specific polyclonal antibodies oriented against $G\alpha_s$, $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{i3}$, all these proteins were determined by quantitative immunoblotting in homogenates prepared from cortex, cerebellum, and brainstem of P0 and adult animals (see chapter 2.2.2.1-7). The levels of all G protein α subunits, namely $G\alpha_{12}$, $G\alpha_{13}$, $G\alpha_{q/11}$, $G\alpha_{i/o}$ and $G\alpha_{i3}$, were already at birth high. Because of the $G\alpha_{i/o}$, $G\alpha_s$ and eventual $G\alpha_{q/11}$ interaction with the $GABA_B$ receptors, our main interest was focused on these three Gα subunits.

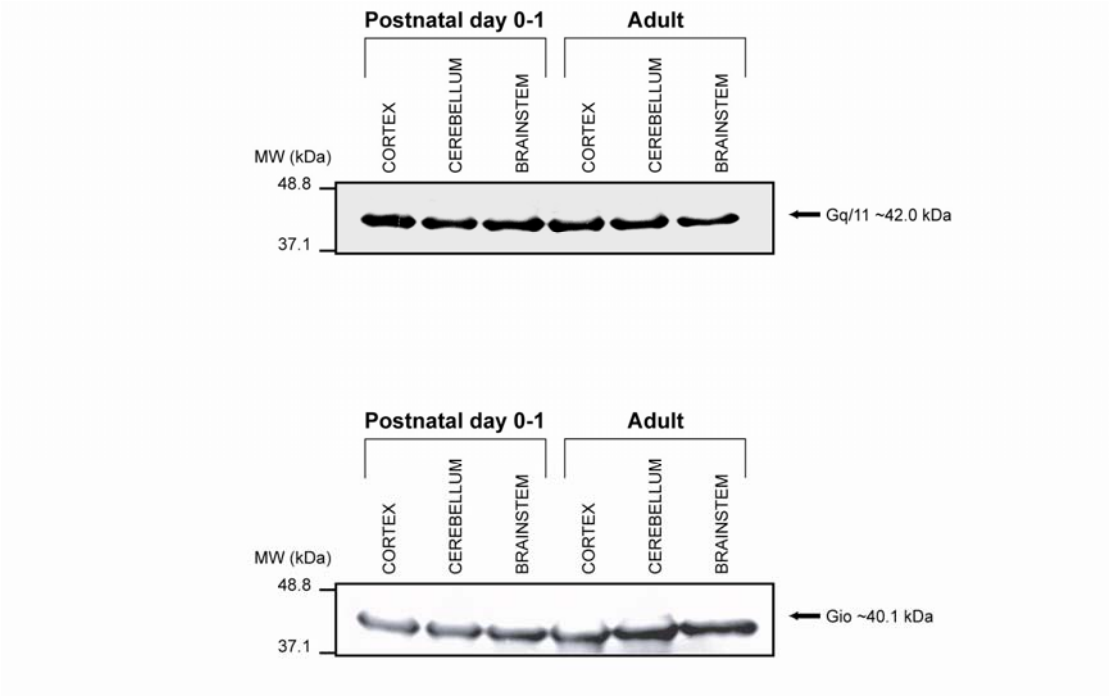


Figure 3.12. The unchanged $G\alpha_{i-o}$ and $G\alpha_{q/11}$ protein levels between different mouse brain areas of adult and newborn mouse. Representative immunoblots showing the distribution of $G\alpha_{i-o}$ and $G\alpha_{q/11}$ in the developing mouse brain areas. Whole cell homogenates (100 µg per lane) were prepared from mouse Cortex, cerebellum and brainstem, resolved by SDS-PAGE and immunobloted with $G\alpha_{i-o}$ or $G\alpha_{q/11}$ antibody (Santa cruz), as described in "Material and Methods".

A representative immunoblot (figure 3.12) shows that expression of $G\alpha_{i\text{-}o}$ and $G\alpha_{q/11}$ in cortex, cerebellum and brainstem was basically unchanged during postnatal development. The graph (figure 3.13) summarises densitometry measurements from four independent immunoblots.

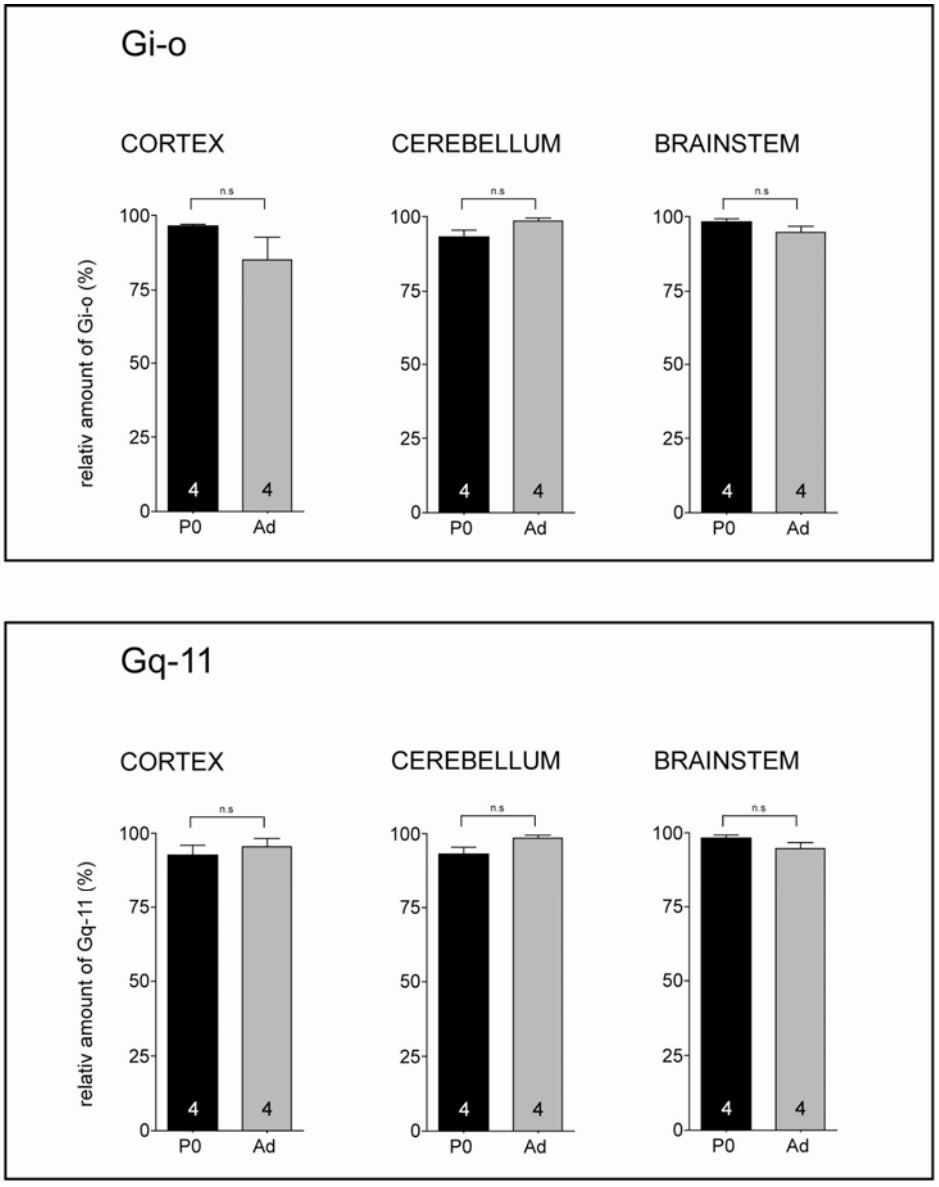


Figure 3.13. No significant changes were observed in the expression of $G\alpha_{i\text{-}o}$ and $G\alpha_{q/11}$ proteins between P0 and Adult mouse cortex, cerebellum and brainstem. The graph summarises the densitometry measurements of the immunoblots (Figure 3.13 for $G\alpha_{i\text{-}o}$ and $G\alpha_{q/11}$). The proteins ($G\alpha_{i\text{-}o}$ and $G\alpha_{q/11}$) were quantified by scanning densitometry and then expressed as mean percentages of the maximal values. Data are from four independent immunoblots and represent mean±S.E.M (two-tailed).

In case of the $G\alpha_{q/11}$ antibody the western blot analysis revealed both $G\alpha_q$ and $G\alpha_{11}$ subunits. For this reason RT–PCR was performed to examine the expression levels of the two Gα subunits in brainarea-specific and age-dependent manner.

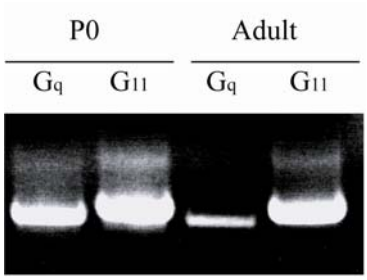


Figure 3.14. Agarose gel electrophoresis of PCR products obtained using the primer pair $G\alpha_q$ forw-rev and $G\alpha_{11}$ forw-rev. $G\alpha_{11}$ showes no age-dependent differences while $G\alpha_q$ shows a reduce of the mouse brainstem RNA during maturation.

Primer Gq	forw:	GCCAAGGAAGCCCGGAGGAT
Primer Gq	rev:	TCAGGATGAATTCTCGAGCTGCCT
Primer G11	forw:	CATCAACGCGGAGATCGAGAAAC
Primer G11	rev:	CTGCGAACACAAAGCGGATGTTC

RNA was isolated from brainstems of P0 and adult mice and analyzed by RT-PCR. For the following PCR two primer pairs were designed to amplify the $G\alpha_q$ (primer forw. GCCAAGGAAGCCCGGAGGAT; rev. TCAGGATGAATTCTCGAGCTGCCT) and $G\alpha_{11}$ DNA fragments (primer forw. CATCAACGCGGAGATCGAGAAAC; rev. CTGCGAACACAAAGCGGATGTTC). After amplification, the products were analyzed on a 1.5 % agarose gel (see figure 3.14). Although no difference was observed in the G11 RNA a significant decrease of the Gq RNA was observed in brain stem during aging.

3.4.2 Distribution of the $G\alpha_s$ protein subtypes in adult and P0 mouse brain areas

Developmental changes in the distribution of $G\alpha_s$ were investigated in the mouse brainstem during postnatal development (P0 and adult) by western blot analyses and densitometry measurements of four independent immunoblots showing above (see chapter 3.4.1).

We screened for α subunit splice variants of the stimulatory G protein family in whole cell homogenate of cortex, cerebellum, and brainstem from mouse and examined the expression of these splice variants during the course of postnatal development. The western blots revealed six bands with a totally different expression pattern during the

development of the mouse brainstem (see figure 3.15). All six bands disappear in control blots containing the blocking peptide for the $G\alpha_s$ antibody.

The protein size of these bands was calculated with the help of molecular weight markers, which were run simultaneously with the samples. In addition, the Bio-1D (Vilber Lourmet) software was used to calculate the fragment sizes of the bands with reference to molecular size markers.

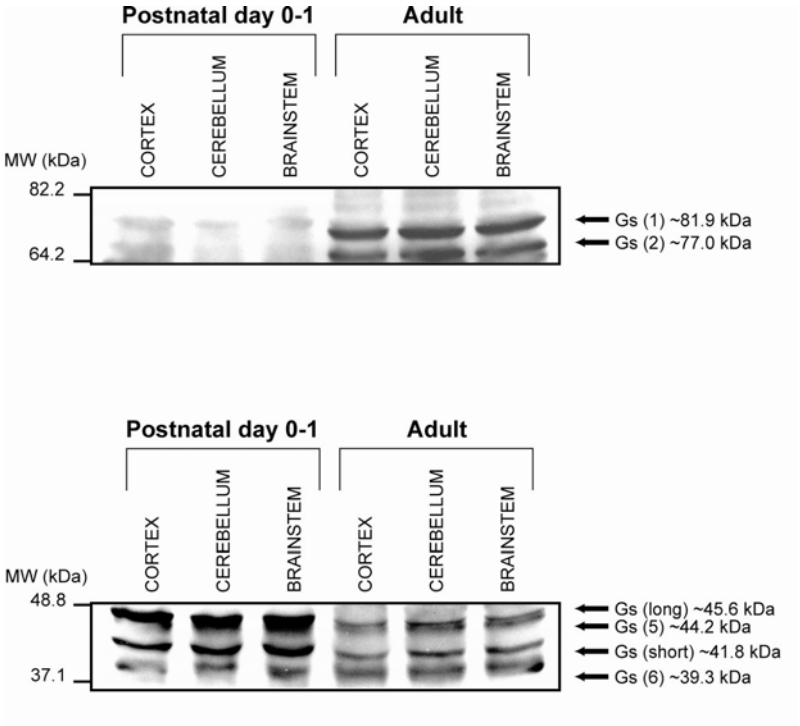


Figure 3.15. There is an age dependent $G\alpha_s$ subtypes expression in mouse brain areas. Representative immunoblots show the distribution of the $G\alpha_s$ isoforms in the developing mouse brain areas. Whole cell homogenates (100 µg per lane) were prepared from mouse cortex, cerebellum and brainstem, followed by immuno-blotting for a commercial $G\alpha_s$ antibody (Santa cruz), as described in "Material and Methods". Immunoblots are representative of four experiments.

The band intensity on the immunoblots was quantified using a scanning densitometer and is expressed as a percentage of the highest value (see figure 3.16).

The complexity of the GNAS locus, the number of alternative splice $G\alpha_s$ variants in combination with the conflicting reports and the continuously new data made it difficult to identify which band size represents which $G\alpha_s$ variant.

The long (GαsS) and short (GαsL) variants of $G\alpha_s$ with a molecular mass of 42 and 45 kDa respectively (see chapter 1.3.1.1), were relativelly easy to identify. Thus, the 41.8 and 45.6 kDa immunoreactive bands were interpreted as being the small (GαsS) and large (GαsL) forms of $G\alpha_s$ respectively. Both splice variants show an increase of the total amount during postnatal development in all three brain areas.

An additional minor band of 44.2 kDa (called Gs 5), between the short and long variant of $G\alpha_s$ was observed. This band has a similar expression pattern to the short and long $G\alpha_s$ variants, with an increase of the total amount during postnatal development. Nevertheless, the size of this band does not correlate to any known $G\alpha_s$ variant.

An other $G\alpha_s$ variant (called 6 in figure 3.15), which size does not represent any till now identified $G\alpha_s$ variant has the size of 39.3 kDa and shows an insignificant slightly increase, during development, in brainstem and a significant increase in cerebellum and cortex.

The two bands ($G\alpha_s$ 1 & 2) with a size between the marker-lines of 64 and 82 kDa were observed mainly in adult brains. Based on band size (approx. 81.9 and 77 kDa) and the binding properties of the $G\alpha_s$ antibody (see figure 3.11) we interpreted them as XLαs isoforms. The exact size as well as the number of the XLαs isoforms is still variate between different reports and data from the GenBank.

There are reports describing the XLαs as a 78 kDa (Klemke, Pasolli et al. 2000; Pasolli, Klemke et al. 2000), 92kDa (Kehlenbach, Matthey et al. 1994), 94 kDa (Bastepe, Gunes et al. 2002),and 98 kDa (Freson, Jaeken et al. 2003) protein. Research done on the GenBank showed that there are at least three different XLαs isoforms:

Name	**GenBank Accession No**	**Size (bp)**	**Size (kDa)**
isoform f	NP_001070975	1119	120.1
Isoforms a	NP_034439	1133	121.5
G protein XLα	AF116268	756	82.3

The size of these proteins was measured with protein calculations software.

In summary, these results demonstrate that expression levels of Gα subtypes in whole cell membrane homogenate in cortex, cerebellum and brainstem do not vary in a region specific manner, although alteration in the expression of the $G\alpha_s$ splice variants

and $G\alpha_q$ is observed. The rest of the investigated Gα (i/o, 11, 12, 13, i3) proteins remains constant also in an age- specific manner.

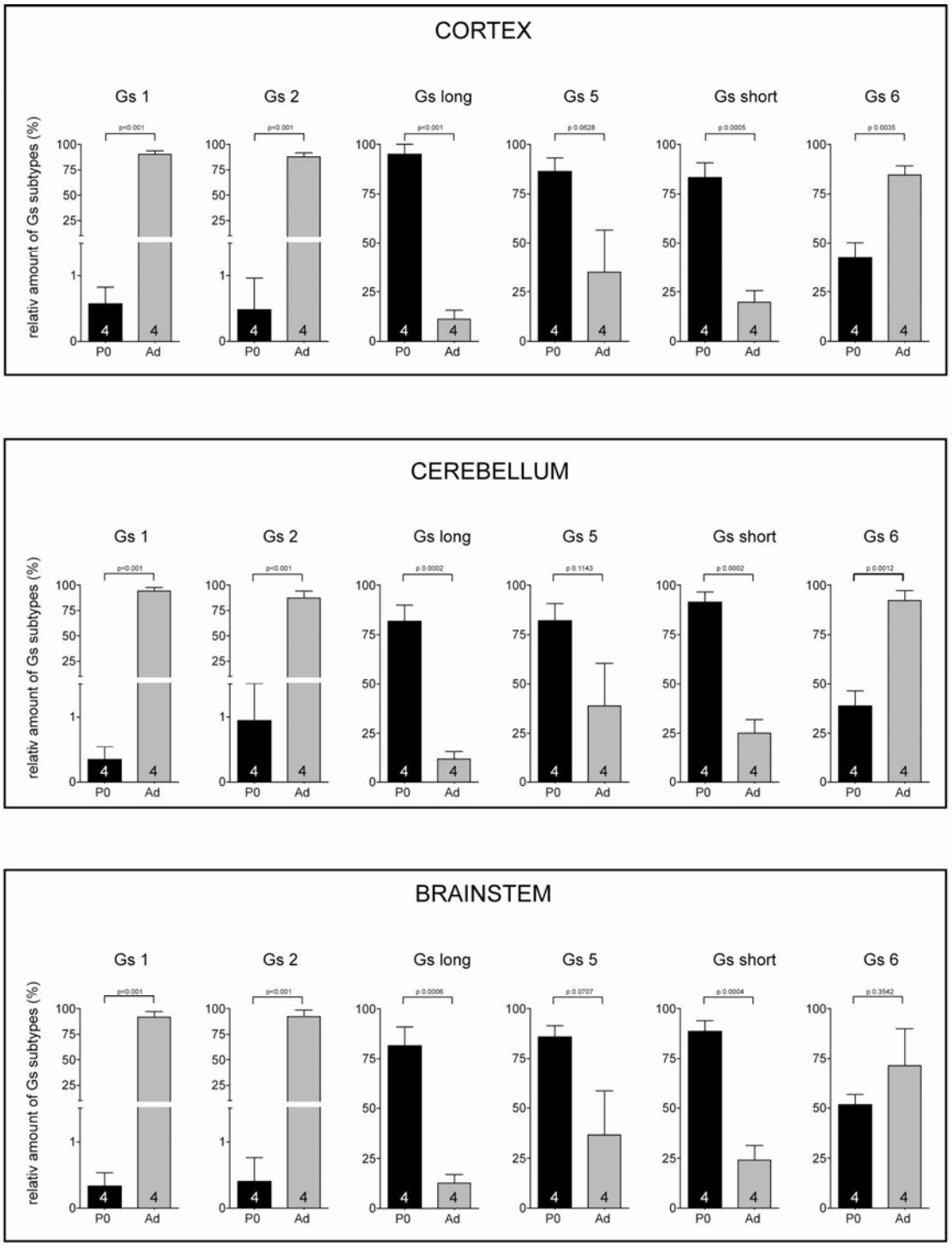


Figure 3.16. The differential distribution of $G\alpha_s$ subtypes between adult and newborn mice, in different brain areas. Immunoblot data (see figure 3.15) were quantified using scanning densitometry, and expressed as mean percentages of the maximal value of each isoform. The data are from four independent experiments and represent mean±S.E.M (two-tailed).

Although the complexity of the GNAS locus and its products did not permit us to distinguish which band exactly correlates with which certain $G\alpha_s$ variant, we show an increase of the short $G\alpha_s$ subtype variants and a sharp elevation in the expression of the long $G\alpha_s$ subtype in newborn animals comparing to adults. The most intense alteration in the expression of $G\alpha_s$ isoforms during maturation was observed in two bands, which were interpreted as XLαs subtypes.

3.5 cAMP levels in mouse brainstem after activation with baclofen

Activation of $G\alpha_s$-protein-coupled receptors is associated with activation of adenylate cyclase, which in turn promotes a cAMP production. Thus, the cAMP levels were measured by enzymeimmunoassay, as described in chapter 2.2.5.

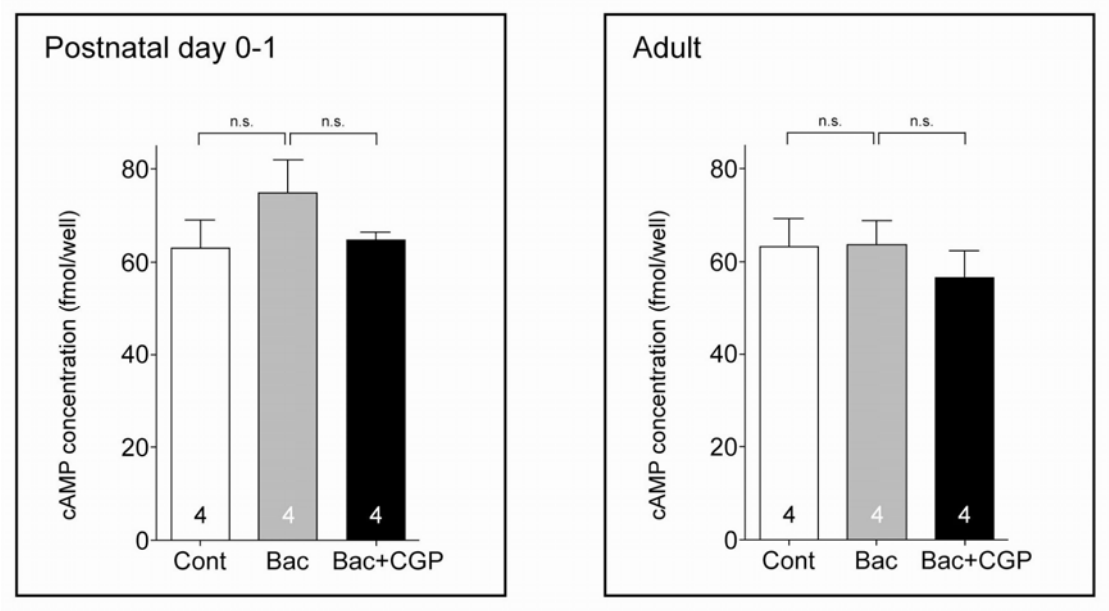


Figure 3.17. Measurement of cyclic AMP (cAMP) in the brainstem of mouse. No significant cAMP production in adult and neonatal mice brainstems after activation of $GABA_B$ receptors was observed. Activation of $GABA_B$ receptors has no influence on the cAMP levels in adult mice brainstems. The new born mice brainstems, after stimulation of $GABA_B$ receptors, show a slight increase of the cAMP levels that was not statistically significant. The cAMP measurement procedure from tissue (mouse brainstem) was determinate as described at "Material and Methods". Tissue from mouse brainstem, were preincubated with baclofen (100 µM) and as second control with CGP55845 (50 µM) and baclofen. The cAMP levels were determined by enzyme immunoassay (cAMP Biotrak; Amersham Biosciences). The average values of four independent experiments are expressed as the mean±S.E.M (two-tailed; unpaired t-test).

As shown in figure 3.17, the increase of cAMP concentration observed between agonist (baclofen), and the absence of agonist, induced stimulation of $GABA_B$ receptors was not statistically significant. Although the effect on $GABA_B$-dependent cAMP production was not significant, there was a slightly increase of baclofen induced stimulation on the $GABA_B$ receptors in neonatal brainstems, and a differential pattern of response between the neonatal and adult mice brainstem.

3.6 Developmental changes of the GABAergig system

Until now, all results were related to the developmental changes mediated by $GABA_B$ receptors. Additional experiments associated with another receptor of the GABAergic system, the $GABA_A$ were performed on the example of Rett syndrome, a disorder of abnormal neuronal maturation which is connected with a synaptic imbalance.

3.6.1 A disruption in the balance between excitatory and inhibitory receptors: neurodevelopmental disorder (MECP2)

One of the symptoms of Rett syndrome patients and mecp2 mutant mice is the breathing abnormalities, like hyperventilation and apnea. For this reason we employed the pre-Bötzinger complex (PBC), an area in the medulla which is important for the generation of inspiratory activity, for further experiments. The mecp2 mutant mice that were used as a model system to study the disease mechanism, were at the age of P7, an age at which the Rett syndrome symptoms are not manifested yet.
Electrophysiological experiments indicate that mecp2 KO mice show, in comparison to the WT mice, a marked depression of inhibitory activity at the cellular level (in press L. Medrihan et al. 2007). In the network of respiratory neurons in the brainstem, GABA and glycin are the two major inhibitory neurotransmitters. Thus, we investigated the expression levels of the $GABA_A$ receptor subtypes as well as those of glycin, at normal (wild type 'WT') and knockout (KO) mice of the same age. To this purpose we performed western blot analyses and densitometry measurements(see figure 3.18).

No change was observed in the expression of the glycin receptors. The electrophysiological experiments also showed no alteration in the glycin-mediated activity (in press L. Medrihan et al. 2007).

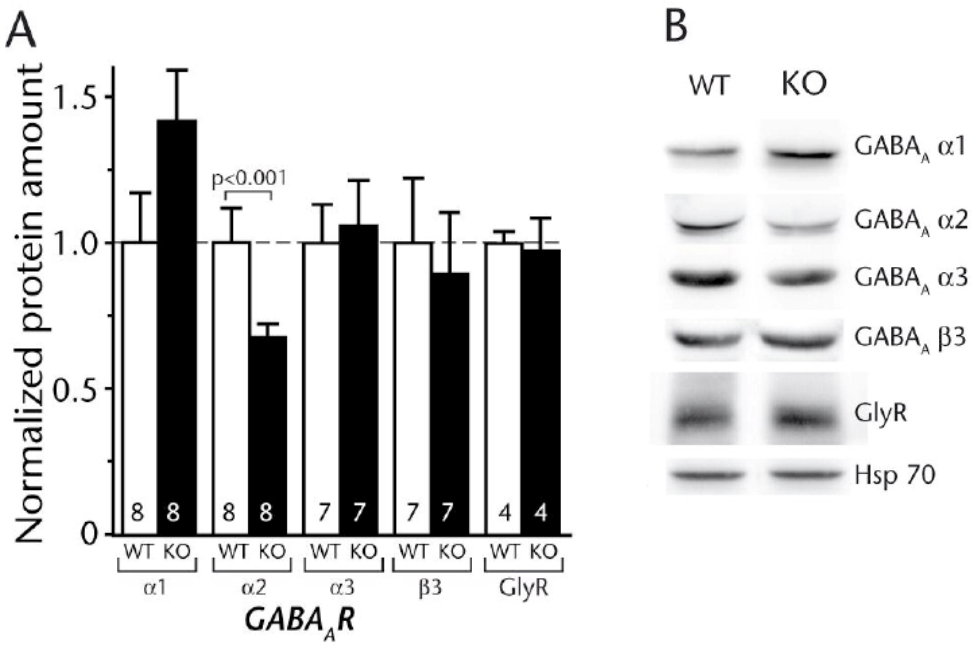


Figure 3.18. Protein levels of postsynaptic $GABA_A$ receptor subunits are changed in early postnatal MeCP2 knockout mice. A) Quantification of protein levels of $GABA_A$ and glycine receptor subunits in brainstem lysates of littermate control (white bars, WT) and MeCP2 deficient mice (black bars, KO) at postnatal days P7. B) Representative immunoblots of $GABA_A$ R α1, α2, α3, and β3 subunits, glycine receptor GlyR, and heat shock protein Hsp70 as input control.

The $GABA_A$α3 and $GABA_A$β3 subunits showed relatively similar expression levels between the KO and WT mouse brainstem. In contrast the $GABA_A$ α2-subunit is expressed at higher levels at WT compared to KO mice. Exactly the opposite becomes visible, in case of the $GABA_A$ α1 subunit, which shows an increase of the expressed levels of the α1-subunit in KO mice compared to the lower levels of the protein in WT.

4 DISCUSSION

4.1 Developmental changes of the GABAergic system

In the central nervous system, γ-aminobutyric acid (GABA) is known to act as an inhibitory neurotransmitter, via ligand-gated $GABA_A$ receptor channels and G protein-coupled $GABA_B$ receptors. Although GABA is best known for his inhibitory effect, more and more data suggest that GABA may be 'inhibitory' to mature neurons, but 'excitatory' to immature neurons which indicates an age-dependent regulation of his function. The majority of available data is from studies of $GABA_A$ receptors (Ben-Ari, Cherubini et al. 1989; Michelson and Wong 1991; Owens, Boyce et al. 1996; Rivera, Voipio et al. 1999; Ruano, Araujo et al. 2000; Ganguly, Schinder et al. 2001; Hubner, Stein et al. 2001; Ben-Ari 2002; Owens and Kriegstein 2002; Leitch, Coaker et al. 2005), while less is known about the developmental inhibitory-excitatory switching in the function of the $GABA_B$ receptors (Harrison, Lange et al. 1988; Gaiarsa, Tseeb et al. 1995; Zhang, Elsen et al. 1999).
In this study, our main interest is the developmental changes of GABA receptor signaling in mice. The brain area of our interest is the brainstem and in some experiments the pre-Bötzinger complex (PBC), a brainstem region. The PBC has been postulated as the centre of respiratory rhythmogenesis (Smith, Ellenberger et al. 1991; Funk and Feldman 1995; Ramirez and Richter 1996; Rekling and Feldman 1998) and it is one region in which the $GABA_B$ mediated signaling shows postnatal developmental changes (Zhang, Elsen et al. 1999). In addition, this brain area, in combination with Rett syndrome, a neurodevelopmental disorder where breathing abnormalities such as hyperventilation and apnea appear and where an indication of an age related depression of the inhibitory activity exist, offers an advantageous model for the investigation of the developmental changes in the $GABA_A$ receptor function.

In this study we investigated MeCP2 knockout mice, an irregular switching from excitation to inhibition. Signaling and subunit expression of the $GABA_A$ receptors were examined in the PBC of mice lacking the transcriptional repressor MeCP2 and compared to normal mice, of the same age (P7).

In case of the developmental switch caused by the $GABA_B$ receptors, almost nothing is known. In this study we investigated which factors could control or influence the developmental shift in the function of the $GABA_B$ receptor during brainstem maturation of newborn and adult mice.

4.1.1 Differential $GABA_B$-receptor-mediated modulation during brainstem maturation

Like all other GPCR (see chapter 1.2), $GABA_B$ receptor signaling diversity may arise from numerous factors, among them the expression of different $GABA_B$-receptor subtypes and the expression of different G protein subtypes which are able to interact with the receptor.

In the present study we investigated two factors that might be responsible for the developmental alteration in the function of the $GABA_B$ receptors:
First, we analysed the existence of a distinct $GABA_BR2$ subtype or any additional change in the molecular structure of this subunit which contains the G protein coupling site(s). Although, various isoforms of the $GABA_BR1$ and $GABA_BR2$ have been described (Kaupmann, Huggel et al. 1997; Jones, Borowsky et al. 1998; Kaupmann, Malitschek et al. 1998; Pfaff, Malitschek et al. 1999; Calver, Medhurst et al. 2000; Clark, Mezey et al. 2000), only the expression of the $GABA_BR1$ splice variants, $GABA_BR1a$ and $GABA_BR1b$, is proven. Thus, we scanned for the expression of different $GABA_BR2$ variants during brain maturation. Investigation of the G protein coupling sites of the $GABA_{B2}$ receptor at different ages revealed no diversity in the expression of the receptor subunit between the mature and immature NMRI mouse brainstems. We demonstrate that at protein level the G protein coupling sites of the $GABA_{B2}$ receptor in brainstem between the newborn and adult mouse are identical.
Second, we considered an alternate explanation for the developmental changes of $GABA_B$ receptor signaling is that the functional coupling between $GABA_B$ receptors to G proteins alters during development. Nowadays, it is general accepted that the downstream effects of $GABA_B$ receptor activation is mediated via the activation of G proteins, and predominantly by G proteins of the $G\alpha_{i/o}$ class (Asano and Ogasawara

1986; Morishita, Kato et al. 1990). So far, it is not known whether the $GABA_B$ receptors are also able to interact with other G proteins, such as $G\alpha_s$, $G\alpha_q$, $G\alpha_{11}$ etc., and activate other signaling pathway. To investigate activation and selective binding of $GABA_B$ receptors to different G alpha proteins in mouse brainstem at different ages we used [^{35}S]-GTPγS- and Eu-GTP- binding assays in combination with immunoprecipitation. The results (see chapter 3.3) reveal that the $GABA_B$ agonist baclofen stimulates GTPγS- and Eu-GTP-binding in the case of Gαi/o at both ages (P0 and adult) and this response is blocked by the $GABA_B$ receptor antagonist CGP 55845A. In contrast neither baclofen-stimulated GTPγS- nor Eu-GTP-binding was observed in adult brainstem tissue in case of the $G\alpha_s$ under similar incubation conditions. However, there was an unexpectedly dramatic increase of the immunoprecipitated $G\alpha_s$ in newborn brainstem homogenates. Stimulation of brainstem $GABA_B$ receptors resulted no GTP-binding to the $G\alpha_{i3}$, $G\alpha i_{12}$ and $G\alpha_{13}$ proteins at both ages. The amount of the immunoprecipitated $G\alpha_{q/11}$ after $GABA_B$ receptors activation in newborn and adult mice was not indicative. Although the results of the GTPγS-binding assay were more intense compared to the new Eu-GTP-binding assay, we developed the Eu-GTP-binding assay, in combination with immunoprecipitation, to increase the number of experiments and to avoid working with radioactivity.

Taken together, these results indicate that in addition to the expected interaction of the $GABA_B$ receptors with the $G\alpha_{i/o}$ proteins in adults we were able to demonstrate that $GABA_B$ receptors interact with the $G\alpha_{i/o}$ proteins in newborn mice brainstem. The most distinct finding was the observation that activated $GABA_B$ receptors couple to $G\alpha_s$ proteins but only in the immature brainstem. In adult mouse brainstem this interaction was non-existant. This developmental regulated interaction between the activated $GABA_BR$ and the $G\alpha_s$ proteins, lead us to investigate the developmental expression of the G alpha s protein subtypes.

4.1.2 Diversity of $GABA_B$ receptor signaling through G protein variability

The heterotrimeric G proteins composed of α, β, and γ subunits with their subunit combinations and their multiple splice variants control diverse signal transduction pathways. The expression of the G protein subtypes in the developing brain is age-

dependent and characteristic for a specific brain region. Qualitative and quantitative analysis of the Gα protein subtypes in mouse brainstem revealed diverse expression patterns for the Gα protein subtypes of our interest. The expression levels of the $G\alpha_{i/o}$ are equal in all three investigated brain areas (cortex, cerebellum and brainstem) at both developmental stages (newborn and adult). The same result was revealed from the analysis of the $G\alpha_{q/11}$. The examination of the $G\alpha_s$ proteins was more complicate because of the numerous isoforms produced by the rather complex genomic locus of GNAS. Western blot analysis with $G\alpha_s$ antibody showed up six bands with a developmental regulated expressions pattern. Two of them, clearly identified as the short and long isoforms of the $G\alpha_s$, showed strong expression levels in the immature brainstem that were decreased during maturation. On the other side, the two isoforms Gs1 and Gs2, which are almost missing in the newborn give a strong signal in adult brainstem, and represent two XLαs isoforms of the $G\alpha_s$. The other two variants Gs5 and Gs6, was not possible to be distinguished. Thus, although the complexity of the GNAS locus and the continuously new data did not permit us to identify all six $G\alpha_s$ isoforms, we could distinguish between the two $G\alpha_s$ (short and long) which show a decreas in expression, and the two extra long $G\alpha_s$ which show an increase in expression during mouse brainstem maturation. Although it is known, like in the case of β2-adrenoreceptor, that the $G\alpha_s$ short and long variants, which differ in only 15 amino acids, have different consequences for the functional properties of a G-protein-coupled receptor (Seifert, Wenzel-Seifert et al. 1998), it is not known how the interaction between the XLαs isoforms and the $GABA_B$ receptor influences the function of the receptor. The paternal expressed XLαs contains all structural domains of $G\alpha_s$, except the long amino-terminal extension encoded within its specific first exon. Even though *in vitro* studies have shown that XLαs forms a heterotrimer with βγ subunits, binds GTP and mediates receptor-stimulated cAMP production, the biological role(s) of XLαs within the cell remains obscure. There are also some conflict reports, based on the ability of XLas to activate a receptor. Moreover, recent findings indicate that the GNAS products are capable of modulating the activity/expression of each other, something that makes more difficult to predict which influence will have these interactions in the $GABA_B$ receptors signaling. For example, it is known that XLαs and $G\alpha_s$ exert antagonistic functions and that the maternal expressed Nesp enhances the expression of XLαs and represses the expression of $G\alpha_s$. Even the paternal expressed protein called Alex, which is a

structurally unrelated protein to the G proteins, and is also encoded in the GNAS locus, has the ability to interact with XLαs, and inhibits its adenylyl cyclase-stimulating function.

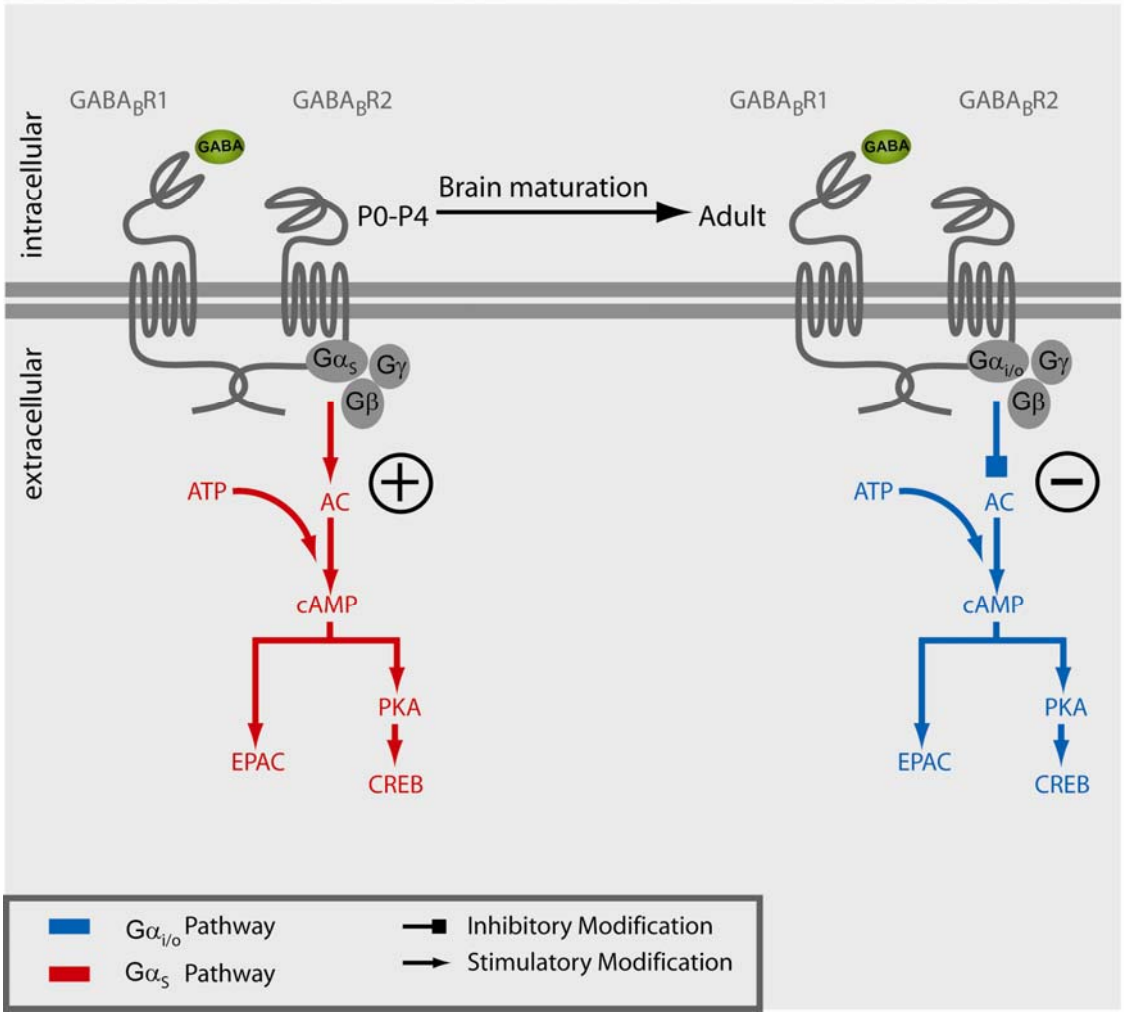


Figure 4.1. Schematic representation of the activation of different signaling pathways through the $GABA_B$ receptors in immature and adult mouse brainstem.

The brainstem cAMP measurement at newborn and adult mice after activation with baclofen, revealed no significant values that could possibly be explained through the antagonistic effect of the GNAS locus products (e.g. Nesp, Alex, XLN1), or the antagonistic function of the Gα and Gβγ subunits, or even through cross-talk with other pathways.

We can only speculate on the $GABA_B$ receptor-mediated signaling through the GNAS locus products. One explanation could be that the $GABA_BR$-$G\alpha_s$ (short and long)

interaction in the immature brainstem is hindered in the adult brainstems through a sterical interference or general an antagonistic function of the XLas variants (or other by other proteins expressed in the GNAS locus).

The distinctive characteristic of $GABA_BR$, the formation of a heterodimers, could influence additional receptor signaling. $GABA_{B1}$ with the two isoforms $GABA_{B1a}$ and $GABA_{B1b}$ which contain the ligand binding site and $GABA_BR2$ with the G protein coupling site(s), regulate the function of the receptor based on the organism, the age, the brain region, the microdomain organisation and the receptor composition. Although it is generally accepted that coexpression of the two $GABA_B$ receptors subunits ($GABA_BR1$ and $GABA_BR2$) is required for a fully functional receptor, thus $GABA_BR2$ is necessary for the $GABA_BR1$ transportation, there is evidence that also $GABA_BR1$ (1a and 1b) and $GABA_BR2$ alone are able to couple to K^+ channels or adenylyl cyclase, although at low efficiency (Kaupmann, Huggel et al. 1997; Kaupmann, Schuler et al. 1998; Kuner, Kohr et al. 1999; Urwyler, Mosbacher et al. 2001). Thus, in our cloning experiments ($GABA_BR1a$ and $GABA_BR2$ expression in HEK 293 cells) because of the weak signal observed in the cells and the $GABA_BR2$ expresion in the intracellular areas of the cell, we can conclude it concerns monomeric $GABA_BR1a$ receptors.

Additionally, although the $GABA_{B1}$ intracellular loops are not necessary for G protein coupling they may be involved in signaling pathways not involving G proteins or in the regulation of subcellular $GABA_B$ receptor localization. For example $GABA_{B1}$ subtypes interact directly with CREB2/ATF4, USF, 14-3-3 and through subtype-selective interacting proteins for the $GABA_{B1a}$ and $GABA_{B2}$ (see chapter 1.6.3.1). These associated proteins are able to regulate subtype-specific expression of the $GABA_{B1a\&b}$ subunits, receptor heterodimerization and cross-talks between different systems, such as Tamalin and NSF.

The distinct subcellular distribution and behaviour of the $GABA_{B1}$ subunits with $GABA_{B1a}$ dominant in neonatal brain and $GABA_{B1b}$ predominant in adults could play an additional role to the $GABA_B$ receptor mediated signaling diversity.

Another layer of complexity arises from the ability of the GPCRs to activate simultaneously multiple G protein pools. Dual coupling to $G\alpha_s$ and $G\alpha_{i/o}$ has been reported, as in the case of the β2-adrenoceptor (Daaka, Luttrell et al. 1997), where the receptor phosphorylation, via PKA, controls the switching from the $G\alpha_s$ to the $G\alpha_{i/o}$

pathway. In some cases, a single receptor has been found to simultaneously activate members of three or even four unrelated classes of G protein ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12}$) (Laugwitz, Allgeier et al. 1996). The interaction of these receptors with multiple G protein classes produces additional interference between the products of each pathway, through the generation of different Gα and Gβγ subunits.
Thus, although we do not know if the interaction of the $GABA_B$ receptors in the immature brainstem to the $G\alpha_s$ and $G\alpha_{i/o}$ proteins is a simultaneous coupling of the two G proteins or if there is a factor that controls a $G\alpha_s$-$G\alpha_{i/o}$ switching, we can conclude that in general the $GABA_B$-receptor binding to $G\alpha_s$ proteins will initiates a new set of signaling events.

These observations together lead us to the conclusion that the interaction of $GABA_B$ receptor to the $G\alpha_s$ and $G\alpha_{i/o}$ proteins in the immature brainstem and only to the $G\alpha_{i/o}$ proteins in the mature brainstem, are probably regulated by additional factors e.g. the $GABA_BR1$ subunit, the way of interaction with the G proteins (simultaneously or switching) and via the organization of all these proteins (e.g. receptors, G proteins, associated proteins) within specialized microdomains.

4.2 Changes in the $GABA_A$ receptor signaling in the developing mouse brainstem in the model of Rett syndrome

The switching of the $GABA_A$ receptor from an excitatory action in immature CNS neurons to the well-established inhibitory action of the adult neurons was recognized in some of the very early studies (Eccles, Schmidt et al. 1963; Obata, Oide et al. 1978).
Since then, the depolarization induced by GABAergic neurotransmission has been observed in immature neural systems, such as the spinal cord (Wu, Ziskind-Conhaim et al. 1992; Reichling, Kyrozis et al. 1994; Nishimaru, Iizuka et al. 1996; Gao and van den Pol 2001), brainstem (Ritter and Zhang 2000; Marchetti, Pagnotta et al. 2002), retina (Huang and Redburn 1996), olfactory bulb (Serafini, Valeyev et al. 1995), hippocampus (Ben-Ari, Cherubini et al. 1989; Cherubini, Rovira et al. 1990; Michelson and Wong 1991; Cherubini, Martina et al. 1998; Ruano, Araujo et al.

2000; Ben-Ari 2002), hypothalamus (Chen, Trombley et al. 1996; Gao and van den Pol 2001), and the neocortex (Luhmann and Prince 1991; Owens, Boyce et al. 1996).

Activation of $GABA_A$ receptors during embryonic life produces depolarization because of a high concentration of intracellular chloride ions. The intracellular accumulation of chloride seems to be generated by a delay in the expression of the chloride exporter KCC2 and through accumulate Cl^-, probably through the Na^+-K^+-Cl^- cotransporter (NKCC1) (Vardi, Zhang et al. 2000). The inhibitory action of the $GABA_A$ receptors in mature neurons is associated with a hyperpolarisation (Rivera, Voipio et al. 1999). Activation of $GABA_A$ receptors leads to an influx of chloride ions but the intracellular concentration of Cl^- decreases with development, probably through the upregulation of a Cl^- extrusion mechanism; the K^+-Cl^- cotransporter (KCC2) (Rivera, Voipio et al. 1999; Payne, Rivera et al. 2003).

The depolarizing action of $GABA_A$ receptors during early development induces an increase in intracellular calcium (Lin, Takahashi et al. 1994; Ben-Ari 2002), in contrast to the decreased intracellular calcium levels in mature neurons. This Ca^{2+}-enhancment in immature neurons may control several developmental phenomena, including synapse formation (Ben-Ari, Khazipov et al. 1997; Cherubini, Martina et al. 1998; Owens and Kriegstein 2002), rate regulation of neurone outgrowth (Maric, Liu et al. 2001), promotion of morphological differentiation (Ben-Ari, Tseeb et al. 1994; Marty, Berninger et al. 1996) and/or neuronal migration (Barker, Behar et al. 1998).

Although, the exact mechanism underlying this shift remains unknown, it has been suggested that the transition from depolarization to hyperpolarisation is associated with $GABA_A$ receptor subunit switching. Developmental changes in the subunit composition modulate the function of the $GABA_A$ receptor (Takayama and Inoue 2004; Lujan, Shigemoto et al. 2005). It has been postulated that the subunit composition with alpha 2 (or alpha 3), which are mainly expressed in immature brain areas, would contribute to the depolarizing $GABA_A$ effect (Hornung and Fritschy 1996), whereas the expression of the alpha1-subunit which is low at birth increase dramatically during brain maturation is involved in synaptic inhibition (Fritschy, Paysan et al. 1994). This $GABA_AR$ subunit switch is conserved across species.

There is only a short time window where many individual neurons show a coexistence of both α1 and α2, which indicates that the alpha 1-subunit gradually replace receptors containing the alpha 2-subunit (MacLennan, Brecha et al. 1991; Fritschy, Paysan et al. 1994; Hornung and Fritschy 1996; Lopez-Tellez, Vela et al. 2004). The time line of

switch from depolarizing to hyperpolarizing GABA transmission and the timing of the replacement of $GABA_A$ receptor alpha 2-subunits by alpha1-subunits take place, in the case of the rat PBC, simultaneously, during the first postnatal week. In addition, the observed increase of intracellular calcium from the $GABA_A$ receptor action in immature neurons disappears when the $GABA_A$ receptor alpha2-subunit is replaced by alpha1-subunit (Lin, Takahashi et al. 1994).

In this study we investigated the imbalance between excitation and inhibition in MeCP2 knockout mice, a model system that allowed us to examine the Rett syndrome. MeCP2 is a protein which binds to DNA and regulates transcriptional repression. It is widely and increasingly expressed during development (Kishi and Macklis 2004). Thus, loss of MeCP2 function leads to improper gene expression programs.

Although many studies suggest that this synaptic imbalance (Dani, Chang et al. 2005; Moretti, Levenson et al. 2006) may cause the Mecp2 observed abnormalities, it is still unknown which transmitter and receptors are predominantly involved.

Electrophysiological experiments in mouse PBC at the age of P7 indicate that mecp2 KO mice show, compared to the WT mice, a marked depression of inhibitory activity which is regulated by the $GABA_A$ receptor (in press L. Medrihan et al. 2007). The investigated age of P7 is a critical period where the symptoms of the syndromes are not manifested.

We investigated the expression levels of the $GABA_A$ subunits and glycin of the mecp2 in the KO mice brainstem at the age of P7 in comparison to the expression levels of the $GABA_A$ receptor subunits at the same age in normal mice brainstem. The results of the experiments demonstrate no difference in the expression levels of glycin, an increase of the α1-subunit and a decrease of the α2-subunit in brainstem of the mecp2 KO mice in comparison to the healthy mice. This expression pattern normally appears in further stages of brain maturation where the $GABA_A$ receptors contribute more to an inhibitory that to an excitatory tone. Collectively the data supports the view that the synaptic imbalance observed in the mouse model of Rett syndrome is related to an irregular time switching of the $GABA_A$ receptor alpha subunits. But it is still and open question as to whether the $GABA_A$ receptor's abnormal switching is the cause or the result of the syndrome.

5 SUMMARY AND CONCLUSIONS

Although, in the adult central nervous system, GABA is known to act as an inhibitory neurotransmitter, recent studies have revealed that the role of the GABAergic system changes developmentally, so that in the immature CNS serves as an excitatory neurotransmitter. In this study, we examined factors that influence the developmental changes of the ionotropic $GABA_A$ and metabotropic $GABA_B$ receptor signaling in mouse brainstem.

We examined the signaling modulation of a GPCR receptor, the $GABA_B$ receptor, in the developing mouse brainstem as function of $GABA_B$ receptor subtype variants, binding properties of $GABA_B$ receptor to different G proteins, and G protein expression variability.

We demonstrated that the developmental alteration of the $GABA_B$ receptor signaling between neonatal and adult mouse brainstem is not associated with expression difference in the G protein coupling sites of the $GABA_BR2$. The investigation of the $GABA_B$ signaling pathways in newborn and adult mouse brainstem revealed that $GABA_B$ receptors couple to $G\alpha_{i/o}$ proteins at both ages. Furthermore, we found that $GABA_B$ receptors interact also with $G\alpha_s$ proteins, and that this coupling disappears with further maturation. Additional experiments demonstrated equal amounts of whole cell $G\alpha_{i/o}$ proteins between neonatal and adult mouse brainstem, whereas the $G\alpha_s$ variants showed an age-dependent alteration in their expression. Analysis of the $G\alpha_s$ variants showed a decrease in the expression of the $G\alpha_s$ short and long variants during maturation of the brainstem whereas the XLαs variants were almost absent in the immature brainstem. The complexity and variability of the $G\alpha_s$ variants as well as their ability to modulate each other, makes it difficult to predict their influence to the $GABA_B$ receptor signaling.

In addition, we investigated the $GABA_A$ receptors in brainstem of MeCP2 knockout mice, a model system which allowed us to examine Rett syndrome. A neurodevelopmental disorder where the balance between excitation and inhibition, which exist in healthy brains and is essential for nearly all functions, is shifted. $GABA_A$ receptors are known to switch from an excitatory action in immature CNS neurons to an inhibitory action in the adult neurons. Although there is no direct

evidence the developmental regulated shift in the function of the $GABA_A$ receptors is correlated with the switching of the alpha $GABA_A$ receptor subunits.

We demonstrated a time shifted switch in the expression of the $GABA_A$ alpha1 and alpha2 subunit in the rat pre-Botzinger complex (PBC) on postnatal day (P) 7 between the knockout and healthy mice, a critical period where the symptoms of the syndromes are not manifested. Brainstem analysis of knockout mice revealed decreased levels of $GABA_A$ alpha1 and increased levels of $GABA_A$ alpha2 comparing to normal mice of the same age (P7). The two subunits have an exceptional role in the regulation of the $GABA_A$ receptor, thus it is proposed that the $GABA_A$ alpha1 subunits which are mainly expressed in the mature brain, are involved in synaptic inhibition, whereas the $GABA_A$ alpha2 subunits which are mainly expressed in immature brain areas, contribute to the depolarizing $GABA_A$ effect. Our data from the $GABA_AR$ subunits expression levels, agree with the observations from electrophysiological experiments (in press L. Medrihan et al. 2007) in mouse PBC at the age of P7, which indicate that mecp2 KO mice show, in comparison to the WT mice, a marked depression of inhibitory activity.

Collectively, our data supports the view that the synaptic imbalance observed in the mouse model of Rett syndrome is related to an irregular time switching of $GABA_A$ receptors alpha subunits.

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