Function and downstream signaling of $GABA_B$ receptors in developing respiratory network of mouse

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Tag der mündlichen Prüfung:

Dedicated to my Parents

Abbreviations

ACSF	Artificial cerebrospinal fluid
ATP	Adenosine tryphosphate
CNS	Central nervous system
CNQX	6-cyano-7 nitroquinoxaline-2, 3-dione disodium salt
DL-AP5	DL-2-Amino-5-phosphonovaleric acid
EDTA	Ethylenedinitrilo-tetraacetic acid
et al.	et alters (and others)
g	Gravitational acceleration, or gram
GABA	γ-aminobutyric acid
GΩ	Giga Ohm
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
KO	Knockout
µ-	micro,-($_x10^{-6}$)
mV	millivolts
m.	minute
NA	Nucleus ambiguus
NGS	Normal goat serum
NH	Nucleus hypoglossus
NMDA	N-methyl-D-Aspartate
N-terminal	at the NH2-terminus of a protein
pA	pico Ampere
PBC	PreBötzinger complex
PBS	Phosphate buffer saline
PFA	paraformaldehyde
pH	Negative logarithm of H^+- concentration
PDZ	Protein interaction domain, acronym for PSD-95, Dlg, ZO-1
PSD	postsynaptic density
RT	Room temperature
s.	second

SDS	Sodium dodecyl sulfate
sIPSC	spontaneous postsynaptic currents
sPSC	spontaneous postsynaptic currents
Tris	Tris-hydroxymethyl-aminomethane
WT	Wild type
5′	5 prime
3′	3 prime

Contents

1. Introduction

The central nervous system (CNS) receives and interprets information about internal and external environment, makes decisions about the information and organizes and carries the actions. The performance of such complex processes results in synapses that are specialized intercellular junctions whose specificity and plasticity provide the structural and functional basis for the formation and maintenance of the complex neuronal network in the brain. The number, location, and type of synapses formed are well controlled, since synaptic circuits are formed in a highly reproducible way. This implies the existence of cellular and molecular properties that determine the connectivity of each neuron in the nervous system. The most common type of a synapse in CNS is the chemical synapse. These synapses are composed of pre- and postsynaptic sites. Presynaptic site of a synapse includes active zone, where the neurotransmitter release occurs, a network of scaffolding proteins (cytomatrix), and neurotransmitter containing synaptic vesicles. Postsynaptic site contains neurotransmitter receptors directly opposed to the active zone. Pre- and postsynaptic sites are separated by extracellular space known as synaptic cleft. Neurotransmitters are released in the process called exocytosis, which requires depolarization of the presynaptic site, leading to an opening of voltage gated Ca^{2+}-channels and the influx of Ca^{2+}-ions into the presynaptic terminal (Zucker et al., 1993; for review Catterall, 1998). This, in turn leads to fusion of neurotransmitter containing vesicles with the plasma membrane and the release of the content into synaptic cleft, thereby activating receptors on the postsynaptic membrane. The synapses can be inhibitory and excitatory. The excitation is mediated mainly by glutamate receptors, while inhibition is mediated by GABA and glycine receptors.

1.1 GABA-ergic neurotransmission

γ-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian brain. There are two distinct categories of target receptors for GABA, each of which mediates synaptic transmission: ionotropic $GABA_A$ and $GABA_C$, and metabotropic $GABA_B$ receptors. $GABA_A$ receptors are ligand-gated Cl^--channels that mediate fast

inhibitory synaptic transmission in the CNS (Barnard et al., 1998; Mehta & Ticku 1999; Vicini 1999). These receptors are pentameric complexes formed by the heteromeric assembly of five different subunits (Barnard et al., 1998; Sieghart & Sperk 2002; Whiting et al., 1999). The brain region-specific distribution and ontogeny-dependent expression of these various subunits give rise to a relatively large number of $GABA_A$ receptor subtypes, which differ in their subunit composition as well as in their physiological and pharmacological properties (Sieghart 1995; Sieghart & Sperk 2002; Whiting et al., 1999). $GABA_C$ receptors, like $GABA_A$ receptors, are pentameric receptor complexes, and are expressed in the retina and in many other regions of the CNS (Qian et al., 1994; ENZ et al., 1996, Euler & Wassle 1998; Lukasiewicz et al., 1998; Shen et al., 2001; Gibbs et al., 2005; Lukasiewicz, 2005). Unlike $GABA_A$ receptors, $GABA_B$ receptors activate second-messenger systems through the binding and activation of guanine nucleotide-binding proteins (G proteins), and mediate the slow inhibitory neurotransmission of GABA. Dysfunction of GABA-mediated synaptic transmission in the CNS is believed to underlie various nervous system disorders, which include epilepsy, spasticity, anxiety, stress, sleep disorders, depression, addiction, pain, schizophrenia (Couve et al., 2000; Bettler et al., 2004).

1.2 The heteromeric nature of $GABA_B$ receptors

$GABA_B$ receptors were first described by Bowery and colleagues in 1981 as bicuculline-insensitive, baclofen-sensitive GABA receptors widely expressed in the mammalian central nervous system (Hill & Bowery, 1981). Although $GABA_B$ receptors have been described early on, they were the last major neurotransmitter receptors to be cloned (Kaupmann et al., 1997). This was due to the difficulties in coupling of $GABA_B$ receptors to effector channels in heterologous cells, which prevented expression of cloning strategies such as those commonly used for the isolation of neurotransmitter receptors. The first $GABA_B$ receptor cDNA was eventually isolated by using a radioligand-binding screening approach. The cloned $GABA_B$ receptor, termed $GABA_{B1}$, has similarity with metabotropic glutamate receptor (mGluRs). It posses seven transmembrane domains with large extracelular N- and intracellular C-

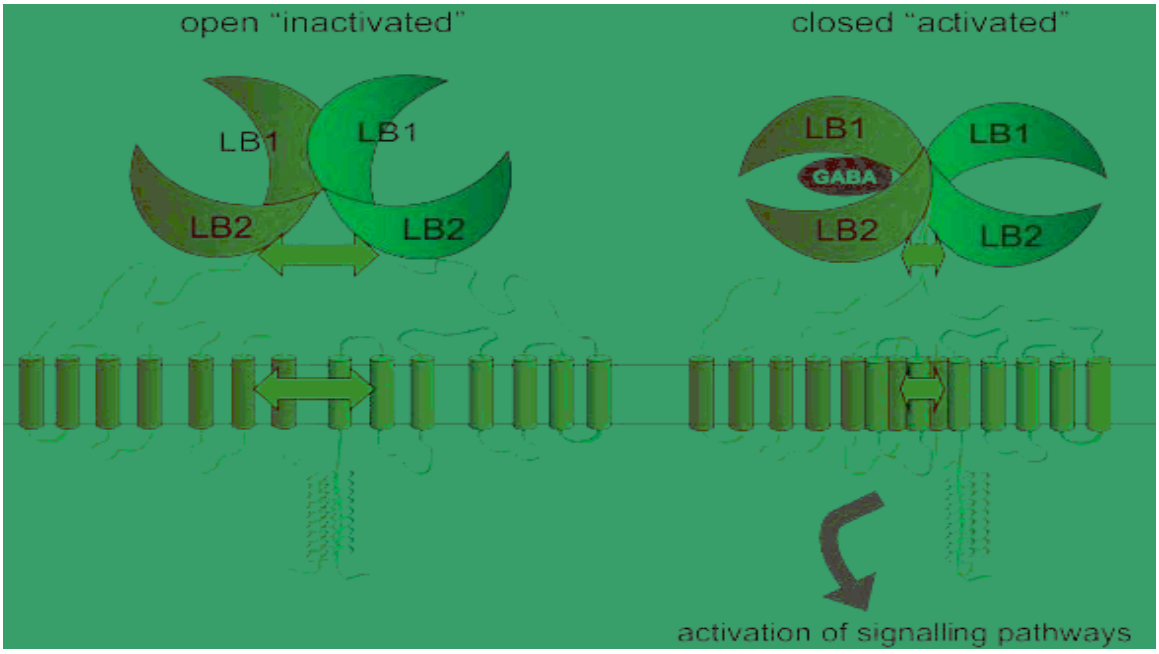


Figure 1.2**:** Agonist binding causes a conformational change in the $GABA_B$ receptor heterodimer resulting in receptor coupling to effector systems. Lobes1 (LB1) and 2 (LB2) make up a single protomer in $GABA_{B1}$ receptor (orange) and $GABA_{B2}$ receptor subunits (green). In the inactivated 'open' state, the ligand-binding pocket situated in the $GABA_{B1}$ receptor extracellular binding domain is open, and the extracellular and transmembrane (TM) domains of $GABA_{B1}$ receptor and $GABA_{B2}$ receptor are apart. Agonist binding to $GABA_{B1}$ receptor induces 'closing' of the ligand-binding pocket and an 'activated' receptor state. This conformational change results in the extracellular and TM domains of $GABA_{B1}$ receptor and $GABA_{B2}$ receptor coming closer together (yellow arrows) that leads to activate downstream signaling cascades (adapted from Calver et al., 2002).

terminuses. However, $GABA_{B1}$ was found to bind GABA with low affinity and couple with less efficiently to effectors than native $GABA_B$ receptors (Kaupmann et al., 1997). It was soon established that $GABA_{B1}$, when expressed alone in heterologous systems, could not traffic efficiently to the cell surface, but was rather retained in the endoplasmic reticulum (ER) due to the presence of an ER retention motif on its intracellular C terminus (Margeta-Mitrovic et al., 2000; Pagano et al., 2001). A second receptor, $GABA_{B2}$, was subsequently cloned and found to be capable to traffic to the cell surface by itself. When $GABA_{B1}$ and $GABA_{B2}$ were co-expressed in

heterologous cells, they were found to form functional surface-expressed receptors with properties similar to those of some native $GABA_B$ receptors (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998). Thus, heterodimerization model has been suggested for $GABA_B$ receptors. In the heterodimer, $GABA_{B1}$ binds the ligand (Galvez et al., 1999), whereas $GABA_{B2}$ is believed to be the primary G protein contact site (Margeta-Mitrovic et al., 2000; Calver et al., 2001; Galvez et al., 2001, Margeta-Mitrovic et al., 2001; Pagano et al., 2001; Robbins et al., 2001; Duthey et al., 2002; Havlickova et al., 2002). It appears, therefore, that the agonist binds to a component of the $GABA_{B1}$ subunit, producing a conformational change in the protein complex that allows $GABA_{B2}$ to engage and activate the G protein coupled signaling system (Figure 1.1). However, the spatial and temporal expression of $GABA_{B1}$ and $GABA_{B2}$ subunits do not always match (Bettler et al., 2004). Therefore, it is possible that functional receptors that exist in neurons lack $GABA_{B2}$. Indeed, some studies suggest that $GABA_{B1}$ subunits can associate into stable homodimers (Villemure et al., 2005).

1.3 Molecular diversity of $GABA_B$ receptors

The cloning of $GABA_{B1}$ uncovered the existence of two alternatively spliced forms of this receptor subunit in human and rodent, $GABA_{B1a}$ and $GABA_{B1b}$ (Kaupmann et al., 1997). These two isoforms differ in their N-terminus by a pair of 'Sushi' domains that is present in $GABA_{B1a}$, but not in $GABA_{B1b}$ (Blein et al., 2004). These domains that are involved in protein-protein interactions are found in other GPCR as well (Grace et al., 2004; Lehtinen et al., 2004). It was suggested that these 'Sushi' domains in $GABA_{B1a}$ bind to auxiliary proteins that modify receptor activity or pharmacology in vivo (Marshall et al., 1999; Mohler & Fritschy, 1999). To note, the two 'Sushi' domains in $GABA_{B1a}$ exhibit strikingly different structural properties (Blein et al., 2004). Therefore it was proposed that they participate in protein interactions with different partners, which could generate, at least partially, the heterogeneity of native $GABA_B$ receptors. Some other splice variants have been also identified for $GABA_{B1}$ in human and rat (Isomoto et al., 1998; Calver et al., 2000; Martin et al., 2001; Pfaff et al., 1999; Schwarz et al., 2000). However, they were not either conserved among different species or the existence of stable protein products were not observed in vivo.

Although initial reports demonstrated cloning of three alternative splice variants for $GABA_{B2}$, it turned out that these variants do not in fact occur in vivo and they were just cloning artifacts (Ng et al., 1999; Martin et al., 2001; Clark et al., 2000). Thus, at the moment there is currently no good evidence for splice variants of $GABA_{B2}$ (Martin et al., 2001).

1.4 Distribution and subcellular localization of $GABA_B$ receptors

In central nervous system, $GABA_{B1}$ and $GABA_{B2}$ have been found to be colocolized at the plasma membrane in agreement with predominantly heteromeric nature of $GABA_B$ receptors (Kaupmann et al., 1998; Kulik et al., 2002; Lopez-Bendito et al., 2002; Kulik et al., 2003; Koyrakh et al., 2005). However, in some brain areas $GABA_{B2}$ is not present, even though the $GABA_{B1}$ and native receptor are present (Durkin et al., 1999; Margeta-Mitrovic et al., 1999; Clark et al., 2000). In addition, there is relatively less $GABA_{B2}$ mRNA in the brain compared to $GABA_{B1}$ (Jones et al., 1998; Clark et al., 2000). Immunoreactivity of both $GABA_B$ receptors was demonstrated in pre- and postsynaptic membranes. Presynaptically, $GABA_B$ receptors are mainly detected in the extrasynaptic membrane and occasionally over the presynaptic membrane specialization of glutamatergic and, to a lesser extent, GABAergic terminals. $GABA_B$ receptors appear to be mostly localized near the active zone, which supports a close link with the release machinery. The splice variants of $GABA_{B1}$, $GABA_{B1a}$ and $GABA_{B1b}$, are differently distributed in the brain (Liang et al., 2000). $GABA_{B1a}$ has been found to be localized mainly at presynaptic terminals, whereas $GABA_{B1b}$ is located predominantly at postsynaptic terminals (Kaupmann et al., 1998b; Billinton et al., 1999; Bischoff et al., 1999; Princivalle et al., 2000; Towers et al., 2000). However, some studies revealed the presynaptic localization of $GABA_{B1b,}$ and postsynaptic localization of $GABA_{B1a}$ (Benke et al., 1999; Princivalle et al., 2001). During postnatal development there is up- and downregulation of both isoforms (Fritschy et al., 2004). $GABA_{B1a}$ has been found to be predominantly expressed in neonatal mice with overlapping regional distribution with $GABA_{B2}$, but with profound distinctions in cellular and subcellular localizations. $GABA_{B1b}$ is expressed in adult mice, together with $GABA_{B2}$. Studies monitoring functional $GABA_B$ responses suggest their presence in

peripheral organs (Bowery, 1993). Northern blot and Western blot analysis has provided direct evidence for $GABA_{B1}$ isoforms and $GABA_B$ receptors distribution throughout the periphery of the rat (Castelli et al., 1999; Calver et al., 2000). However, the $GABA_{B2}$ subunit was not always present with $GABA_{B1}$, such as in uterus and spleen (Calver et al., 2000). $GABA_B$ receptors have been found in heart myocytes. $GABA_{B1a}$ and $GABA_{B1b}$ show different distribution pattern in the periphery as well. Thus, $GABA_{B1a}$ is present in the adrenals, pituitary, spleen, and prostate, whereas $GABA_{B1b}$ is found in the rat kidney and liver (Belley et al., 1999). Furthermore, it is widely believed that the development of pharmacological agents that selectively modify the function of specific pre- and postsynaptic $GABA_B$ receptor populations may provide beneficial therapies with limited side effect profiles for these ailments.

1.5 Downstream signaling of $GABA_B$ receptors

Effector systems of $GABA_B$ receptor signaling are the adenylate cyclase system and Ca^{2+}- and K^+-ion channels (Hill et al., 1984; Karbon et al., 1984; Hill, 1985; Inoue et al., 1985; Andrade et al., 1986; Xu & Wojcik, 1986; Dolphin et al., 1990; Bindokas & Ishida, 1991; Gage, 1992; Zhang et al., 1998). Activation of $GABA_B$ receptors by its agonists causes inhibition of basal and forskolin stimulated adneylyl cyclase activity via pertussis toxin-sensitive G proteins, in particular $G_{\alpha i/o}$ (Odagaki et al., 2000; Odagaki & Koyama, 2001). The functional consequences of adenylyl cyclase inhibition via $GABA_B$ receptors are poorly understood, but reports demonstrate the involvement of transcription factors (Steiger et al., 2004) and kinases (Diverse-Pierluissi et al., 1997; Couve et al., 2002; Ren & Mody, 2003). However, some pertussis toxin-insensitive effects of $GABA_B$ activation have been described (Noguchi & Yamashita, 1999; Cui et al., 2000), particularly the presynaptic $GABA_B$ receptors are insensitive to pertussis toxin (Harrison et al., 1990). Moreover, it has also been found that $GABA_B$ activation causes in an increase in guanosine triphosphate (GTP) binding in young rats, which was not obtained in older animals, which would suggest that there may be a developmental change in the coupling of $GABA_B$ receptors and G proteins (Moran et al., 2001).

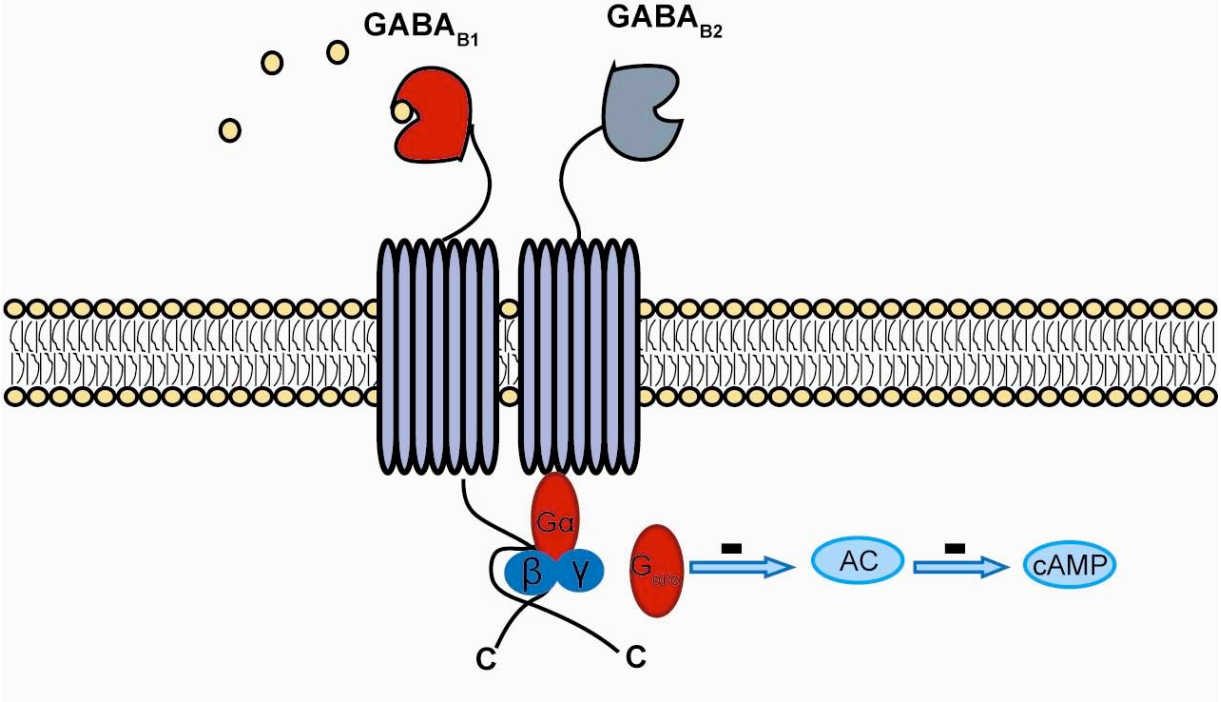


Figure 1.4: Activation of $GABA_B$ heterodimer leads to activation of heteromeric (αβγ) Gαi/o -proteins which results in dissociation of G_α from $G_{\beta\gamma}$ subunits. Gαi/o leads to inhibition of adenlyly cyclase (AC) activity, which consequently inhibits cAMP.

Other effector systems, such as Ca^{2+}- and K^+-channels, have been extensively studied by electrophysiological techniques. These studies revealed that $GABA_B$ receptors modulate these channels both at pre- and postsynaptic sites via the $G_{\beta\gamma}$ subunits of G-proteins (Bowery et al., 2002; Calver et al., 2002; Bettler et al., 2004). Presynaptic $GABA_B$ receptors suppress synaptic transmission via inhibition of voltage sensitive Ca^{2+}-channels, (Mintz & Bean, 1993; Thompson et al., 1993; Poncer et al., 1997). This effect on Ca^{2+}-channels appears to be primarily associated with presynaptic P/Q- and N-type channels (Santos et al., 1995; Lambert & Wilson, 1996; Chen & van den Pol, 1998; Takahashi et al., 1998; Bussieres & El Manira, 1999; Barral et al., 2000), although facilitation of L-type has also been described (Zhang et al. 1998; Shen and Slaughter, 1999). It was also proposed that $GABA_B$ receptors are directly involved in vesicle priming (Sakaba & Neher, 2003).

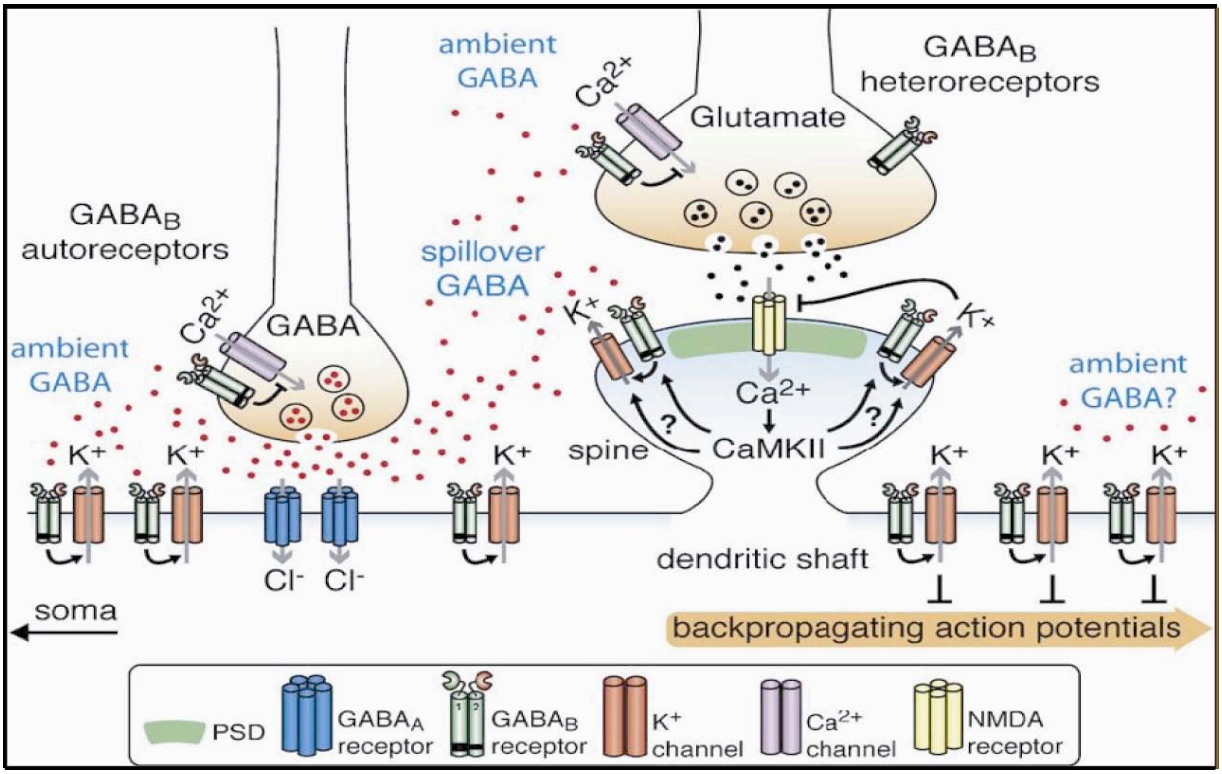


Figure 1.4: Localization and physiological roles of $GABA_B$ receptors. $GABA_B$ receptors are located on presynaptic, postsynaptic and extrasynaptic membranes. Presynaptic $GABA_B$ receptors suppress neurotransmitter release by decreasing Ca^{2+}-currents or by a direct inhibition of the release machinery. $GABA_B$ autoreceptors inhibit the release of GABA, whereas $GABA_B$ heteroreceptors inhibit the release of glutamate and several other neurotransmitters. Some $GABA_B$ heteroreceptors are activated by ambient GABA, others probably by GABA spillover from inhibitory terminals. Postsynaptic $GABA_B$ receptors induce sIPSCs by activating K^+-channels, which hyperpolarizes the membrane, favors voltage-sensitive Mg^{2+} block of NMDA receptors and shunts excitatory currents. $GABA_B$ receptors in spines and dendritic shafts are activated by spillover of GABA from adjacent terminals during population oscillations or during epileptiform activity, which may serve to regulate the excitability of the network and to counteract excess excitation. Dendritic $GABA_B$ receptors inhibit backpropagating action potentials through activation of K^+-channels, which may influence synaptic plasticity processes and action potential generation at the axon hillock. During high-frequency transmission GABA depresses its own release by an action on $GABA_B$ autoreceptors, which permits sufficient NMDA receptor activation for the induction of LTP (adapted form Bettler et al., 2006).

The postsynaptic $GABA_B$ receptors activate K^+-channels, which in turn hyperpolarize the membrane, thus mediating slow postsynaptic currents (sIPSC) and shunts excitatory currents (Wagner & Dekin, 1993, 1997; Lüscher et al., 1997; Harayama et al., 1998). Recent studies have implicated the role of $GABA_B$ receptors in the modulation of synaptic plasticity (Davies et al., 1991; Patenaude et al., 2003; Huang

et al., 2005), heterosynaptic depression (Vogt & Nicoll, 1999), population burst firing and inhibition of backpropagating action potentials (Zilberter et al., 1999; Leung & Peloquin, 2006).

1.6 Postnatal development of $GABA_B$-ergic inhibition

$GABA_B$ receptors together with $GABA_A$ and glicine receptors play crusuial role in synaptic inhibition in adult mice. However, the roles of GABA-ergic transmission change during postnatal development. $GABA_A$ mediated inhibition is not present in many brain areas at early stages of development (Ballanyi & Grafe, 1985; Cherubini et al., 1991; Hara et al., 1992; Gaiarsa et al., 1995; Ritter & Zhang, 2000). Morever, the chloride reversal potential in PBC neurons during the first postnatal week is more depolorizing than the resting membrane potential (Ritter & Zhang, 2000). Till the end of the first postnatal week, concomidantly with the appearance of chloride mediated inhibition, the blockade of $GABA_A$ receptors abolishes respiratory rhythm and seizure-like activity in respiratory network (Brockhaus & Ballanyi, 1998; Ritter & Zhang, 2000). Studies have demonstrated that at the very early stage of postnatal development (P0-P4) $GABA_B$ receptor mediated postsynaptic modulation plays an important role in PBC, while the $GABA_B$ mediated presynaptic modulation developes with longer latency and becomes predominant within the first postnatal week (Zhang et al., 2002).

1.7 The structure and function of I_h channels

Hyperpolarization activated cation currents, or in other term pacemaker currents are generated by pacemaker channels that belong to the superfamily of voltage-gated ion channels but form a distinct subgroup that is closely related to voltage-independent, cyclic nucleotide-gated channels. Because of their activation upon membrane hyperpolarization, pacemaker currents were referred as I_f for “funny current” in the heart (Brown et al., 1979), or I_q for “queer current” in the brain (Halliwell et.al., 1982) when they were originally discovered. This unique property of pacemaker currents gave rise to now widely used name h-current where “h” stands for hyperpolarization. Upon hyperolarization, the channels are permeable to both Na^+-and K^+-ions (permeability ratio Na^+: K^+=0,2-0,4). However, imaging techniques allowed identifying

permeability to Ca^{2+}-ions as well (Yu et al., 2004).

Activation of I_h current is slow, with activation time constants ranging between hundreds of milliseconds and seconds. However, in some pyramidal neurons from hippocampus, cortex, and cerebellum, activation is complete within tens of milliseconds (Frère et al., 2004). I_h, mostly, is extremely sensitive to intracellular concentration of cyclic adenosine monophosphate (cAMP). The sensitivity to cyclic guanosine monophosphate (cGMP) has been also reported, although to much weaker extent (Kaupp et al.; 2001; Robinson et al., 2003).

I_h current plays an important pacemaker role in controlling cellular excitability. For example, in thalamic circuits I_h regulates the periodicity of network oscillations generated by thalamic relay neurons (Luthi & McCormick, 1998; Luthi et al., 1998). At present, I_h currents are implicated in numerous additional cellular functions that include contribution to neuronal resting membrane potentials, presynaptic modulation of neurotransmitter release (Pape, 1996; Beaumont &Zucker, 2000; Southan et al., 2000) and modulation of the dendritic integration of inhibitory and excitatory synaptic inputs (Schwindt & Crill, 1997; Magee, 1998; 1999).

To date, four mammalian HCN subunits have been cloned (from human, rat, rabbit and mouse), which have been termed HCN1–4 (Santoro et al., 1997; 1998; Ludwig et al., 1998; Seifert et al., 1999; reviewed by Kaupp & Seifert, 2001). HCN channels display the membrane topology of voltage gated K^+-channels, with six transmembrane domains S1-S6 (Figure 1.3). Currents, mediated by HCN1, -2, and -4 genes in heterologous expression systems have properties typical for I_h, whereas HCN3 mediated currents have not been described (Much et al., 2003). Homomeric or heteromeris assembly of HCN1, -2 and -4 subunits gives rise to channels that display different characteristics. Channels, composed of HCN1 subunits, are activating rapidly (tens of milliseconds at voltages bellow 100mV) and are weakly sensitive to cAMP. On contrast to HCN1, HCN2- and -4 subunits give rise to channels that are slow activating (hundreds of milliseconds at voltages bellow 100mV) and are extremely sensitive to cAMP. HCN ion channels are widely expressed on both neuronal and nonneuronal cells. HCN1 is predominantly expressed in the cortical, hippocampal, and cerebellar regions (Robinson et al., 2003, Santoro et al., 1999;

Moosmang et al., 1999, Kaupp et al., 2001). HCN2 and HCN4 are widely expressed in regions, where they function as pacemakers (Franz et al., 2000; Monteggia et al., 2000; Santoro et al., 2000). Thus, the current mediated by these subunits (whether expressed alone or in different heteroligomeric combinations or natively) can have different properties.

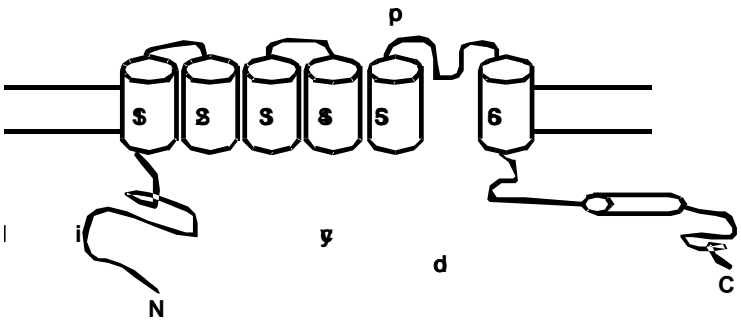


Figure 1.3: Transmembrane topology of the cloned HCN channels. S1-S6 symbolize the six transmembrane-spanning domains of the channels; N- and C-terminus, respectively. The box at the C-terminus represents the cyclic-nucleotide-binding domain, which is connected to the channel via a C-linker domain (Wang et al., 2001) that is important in coupling the binding of cyclic nucleotide to the alterations in voltage-gating of the channel. The number of amino acids at both termini varies for the four HCN subunits.

Many mechanisms that include changes in intracellular levels of cAMP do, therefore, modulate I_h channels due to unique property of these channels to be directly gated by cAMP (Tokimasa and Akasu, 1990). Thus, neurotransmitters that alter the basal activity of adenylyl cyclases, have been shown to modulate I_h (Banks et al., 1993; Bobker and Williams, 1989; DiFrancesco and Tromba, 1988).

1.8 Aim of the study

The present study endeavors to gain more insights into the function and downstream signaling of $GABA_B$ receptors in brainstem respiratory network of neonatal mice.

As a first step, we have used mutant mice with ablations in the $GABA_{B1}$ gene (lacking $GABA_{B1a}$ and $GABA_{B1b}$ subunits) to see what functional consequences would have deletion of $GABA_{B1}$ in respiratory network of neonatal mice.

Next, we have addressed a question of downstream signaling of $GABA_B$ receptors that are negatively coupled to cAMP synthesis by studying the regulation of I_h in neonatal mice. In particular, we were interested to find out the pathway or the pathways and the responsible molecules involved in such regulation, and whether this regulation undergoes changes during postnatal development.

2. Materials and Methods

2.1 Electrophysiology

2.1.1 Slice preparation

The preparation of brainstem slices followed the general procedure described in detail in Zhang et al. (1999). Briefly, postnatal day 0 (P0) to P11 male or female NMRI (Charles River Laboratories, USA) and Balb/c (present from the University of Basel, Switzerland) mice were decapitated at C3-C4 spinal level. The whole brain was carefully removed from the skull and was immediately placed in the ice-cold artificial cerebrospinal fluid (ACSF, composition described later in this chapter), bubbled with carbogen (95% O_2 and 5% CO_2). The cerebellum and forebrain were removed to expose the brainstem. The brainstem was glued with the dorsal ste up by Cyanoacrylat (Loctite, Germany) onto the agarblock. The brainstem was sectioned by a vibratome slicer (752M Vibroslice, Campden Instruments, UK) from rostral to caudal until the nucleus ambiguous (NA) and inferior olive (IO) were seen at the rostral boundary of the PBC. Afterwards 200μm slices were cut, transferred into incubation chamber, which was superfused by ACSF. A schematic drawing of a slice used for recordings is shown in Fig. 2.1.

2.1.2 Electrophysiological Recordings

For electrophysiological recordings, the slices were placed into the glass bottomed recording chamber. To prevent the slices from any dislocation during recordings, they were fixed by a platinum wire with a grid of parallel nylon threads. During experiments, slices were continuously perfused with extracellular solution by using a pump (Watson Marllow,). The slices were visualised by a Axioscope microscope (Zeiss, Germany) using a 5x objective. The neuronal bodies of PBC were identified under infrared gradient contrast illumination (C2400, Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany) with a 40x water immersion objective. The recordings were performed using an Axopatsch 200 amplifier (Axon Instrument

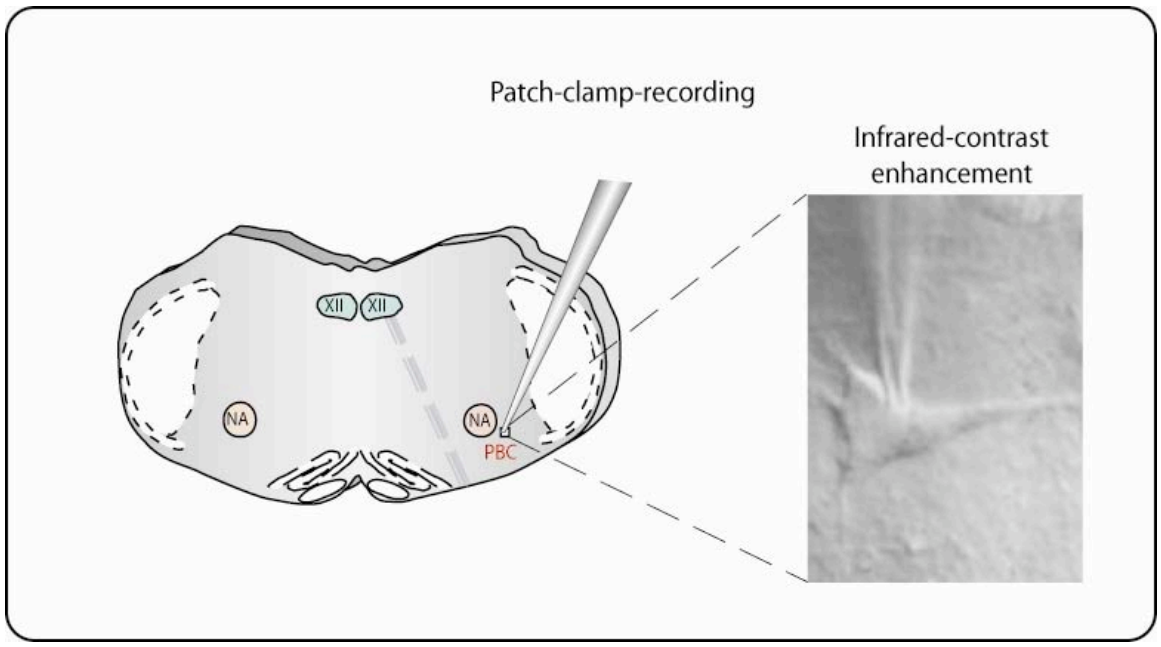


Figure 2.1: Schematic representation of brainstem slices containing PBC used for electrophysiological experiments: Abr. PBC; pre-Bötzinger complex, NA; nucleus ambiguous.

Inc., USA). Recording electrodes (resistance 2-4MΩ) were prepared by pulling borosilicate glass micropipettes (GC150-10F, Clark Electromedical Instruments, UK) on a multistage puller (P87, Sutter Instrument Co., Novato, USA). Experiments were carried out in voltage-clamp mode of whole-cell configuration. Whole cell recording configuration can be applied to measure the currents that result from ion movements across the membrane. The first step in achieving this configuration is to obtain a high resistance contact between the pipette and the cell membrane (gigaseal). The patch of membrane under the pipette is ruptured by application of a short pulse of negative pressure. The tight seal between pipette glass and cell membrane persists and low resistance route for current flow is now into the cell and across entire cell surface membrane. In the whole cell-recording pipette solution forms a continuum with the cell cytoplasm. Thus the solution filling the patch pipette will enter into and equilibrate with the cell interior. The potential on the outside surface is 0 mV (bath potential). After establishing the whole-cell configuration, the holding potential was set at -70mV.

The membrane currents were filtered by a four-pole Bessel filter set at a corner frequency of 1 kHz and digitized at a sampling rate of 5 kHz using a DigiData 1200 interface (Axon Instrument Inc., USA). Leakage currents were corrected by applying

four leakage-subtraction pulses immediately before the main command step and subtracting the accumulated calibrating responses from the test responses (P/4 protocol). The capacitance and resistance was compensated 80 % according to manufactures recommendation. All the experiments were conducted at the 35°C.

2.1.3 Capacitance and series resistance calculation

The passive properties of PBC neurons were estimated by determining membrane capacitance and series resistance for each recorded neuron before subsequent compensation. Capacitance and series resistance were calculated from the integral of the current transients induced by 20 mV hyperpolorarizing voltage commands from a holding potential of –70 mV immediately after rupture of the cell membrane according to these formulas:

$$C=I\tau/20$$

$$Rs=\tau/C$$

Where C is the capacitance, τ is the decay, I is the current, Rs is the series resistance. Cells with series resistance higher than 20MΩ and were not used for data analysis.

2.1.4 I_h current and spontaneous postsynaptic current measurements

Hyperpolarization activated cation currents were evoked by applying hyperpolarizing steps of 1500ms from -60 to -120mV. Patch-clamp electrodes were filled with pipette solution INLOW (see Solutions). For voltage protocols pClamp 6,0 software (Axon Instrument Inc., USA) was used. The measurement and analysis of I_h current will be discussed in detail in Results part. Because the total whole cell current is dependant on the total number of expressed channels, we estimated current density as a cell size independent parameter according to following formula:

$$I=I_1/C$$

Where I=current density (pA/pF), I_1=whole cell current (pA), and C=capacitance (pF).

Spontaneous postsynaptic (sPSC) and spontaneous postsynaptic inhibitory currents (sIPSC) were recorded from neurons of preBötzinger complex at about 0mV.

2.1.5 Ventilation Recordings

Ventilation patterns were recorded by whole-body plethysmography. Briefly, unanaesthetized newborn pups were placed in a chamber (15ml), which was closed. The chamber was connected to a differential pressure transducer (CD15 Carrier Demodulator, ValiDyne). The analog signal of ventilation-related changes of air pressure was amplified and digitized using an A/D-converter (DigiData 3200, Axon Instruments) and analysed using Clampex 9 (Axon Instruments).

2.1.6 Data acquisition and analysis

For the data acquisition and analysis pClamp 6,0 software (Axon Instrument Inc., USA) was used. Only tests of a single neuron in each slices was used for data analysis. Unless stated, data are reported as mean±SEM. Statistical significance was evaluated by Students t test (Prism 4 software, Graphpad, USA).

2.1.7 Solutions and drugs used for electrophysiological experiments

Experiments were carried out in the ASCF containing (in mM): NaCl, 118; KCl, 3; $CaCl_2$, 1.5; $MgCl_2$, 1; $NaHCO_3$, 25; NaH_2PO4, 1; Glucose 5, equilibrated with carbogen at 27-29°C (pH 7.4, Osm. ca. 324). For IH current measurements pipettes were filled with INLOW solution containing (mM): KGluconate, 140; $CaCl_2$, 1; EGTA, 10; $MgCl_2$, 2 Na_3ATP, 4; Na_3GTP, 0,5; HEPES-KOH, 10 (pH 7.3, osm ca. 310). For measurement of synaptic transmission pipettes were filled with INK solution containing (in mM): 140; KCl, 1; $CaCl_2$ x $2H_2O$, 10; EGTA, 2; $MgCl_2x6H_2O$, 0,5 Na_2GTP, 4; Na_2ATP, 10; HEPES (pH 7,2, osm ca. 310). Potassium D-gluconat, Calcium chloride dehydrate, Ethylene glycol-bis (2-aminoethylether)-N,N,N_,N_-tetraacetic acid, $MgCl_2x6H_2O$, Guanosine 5´-triphospate sodium salt hydrate, Adenosine 5´-triphospate disodium salt, HEPES, Potassium chloride, Cesium chloride, Tetraethylammonium chloride were purchased from Sigma-Aldrich,

Germany. Natrium chloride, Natriumhydrogencarbonate, Natrium dihydrogen phosphate Monohydrate, Calcium chloride dehydrate, α-D (+)-Glucose Monohydrate were purchased from Roth; Germany. Magnesium chloride hexahydrate and Potassium chloride were purchased from Sigma-Aldrich, Germany.
Bellow are listed the pharmacological compounds that were used for electrophysiological experiments.

Substance	**Concentration**	**Purchased from**
R-Baclofen	5μM, 30μM	Tocris
CGP55845A	5μM	gift from Novarits
ZD7288	100μM	Tocris
Rp-cAMP	10μM	Tocris
SQ *22,536*	200μM	Alexis
Pertussis toxin (PTX)	0,04μg/ml	Sigma-Aldrich
Bovine brain Gβγ subunit	20nM	Callbiochem
SPβγ	200μM	synthesized by SeqLab
FVII	200μM	synthesized by SeqLab
CNQX disodium salt	100μM	Tocris
DL-AP5	10μM	Tocris
Anti-Gαs antibody	1:10	Santa-Cruz
Anti-Gi3 antibody	1:10	Santa-Cruz

Peptide sequences were as follows:

SPβγ: DALRIQMEERFMASNPSKVSYEPIT(Ma et al., 1997)

FVII: YEDSYEDISAYLLSKNNAIPR (Ma et al., 1997)

Peptides were prepared in water and kept at -20°C.
The above mentioned substances were added from the higher concentrated stock solutions either extracellular or intracellular. (R)-Baclofen, ZD7288, Rp-cAMP, SQ *22,536*, CGP55845A, and CNQX were made in H_2O. The stock solution of DL-AP5 was made in NaOH. Pertussis toxin was included in pipette solution. Bovine brain $G_{\beta\gamma}$ subunit was aliquoted and kept at -80°C. The effects of different G-protein antibodies,

$G_{\beta\gamma}$ subunits and peptides were assessed by introducing them directly into the cell by intracellular dialysis from the recording pipette. For all recordings in the presence of G-protein antibodies and peptides the tip of recording pipette was filled with to 1-2mm with standard recording solution, and the pipette was back-filled with the experimental solution. In this way the onset of activation of antibody loading was delayed, which permitted the measurement of control responses within the first few minutes of whole cell recording. Absence of protein-containing mixtures in the tip of the recording pipette facilitated the formation of gigaohm seals and helped prevent clogging of the pipette tip after patch rupture. For the antibody experiments intracellular solution was modified slightly for its normal composition including 0, 5% bovine serum albumin and increasing the concentration of GTP (1mM).

2.2 Fluorescence immunohistochemistry

2.2.1 Brain tissue preparation

The tissue for immunohistochemistry was prepared as follows. Postnatal NMRI mice were deeply anaesthetized with TBE (tribromethanol) until they were unresponsive to painful stimuli. A thoracotomy was perormed and animals were perfused through the aorta with 0.9% sodium chloride followed by 100ml 4 % paraformaldehyde in 0.1 M phosphate puffer. The whole brain was removed, post-fixed for 1 hour in the same fixative at 4 ºC. The tissue was cryoprotected in 30% sucrose overnight at 4 ºC. Series of transverse sections of brainstem with a thickness of 14 µm were cut using a cryostat (Leica). Each section was quickly placed on the slide. After sectioning the slides were kept at -20ºC.

2.2.2 Immunofluorescence staining

The slices were washed three times for 10 min. with PBS. Non-specific binding sites were blocked and permeabilisiation was done by incubating slices in 2% NGS and 0, 2 % Triton X-100 in phosphate-saline buffer (PBS) for 20-30 min at RT (room temperature). Sections were incubated overnight at 4ºC in primary antibody solution dissolved in PBS containing 2% NGS and 0,2% Triton X-100. Afterwards the sections

were washed 3 times for 10 min. in PBS. Then sections were incubated for 1 Hour at RT in the dark with species-specific flurochrome-conjugated secondary antibodies, followed by three washing steps for 10 min each. Finally, sections were slightly air-dried and coverslipped with fluorescent mounting medium (DAKO). Primary antibody was rabbit anti $GABA_{B12}$ subunits (Chemicon). Secondary antibody was goat anti rabbit Cy3 antibody (Jackson Immunoresearch). Sections were visualized by confocal laser scanning microscopy (Zeiss LSM510). Typically, images (1024x1024 pixel) at a zoom factor 4 spaced by 0,38-0,42µm were taken, using a 63xoil-immersion objective (A=550, E=570).

2.2.3 Solutions and chemicals

TBE (tribromethanol)
1 ml TBE, 4 ml ethanol, 45 ml 0.9% NaCl

PFA 4% (for 1 L)
80g paraformaldehyde in 0,1 mM PB

PB (phosphate buffer for 2 L)
Buffer 1: 0.2 M NaH_2PO_4 (27, 6 g $NaH_2PO_4xH_2O$)
Buffer 2: 0.2 M Na_2HPO_4 (71, 7 g $NaH_2PO_4x12H_2O$)

PBS (for 1 L)
10 mM PB; 150 mM NaCl; 2,7mM KCl
(50 ml PB; 8,77 g NaCl; 200 mg KCl; 900 ml H_2O)

NaCl, $NaH_2PO_4xH_2O$ and $NaH_2PO_4x12H_2O$ were purchased form Roth, KCl and NGS (normal goat serum), were purchased from Sigma-Aldrich.

2.3 $GABA_{B1}$ knockout mice

2.3.1 Generation and breeding of $GABA_{B1}$ knockout mice

$GABA_{B1}^{-/-}$ mice were generated by Novartis group (Basel, Switzerland), and 3 pairs were generously provided by Prof. Bettler (University of Bassel, Switzerland). Briefly, $GABA_{B1}$ null mutant mice were generated by using Balb/c embryonic stem cells, as illustrated in Figure 2.2. In these mice two known $GABA_{B1a}$ and $GABA_{B1b}$ alleles were deleted. The breeding was done in our Animal Facility (Center Physiology and Pathophysiology, University of Göttingen) by crossing heterozygous $GABA_{B1a/b}^{+/-}$ males with $GABA_{B1a/b}^{+/-}$ females. The mice were obtained at the predicted Mendelian ratio.

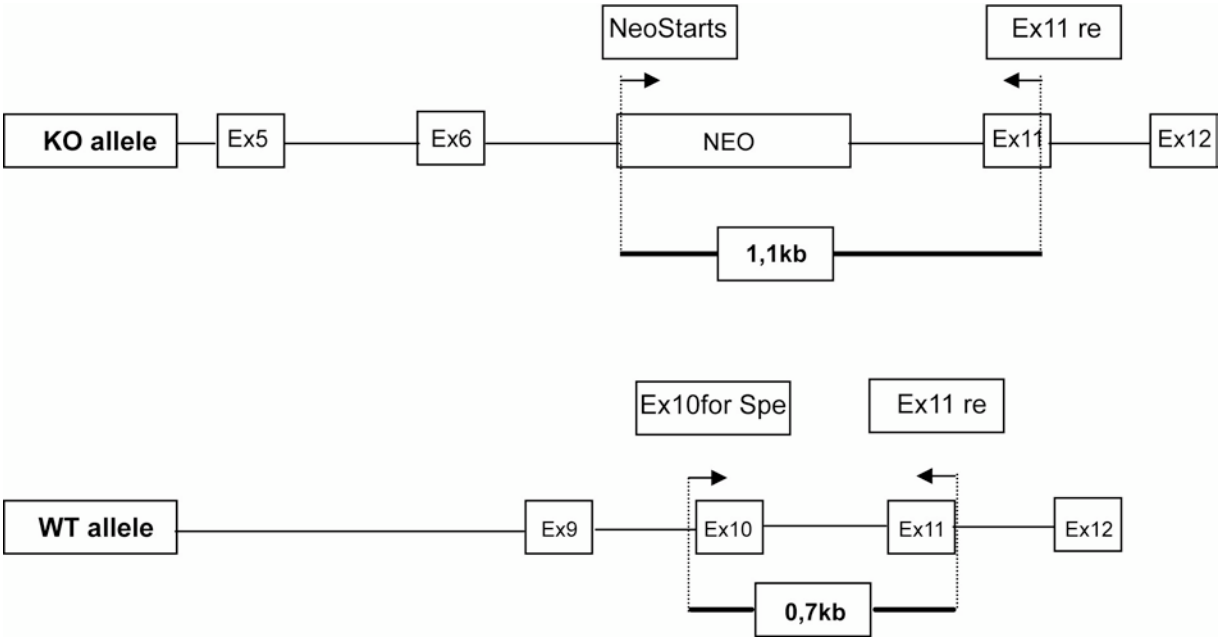


Figure 2.2: Situation of the KO and wild type alleles in the mouse genome.

2.3.2 Diagnostic PCR

Diagnostic PCR was performed in order to distinguish between wild type $GABA_{B1a/b}^{+/+}$, heterozygote $GABA_{B1a/b}^{+/-}$ and homozygote $GABA_{B1a/b}^{-/-}$ mice. For DNA extraction mouse tails were incubated overnight at 55°C with agitation (850 rpm) in eppendorf tubes in 0, 5 ml lysis buffer containing Proteinase K. Afterwards eppendorf tubes were

centrifuged for 10 minutes at 12'000 rpm (Eppendorf centrifuge). 10 µl of supernatant was taken and diluted into 100 ml water. 2µl of the dilluted supernatant was taken and used for 25µl PCR reaction. The DNA was amplified in the thermocycle (GeneAmp, PCR System 9700, Applied Biosystems, Germany).

The following steps have been chosen:

1. 93°C 10min
2. 93°C 30sec
3. 56°C 45sec
4. 65°C 2 min
   Repeat the last three steps for 40 times.
5. 65°C 10 min
6. 4°C

2.3.3 Solutions and chemicals

Lysis Buffer (mM)

100 mM	Tris-Cl pH 8.5
5 mM	EDTA pH 8.0,
200 mM	NaCl

0,2% SDS.

Proteinase K (Roche, Germany). (It was added just before the digestion to a final concentration of 100 mg/ml. Stock solution was made in water and aliquots were kept at –20^{o}C).

For PCR reaction the following substances with the final concentration were taken:

Taq DNA Polymerase	0,05units/µl (Sigma-Aldrich, Germany)
1xPCR buffer	(delivered together with *Taq* DNA Polymerase)
200µM	Deoxynucleotide Mix (Sigma-Aldrich, Germany)
5OD	primer mix
H_2O	

Primers were designed by Novartis Group and were synthesized by Applied Biosystems (Göttingen).

To detect WT allele following primers were used:

Ex10	5’AGC TGA CCA GAC CTT GGT CAT 3’
Ex11re (21mer)	5'AAC TGG CTT CTC CCT ATG TGG 3’

To detect KO allele the following primers were used:

NeoStart	5' ATG GGA TCG GCC ATT GAA CAA 3’
Ex11re (21mer)	5' AAC TGG CTT CTC CCT ATG TGG 3’

3. Results

3.1 PBC neurons express $GABA_B$ receptors

First, we tested whether PBC neurons express $GABA_B$ receptors. For this reason we have used an antibody against $GABA_{B1}$. Immunofluorescence staining of neonatal mice brain sections revealed a widespread $GABA_{B1}$ staining in pre-Bötzinger complex. As illustrated in Figure 3.1, the neurons in pre-Bötzinger complex exhibit diffuse somatodedritic staining of $GABA_{B1}$.

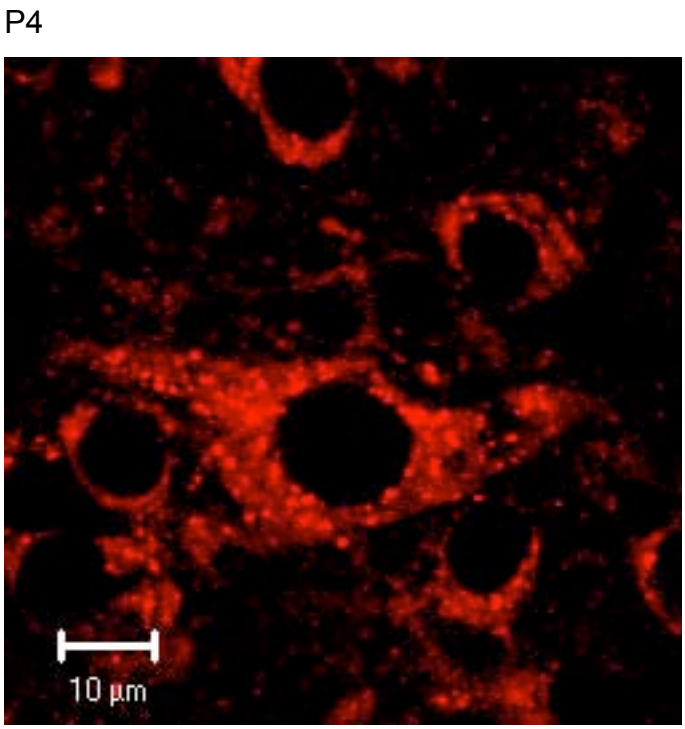


Figure 3.1: Expression of $GABA_{B1}$ receptors In PBC. Immunofluorescence detection of mouse transverse sections by using an antibody against $GABA_{B1a/b}$. Scale bar, 10µm

3.2 Consequencies of $GABA_{B1}$ deletion in respiratory network

3.2.1 Respiration is not affected in $GABA_{B1a/b}^{-/-}$ KO mice

In these series of experiments we used a knockout approach to analyze the functional consequences of deletion of $GABA_{B1}$ subunit in vivo. The $GABA_{B1}$ knockout mice were generated, in which the two known $GABA_{B1}$ subunits, $GABA_{B1a}$ and $GABA_{B1b}$,

were deleted. $GABA_{B1a/b}^{-/-}$ KO mice were viable. Whole-body plethysmography demonstrated that the lack of $GABA_{B1}$ receptors has no essential role in respiration. The representative ventilation traces, presented in Figure 3.2A, obtained from WT and $GABA_{B1a/b}^{-/-}$ KO mice showed no difference in breathing between two genotypes. Averaged ventilation frequencies were 3,423±0,2313 Hz (n=6) and 3,376±0,6376 Hz (n=3) in WT and in $GABA_{B1a/b}^{-/-}$ KO mice, respectively (Figure 3.2B).

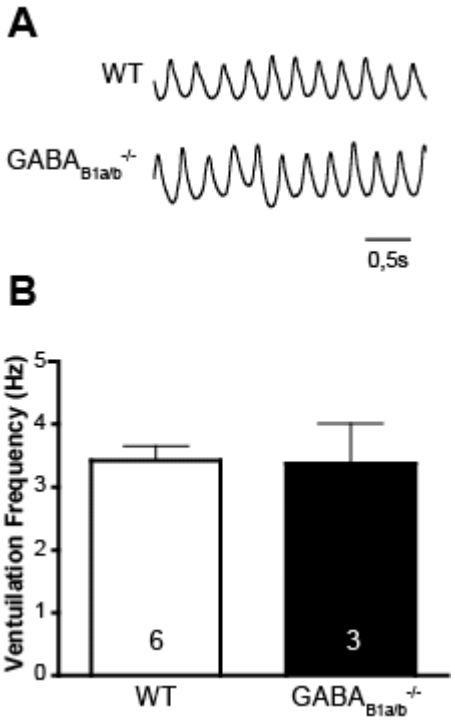


Figure 3.2: $GABA_{B1}$ receptors have no essential role in respiration. A: Representative ventilation traces in WT and $GABA_{B1a/b}^{-/-}$ KO mice. B: Averaged ventilation frequencies in WT and $GABA_{B1a/b}^{-/-}$ KO mice. Numbers within the bar graphs indicate the number of mice tested for each genotype. Data are shown as means±SEM.

3.2.2 Synaptic transmission is impaired in $GABA_{B1a/b}^{-/-}$ KO mice

Next, we examined whether the lack of $GABA_B$ would have any effect in synaptic transmission. Therefore we measured spontaneous postsynaptic currents (sPSC) in acute brainstem slices containing pre-Bötzinger complex of neonatal mice (P0-P3). The synaptic transmission was significantly impaired in $GABA_{B1a/b}^{-/-}$ mice compared to WT mice. In Figure 3.3A are presented representative traces of sPSC obtained from

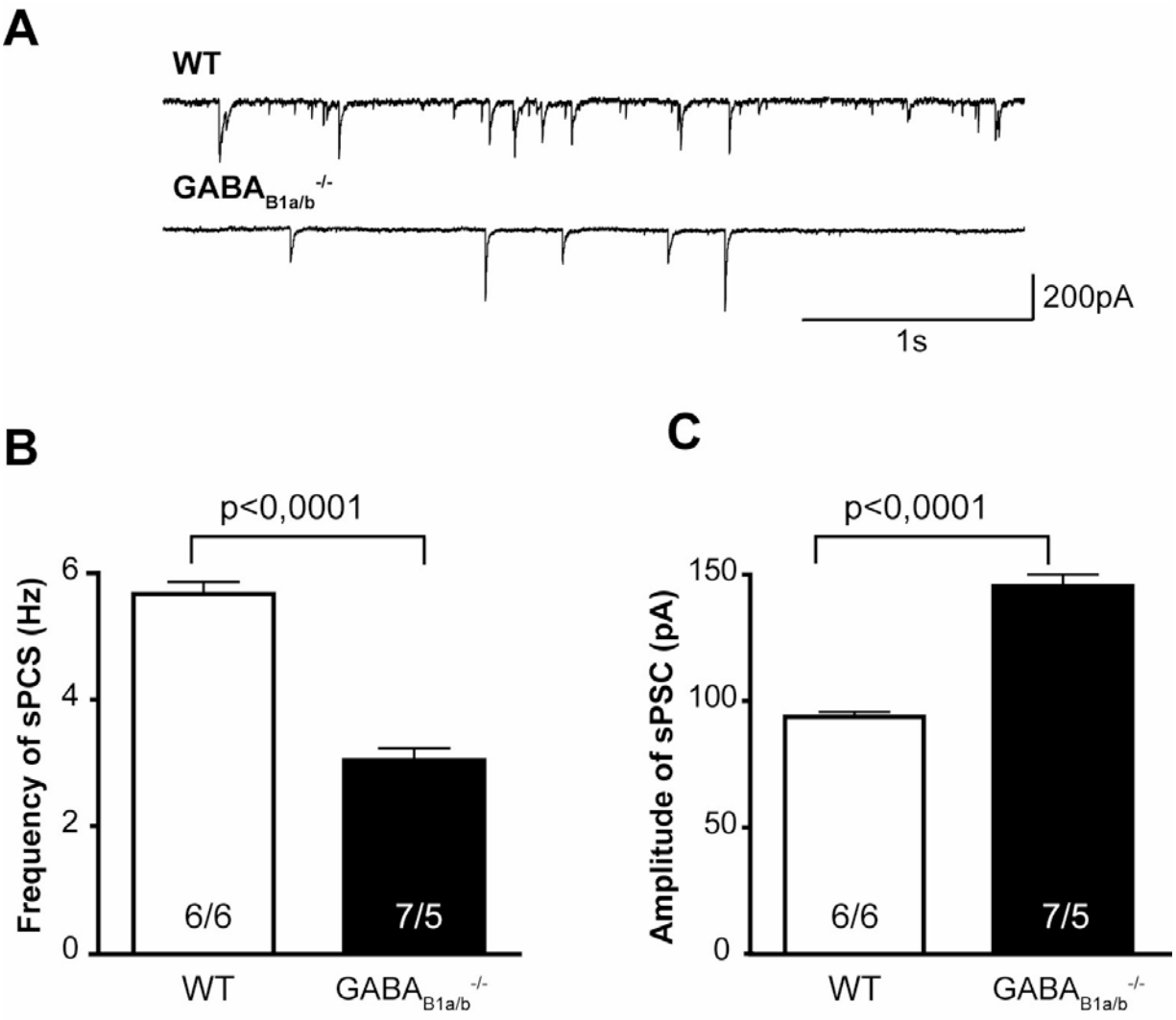


Figure 3.3: Impaired synaptic transmission in $GABA_{B1a/b}^{-/-}$ KO mice. sPSC were measured under whole-cell voltage clamp mode in pre-Bötzinger complex of neonatal mice (P0-P3). A: Representative traces of sPCS obtained from WT and $GABA_{B1a/b}^{-/-}$ KO mice, respectively. B: Averaged sPSCs frequency: C: Averaged sPSCs amplitude. Numbers in the bar graphs indicate the neuron and mice numbers tested for each genotype, respectively. Statistics was done by unpaired student's t-test. Data are shown as events±SEM.

WT and $GABA_{B1a/b}^{-/-}$ KO mice. The averaged mean frequency of sPSC was 5,670±0,1897 Hz in case of WT, whereas it was 3,041±0,1949 Hz in case of $GABA_{B1a/b}^{-/-}$ KO mice (Figure 3.3B). The amplitude of sPSC was also significantly affected in $GABA_{B1a/b}^{-/-}$ KO mice. The averaged amplitude of sPSC was 93,74±1,98734pA and 145,3±4,630pA (n=7) in WT and $GABA_{B1a/b}^{-/-}$ KO mice,

respectively (Figure 3.3C).

3.2.3 $GABA_B$ mediated responses are lacking in $GABA_{B1a/b}^{-/-}$ KO mice

Next we tested whether functional $GABA_B$ receptors are present in pre-Bötzinger complex of $GABA_{B1a/b}^{-/-}$ mice. For this reason, we recorded spontaneous inhibitory postsynaptic currents (sIPSC) in the presence of ionotropic and metabotropic glutamate receptor antagonists (100µM CNQX, 10µM AP5). In these neurons, application of baclofen caused the expected marked depression of sIPSC in WT mice. However, baclofen was not able to inhibit sIPSC in $GABA_{B1a/b}^{-/-}$ mice indicating that in contrast to WT mice no functional $GABA_B$ receptors are operational. In Figure 3.4 A, B are shown the representative traces of recordings of sIPSC in WT and In $GABA_{B1a/b}^{-/-}$ mice during subsequent application of 30µM baclofen and 5µM CGP55485A, a specific $GABA_B$ receptor antagonist. In WT mice baclofen caused an inhibition of the frequency of sIPSC from 7,655±0,2185 Hz to 2,338±0,1268 Hz. In these neurons CGP55485A application antagonized the effect of baclofen by increasing the frequency to 7,268±0,2022Hz (Figure 3.4C). In case of $GABA_{B1a/b}^{-/-}$ mice the mean frequency of sIPSC was 4,653±0,1200 Hz (Figure 3.4D). After baclofen application it remained unchanged and was 4,430±0,1145 Hz. CGP55845A application caused a slight increase of the frequency of sIPSC to 5,245±0,1508 Hz. Likewise, baclofen caused a significant decrease of the amplitude of sIPSC in WT, but not in $GABA_{B1a/b}^{-/-}$ mice. In Figure 3.4 E, F are shown the summary bar graphs of the amplitude of sIPSC obtained from WT and $GABA_{B1a/b}^{-/-}$ mice, respectively. In detail, baclofen caused a decrease of the amplitude from 126,6±2,109pA to 92,57±2,656pA in WT mice. However, in contrast to the frequency, the amplitude was not increased by CGP55845A application in WT mice (87,12±1,612pA).

In $GABA_{B1a/b}^{-/-}$ mice the amplitude of sIPSC was 136,2±2,523 pA, and after subsequent application of baclofen and CGP55845A it became 127,2±2,294pA and 126,3±2,566pA, respectively.

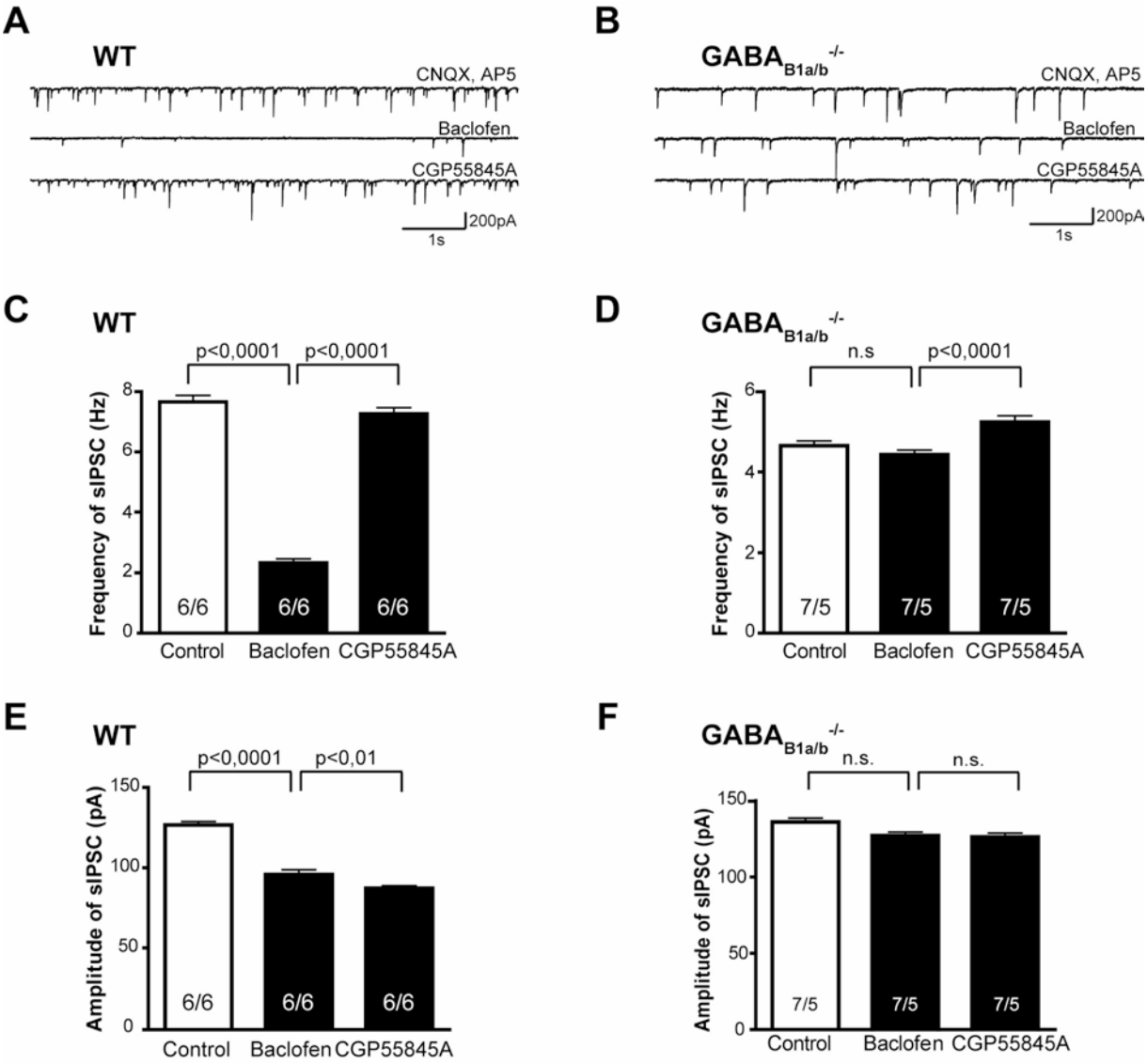


Figure 3.4: sIPSC measurements of $GABA_{B1a/b}^{-/-}$ mice: Experiments were performed under whole-cell voltage clamp mode in pre-Bötzinger complex: A: Representative traces of sIPSCs in WT mouse recorded in the presence of CNQX, AP5, and after subsequent application of 30µM baclofen and 5µM CGP55845A, respectively. B: Representative traces of sIPSC in $GABA_{B1a/b}^{-/-}$ mouse in the presence of CNQX, AP5 and after subsequent application of 30µM baclofen and 5µM CGP55845A respectively. C, D: Summary bar graphs of sIPSC frequency during application of baclofen and CGP55845A in WT and $GABA_{B1a/b}^{-/-}$ mice, respectively: E, F: Summary bar graphs of sIPSC amplitude during application of baclofen and CGP55845A respectively. Numbers in the bar graphs indicate the cell and mice numbers tested for each genotype. Statistics was done by unpaired student's t-test. Data are shown as events±SEM.

3.1.5 The important role of $GABA_B$ receptors in the network sensing extracellular pH

To explore the potential role of $GABA_B$ receptors in the network sensing extracellular pH, we first asked whether changing of extracellular pH will alter synaptic transmission in $GABA_{B1a/b}^{-/-}$ mice. For this reason, we first recorded sPSC in acute brainstem slices of $GABA_{B1a/b}^{-/-}$ mice (P5-P8) superperfused with an extracellular solution with pH7,4. Afterwards, the slices were superfused for a minute with an extracellular solution with lower pH: pH7,2. Afterwards, sPSC were recorded. In Figure 3.5A,B are illustrated the representative sPSC traces obtained from WT and $GABA_{B1a/b}^{-/-}$ mice, respectively. As is seen from the figure, the frequency of sPSC is decreased when the extracelluar pH was 7,2 in WT, but not in $GABA_{B1a/b}^{-/-}$ mouse. The quantification of mean frequency in WT mice were as follows: 7,460±0,2192 Hz in control (pH7,4) and 5,536±0,1844 Hz in pH7,2 (n=10/8, p<0,0001, Figure 3.5C). In case of $GABA_{B1a/b}^{-/-}$ mice the mean frequency in control (pH7,4) was 5,633±0,1436 Hz and 5,952±0,1440 Hz in pH7,2 (n=11/8, n.s; Figure 3.4D). Furthermore, the analysis showed a decrease of mean amplitude in both genotypes. In detail, the mean amplitude of sPSCs in WT was 119,4±2,938 pA in control and 95,16±2,495 pA in pH7,2 (n=10/8, p<0,0001, Figure 3.5E). The mean amplitude of sPSC in $GABA_{B1a/b}^{-/-}$ mice was 115,4±2,434 pA in control and 98,64±1,932 pA in pH7,2 (n=11/8, p<0,0001, Figure 3.5G).

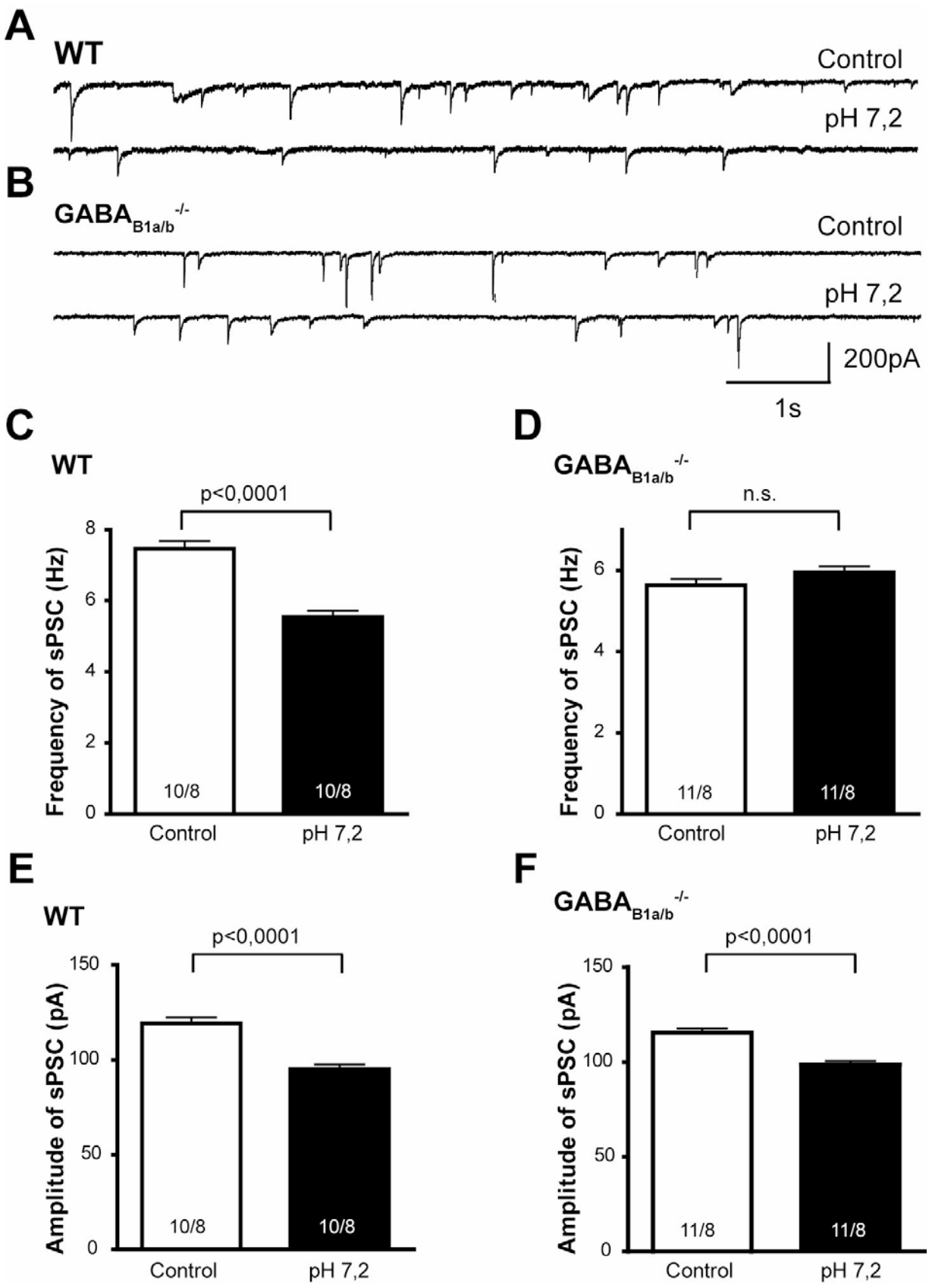

A
WT
Control
pH 7,2
B
GABA B1a/b -/-
Control
pH 7,2
200pA
1s
C
WT
p<0,0001
Frequency of sPSC (Hz)
8
6
4
2
0
10/8
10/8
Control
pH 7,2
D
GABA B1a/b -/-
n.s.
Frequency of sPSC (Hz)
6
4
2
0
11/8
11/8
Control
pH 7,2
E
WT
p<0,0001
Amplitude of sPSC (pA)
150
100
50
0
10/8
10/8
Control
pH 7,2
F
GABA B1a/b -/-
p<0,0001
Amplitude of sPSC (pA)
150
100
50
0
11/8
11/8
Control
pH 7,2

Figure 3.5: The role of $GABA_B$ receptors in pH sensing: Experiments were performed under whole-cell voltage clamp mode in pre-Bötzinger complex: A; B: Representative traces of sPSC obtained from WT and $GABA_{B1a/b}^{-/-}$ mice in control condition (pH 7,4) and after superfusion of the slices in ACSF with lower pH (pH,2). C; D: Summary bar graphs of sPSC frequency in control condition and in lower pH obtained from WT and $GABA_{B1a/b}^{-/-}$ mice, respectively: E,F: Summary bar graphs of sPSC amplitude in control condition and in lower pH obtained from WT and $GABA_{B1a/b}^{-/-}$ mice, respectively. Numbers in the bar graphs indicate the neuron and the mice numbers, respectively, tested for each genotype. Statistics was done by unpaired student's t-test. Data are shown as events±SEM.

3.2.5 The atypical effects of baclofen in sPSC in low extracellular pH

Next, we tried to see whether baclofen will affect synaptic transmission in $GABA_{B1a/b}^{-/-}$ mice when the extracellular pH is low (pH 7,2). In Figure 3.6A,B are presented the representative traces of sPSC, obtained from WT and $GABA_{B1a/b}^{-/-}$ mice, respectively. Surprisingly, baclofen decreased the frequency in both genotypes, although not to the same extent. In contrast to WT mice, application of CGP55845A did not antagonize the effect of baclofen in $GABA_{B1a/b}^{-/-}$ mice. Quantification of mean frequency showed that 30μM baclofen almost blocked frequency from 5,300±0,2397 Hz to 0,2967± 0,07527 Hz (n=7/6, p<0,0001, Figure 3.6C), while 5μM CGP55845A increased the frequency to more than control (8,280±0,3478 Hz, n=7/6, p<0,0001) in WT mice. Baclofen administration in $GABA_{B1a/b}^{-/-}$ mice caused an inhibition of frequency from 3,005±0,1031 Hz to 2,308± 0,1089 Hz (n=6/5, p<0,0001, Figure 3.6D). However, 5μM CGP55845A application did not change the frequency (2,283±0,1426 Hz, n=6/5, n.s). Quantification of amplitude showed that baclofen does not decrease the amplitude of sPSC in low pH in WT mice. The summary of amplitude of WT mice is presented in Figure 3.6E. The amplitude was 51,81±0,9004pA in pH7,2, 54,01±3,977pA after baclofen and 50,23±0,9337pA after CGP55845A application (n=6/5, n.s.). In case of $GABA_{B1a/b}^{-/-}$ mice the mean amplitude in pH7,2 was 98,61±2,612 pA, after baclofen 79,60±2,506 pA (n=6/5, p<0,0001) and 77,95±2,538 pA CGP55845A application (n=6/5, n.s).

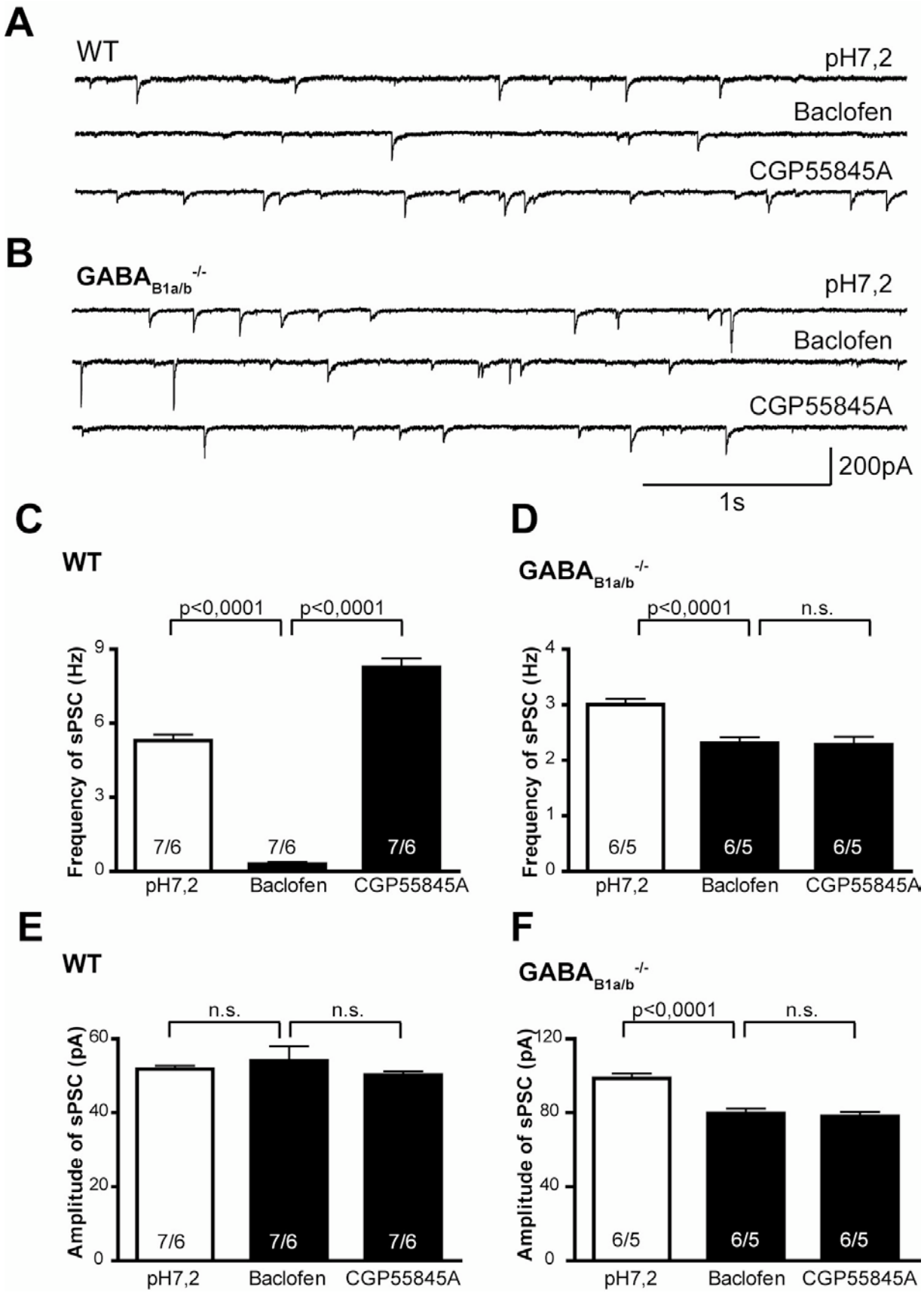

A
WT
pH7,2
Baclofen
CGP55845A
B
GABA B1a/b -/-
pH7,2
Baclofen
CGP55845A
200pA
1s
C
WT
p<0,0001
p<0,0001
Frequency of sPSC (Hz)
9
6
3
0
7/6
7/6
7/6
pH7,2
Baclofen
CGP55845A
D
GABA B1a/b -/-
p<0,0001
n.s.
Frequency of sPSC (Hz)
4
3
2
1
0
6/5
6/5
6/5
pH7,2
Baclofen
CGP55845A
E
WT
n.s.
n.s.
Amplitude of sPSC (pA)
60
40
20
0
7/6
7/6
7/6
pH7,2
Baclofen
CGP55845A
F
GABA B1a/b -/-
p<0,0001
n.s.
Amplitude of sPSC (pA)
120
80
40
0
6/5
6/5
6/5
pH7,2
Baclofen
CGP55845A

Figure 3.6: The effect of baclofen when the extracellular pH is low: Experiments were performed under whole-cell voltage clamp mode in pre-Bötzinger complex: A; B: Representative traces of sPSCs obtained from WT and $GABA_{B1a/b}^{-/-}$ mice after superfusion of the slices in ACSF with lower pH7,2, 30µM baclofen, 5µM CGP55845A. C; D: Summary bar graphs of sPSCs frequency obtained from WT and $GABA_{B1a/b}^{-/-}$ mice, respectively: E,F: Summary bar graphs of sPSCs amplitude obtained from WT and $GABA_{B1a/b}^{-/-}$ mice, respectively. Numbers in the bar graphs indicate the neuron and the mice numbers, respectively, tested for each genotype. Statistics was done by unpaired student's t-test. Data are shown as events±SEM.

3.3 I_h channels in pre-Bötzinger complex

3.3.1 Identification of I_h current in mouse pre-Bötzinger complex

The properties of hyperpolarization activated cation current, I_h, were investigated under voltage-clamp mode in mouse pre-Bötzinger complex (PBC). The isolation of I_h, current from other membrane responses was done by applying hyperpolarization voltage step from -60mV to -120mM. The representative trace of I_h current is shown in Figure 3.7A. The 'instantaneous' current jump ($\boldsymbol{I_{inst}}$) was measured immediately following the capacitive transient. The 'steady state' current ($\boldsymbol{I_{steady}}$) was measured at the end of 1,5 s hyperpolarizing voltage step. The difference between $\boldsymbol{I_{steady}}$-$\boldsymbol{I_{inst}}$ can be defined as $\boldsymbol{I_h}$ current. Applying series of hyperpolarizing voltage steps from -60mV to -120mV with 10mV increments allowed to plot I_{inst} and I_{steady} against membrane potential (Fig 3.7B). Since I_h current was reported to be dependant on bath temperature (DiFrancesco and Ojeda, 1980, Watts et al., 1996), we conducted all experiments at 35°C. Next, we tested the sensitivity of I_h current to ZD7288, which is a commercially available antagonist of I_h channels (Maccaferri and MacBain, 1996). In our experiments, bath application of 100 µM ZD7288 caused an inhibition of I_h current amplitude (Figure 3.8A). The mean amplitude of I_h current was 315,3±59,39pA (n=19) before ZD7288 application and 55,50±21,19 (n=10) after ZD7288 application (n=10, $p<0,01$) (Figure 3.8 B). These experiment shows that ZD7288 blocks I_h current in PBC neurons.

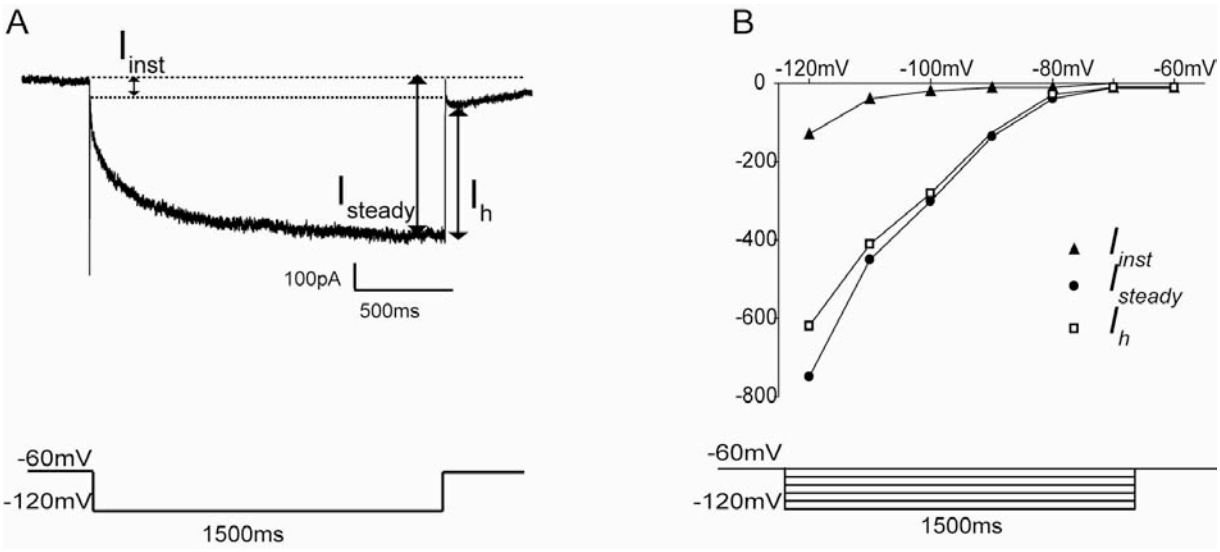


Figure 3.7: Identification of I_h current in PBC complex. A. representative current trace evoked by hyperpolarizing voltage step from a holding potential of -60mV as indicated. B. *IV* relationship: Instantaneous current, measured at the end of capacitive transient (filled triangle), the steady state current, measured at the end of voltage step (filled cycles) and the difference between them (empty squares), which is referred to as I_h.

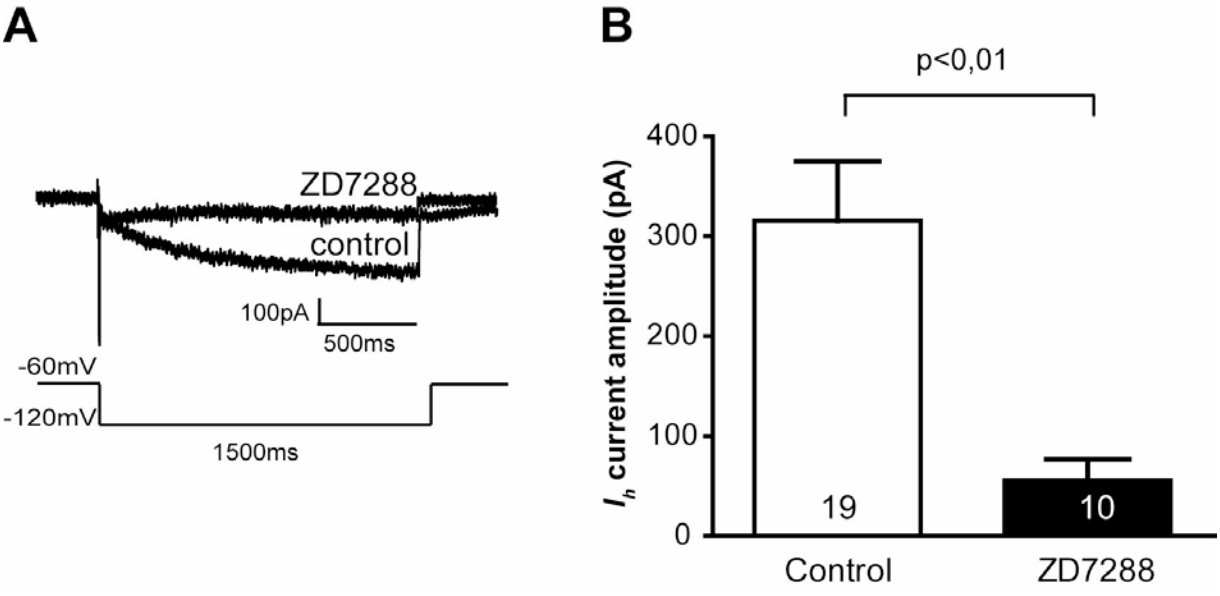


Figure 3.8: Sensitivity of I_h current ot ZD7288. I_h current was measured under voltage clamp by giving hyperpolarizing step of 1,5s duration from the holding potential of -60mV to -120mV as indicated. A: Representative traces recorded before and after ZD7288 application. B: Mean I_h current amplitude before and after ZD7288 application. Data are expressed as Mean±SEM. Numbers in the bar graphs indicate the number of the cells tested.

3.3.2 Developmental changes of I_h current in PBC neurons

To determine whether I_h undergoes changes during early postnatal development in PBC, we compared I_h current between two different age groups: P0-P4 and P5-P11. The recordings were preformed in PBC neurons under voltage clamp mode by giving series of hyperpolarizing steps from the holding potential of -60mV to -120mV. In Figure 3.9A are shown the representative traces of I_h current in P0 and P11 old mice. It is apparent from the traces that I_h current amplitude is significantly larger in P11 old mouse compared to P0 old mouse. Consequently, mean I_h current amplitude, presented in Figure 3.9B, was as much larger in older mice as compared to younger mice. The mean amplitude of I_h current was only 165,4±21,96pA in the age group of P0-P4 (n=11), while it was 418±23,27pA in the age group of P5-P11 (n=108; p<0,005). Further, we characterized mean I_h current density, which was 3,961±0,5935pA/pF in the age group of P0-P4 (n=11), and 7,297±0,3666 pA/pF (n=108, p<0,005) in the age group of P5-P11 (Figure 3.9D). Our results demonstrate that both I_h current amplitude and density increase significantly during postnatal development. In addition, we found that not only I_h current amplitude and density increase during postnatal development, but also the number of the cells having I_h current as shown In Table 3.1. While the number of the cells having I_h current was 22,66% in the age group of P0-P4, it was significantly more in the age group of P5-P11 (p<0,01).

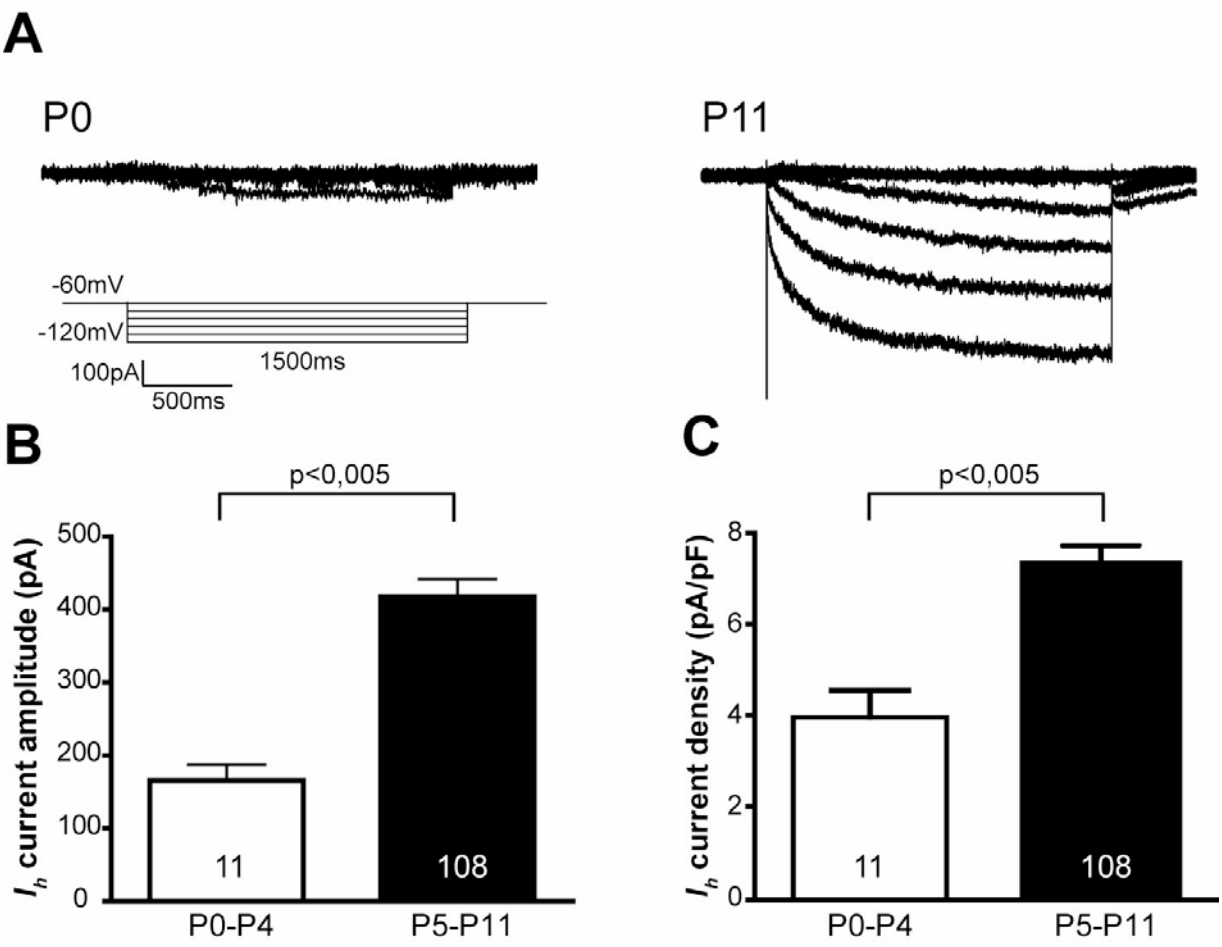


Figure 3.9: Postnatal changes of I_h current in mouse PBC. I_h current was measured under voltage clamp by giving series of hyperpolarizing steps of from the holding potential of -60mV to -120mV with 10mV increments as indicated. A: Representative traces of I_h current in P0 and P11 old mice. B: Averaged mean I_h current amplitude in the age group of P0-P4 and P5-P11, respectively C: Averaged I_h current density in the age group of P0-P4 and P5-P11, respectively. Data are shown as mean±SEM. Numbers in the bar graphs indicate the number of the cells tested.

Table 3.1

Age	Cells that have I_h current (%)
P0-P4	22, 66%±9,458
P5-P11	67, 38%±8,398

Data are expressed as mean±SEM

3.4 $GABA_B$ modulation of I_h current

I_h can be modulated by a variety of mechanisms including changes in intracellular levels of cAMP (Tokimasa and Akasu, 1990). Neurotransmitters that alter the basal activity of adenylyl cyclase, have been shown to modulate I_h (Banks et al., 1993; Bobker and Williams, 1989; DiFrancesco and Tromba, 1988). Since $GABA_B$ receptors are negatively coupled to cAMP activity, we tested whether activation of $GABA_B$ receptors can modulate I_h in neurons of pre-Bötzinger complex during postnatal development. The experiments were performed under voltage clamp mode, and I_h currents were evoked by a single hyperpolarization step from the holding potential of -60mV to -120mV. Detailed analysis showed that when $GABA_B$ receptor is activated by its selective agonist baclofen, it causes different effects on I_h depending on age. We found that in younger mice, age group of P0-P4, baclofen has no effect on I_h. Figure 3.10A shows example traces of a recorded neuron (P0) superfused with ACSF and 5 µM baclofen. As is seen from the Figure 3.10A application of 5µM baclofen did not have any effect on I_h current amplitude. Averaged current amplitude was 186,1±27,56pA (n=9) and 173,9±18,50pA (n=9, n.s) after baclofen application (Figure 3.10B). Indeed, further analysis of I_h current density also showed no effect of baclofen on I_h. Averaged mean density was 4,662± 0,8006pA/pF (n=9) and 4,709±0, 8868 pA/pF (n=9, n.s). This data indicates that $GABA_B$ receptor activation in the age group of P0-P4 has no effect on I_h. In older mice, age group of P5-P11, $GABA_B$ receptor activation by baclofen showed biphasic effects on I_h. 40% of the tested neurons responded to baclofen with a decrease of I_h current amplitude, while in 60% of the tested neurons baclofen increased I_h current amplitude. Figure 3.11A shows an example of the effect of baclofen on I_h recorded from a cell of pre-Bötzinger complex at the age of P9. Application of 5µM baclofen decreased the amplitude of I_h. *IV* relationship of a neuron shows no change in voltage dependence in control condition and after baclofen application (Figure 3.11B). Averaged peak current amplitude was reduced from 604, 2± 71,08pA (n=12) to 472, 9±45, 56 pA (n=12, $p<0,005$) with the application of baclofen (Figure 3.11C). Furthermore, baclofen decreased I_h current density from 8,696±1,059pA/pF (n=12) to 6,794±0,7616pA/pF (n=12, $p<0,005$, Figure 3.11D).

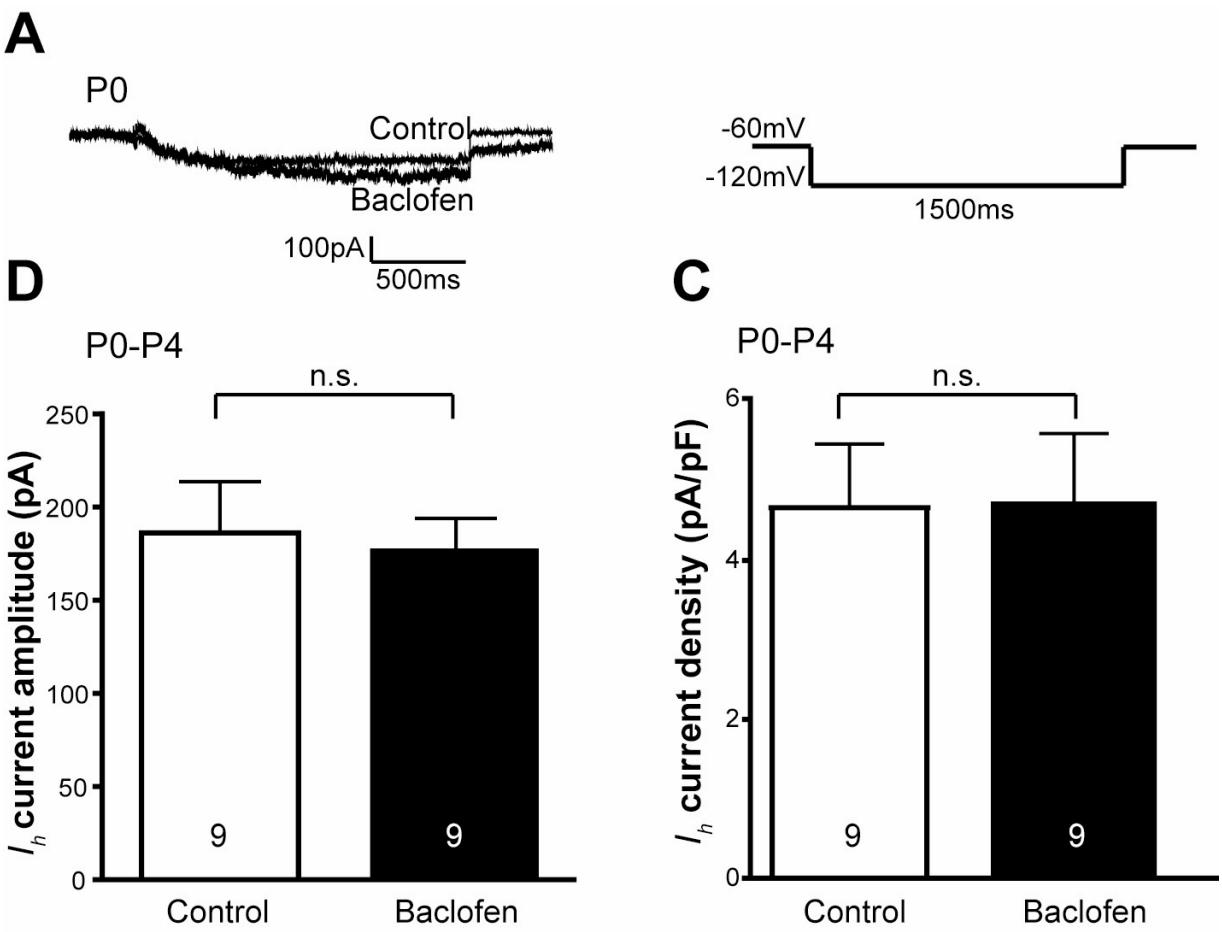


Figure 3.10: Activation of $GABA_B$ receptors has no effect on I_h: I_h current was elicited by a hyperpolarizing step from the holding potential of -60mV to -120mV as indicated. A: Representative traces of I_h current before and after $GABA_B$ receptor activation by 5 µM baclofen administration: B: Mean amplitude of I_h current before and after baclofen application: C; Averaged I_h current density before and after baclofen application. Peak amplitude of I_h current was divided by cell capacitance to obtain current density. Data are expressed as mean±SEM. Numbers in the bar graphs indicate the number of the neurons tested.

The reduction of both, I_h current amplitude and density was considered very significant ($p<0,005$, both cases). As we already mentioned, in majority of the recorded neurons application of 5µM baclofen caused an enhancement of the amplitude of I_h current. Example traces of a neuron that responded to baclofen with an increase of I_h current amplitude are presented in Figure 3.12A. *IV* relationship of a neuron shows no change in voltage dependence in control condition and after baclofen application (Figure 3.12). The mean I_h current amplitude in control was 572,2± 46,10 pA (n=18), while it was increased to 757,2± 53,42 pA due to baclofen

application (n=18, p<0,0001, Figure 3.12C). The current density also increased from 8,808± 1,062 pA/pF (n=18) to 11,33± 1,363 pA/pF (n=18, p<0,0001, Figure 3.12D).

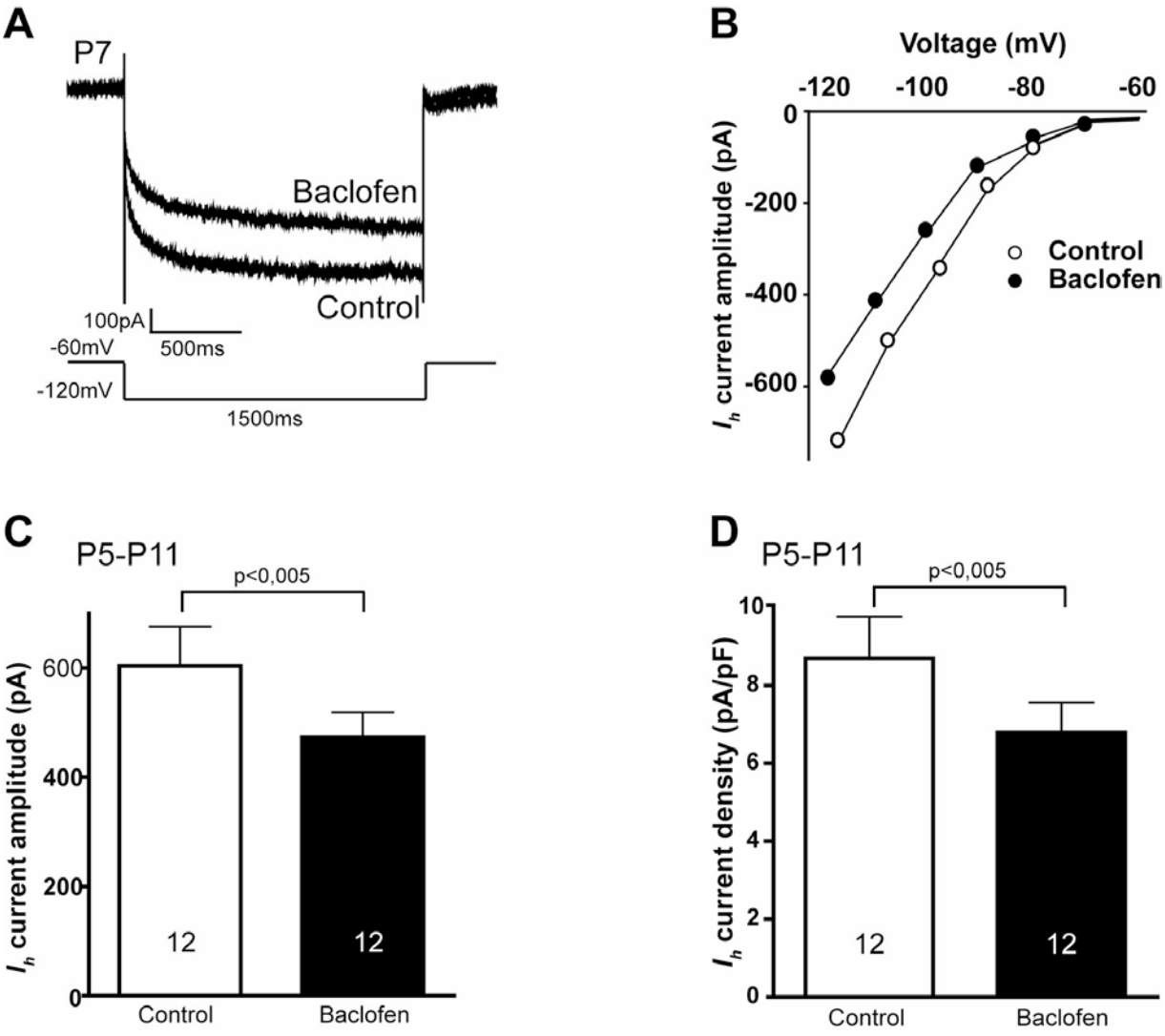


Figure 3.11: Activation of r $GABA_B$ receptors decreases I_h current: I_h current is elicited by a hyperpolarization step from the holding potential of -60mV to -120mV as indicated. A: Representative traces of I_h current before and after $GABA_B$ receptor activation by 5 µM baclofen: B: *IV* relationship, here Ih current is elecited by series of hyperpolarization steps from holding potential of -60mV to -120mV with 10mV increments: C; Mean amplitude of I_h current before and after baclofen application (p<0,005): D; Averaged I_h current density before and after baclofen application. Data are expressed as mean±SEM. Numbers in the bar graphs indicate the number of the cells tested.

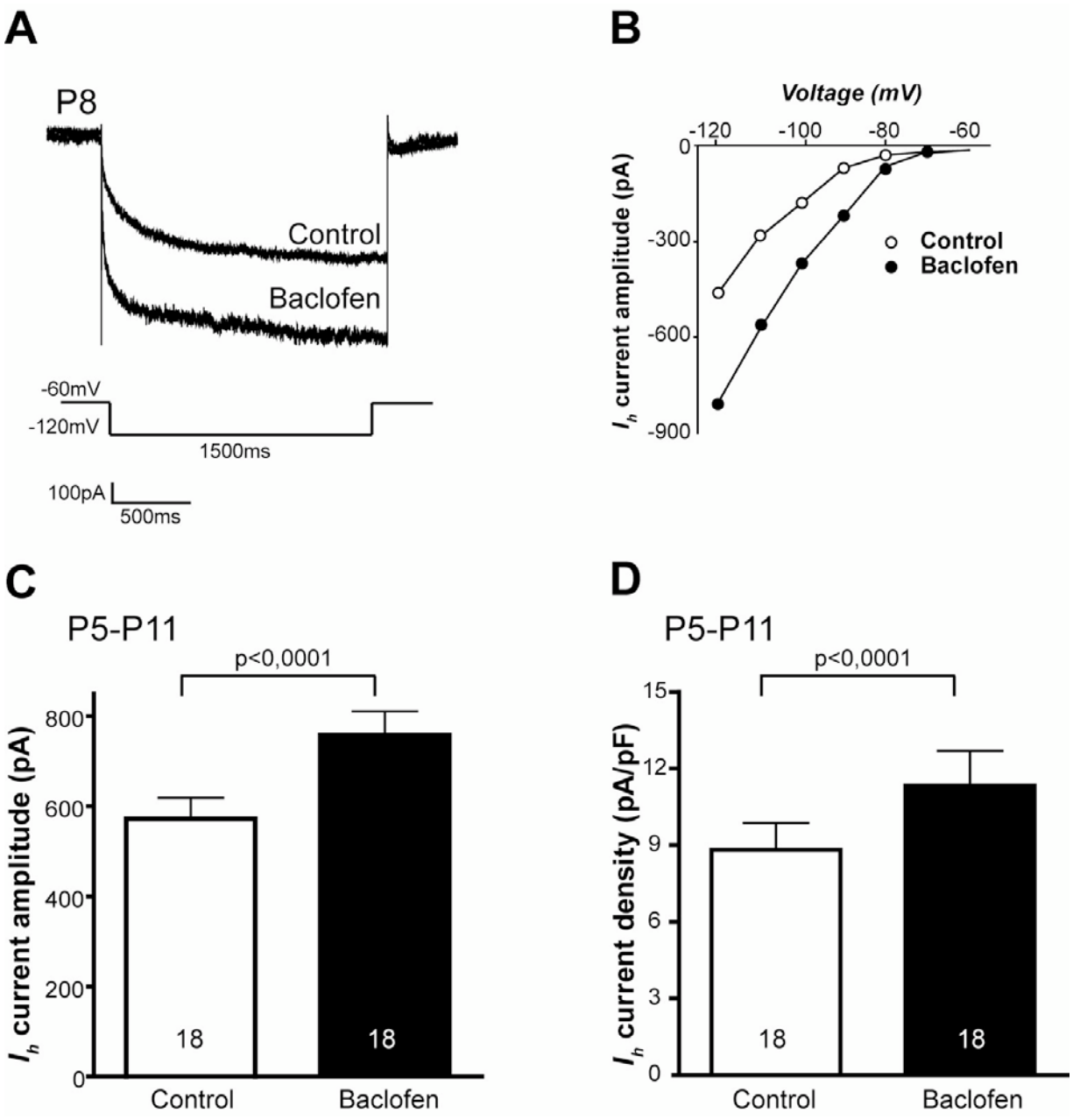


Figure 3.12: Activation of r $GABA_B$ receptors increases I_h current: I_h current is evoked by a hyperpolarizing step from the holding potential of -60mV to -120mV as indicated. A: Representative traces of I_h in control condition and after $GABA_B$ receptor activation by 5 µM baclofen: B: *IV* relationship, here I_h current is elicited by series of hyperpolarizing steps from the holding potential of -60mV to -120mV with 10mV increments: C; Mean amplitude of I_h current in control and after baclofen application ($p<0,005$): D; Averaged I_h current density in control and after baclofen application. Data are expressed as mean±SEM. Numbers in the bar graphs indicate the number of the cells tested.

Next we tested whether the observed biphasic effects of baclofen on I_h are simply due to two different cell populations with different passive properties. For this reason, we quantified and compared the cell capacitance between two group of neurons, and found that there was no difference in mean capacitance between cell group of decrease and the cell group of increase to baclofen application, being 55,42±4,641pF (n=12) and 60,43±3,674pF (n=18), respectively (Tabelle 3.2).

Tabele 3.2

	Neurons with decresing response to baclofen	Neurons with increasing response to baclofen
Cell capacitance	55,42±4,641pF	60, 43± 3,674pF
Cell number	12	18

Considering the fact that $GABA_B$ receptor activation may lead to activation of other membrane conductances, we performed experiments in the continuous presence of I_h channel blocker ZD7288. In Figure 3.13A are shown the representative traces of an experiment performed in neurons of pre-Bötzinger complex in the age group of P5-P11 mice. It shows clearly that the remaining I_h current amplitude was almost unchanged after baclofen application. The averaged mean of I_h current amplitude was 55,50±21,19pA in the presence of 100µM ZD7288 (n=6), and after baclofen application it was 50,00±40,41 pA (n=6, Figure 3.13). Our results demonstrate that $GABA_B$ receptors affect only I_h churrents, since when I_h channel is blocked baclofen has no effect.

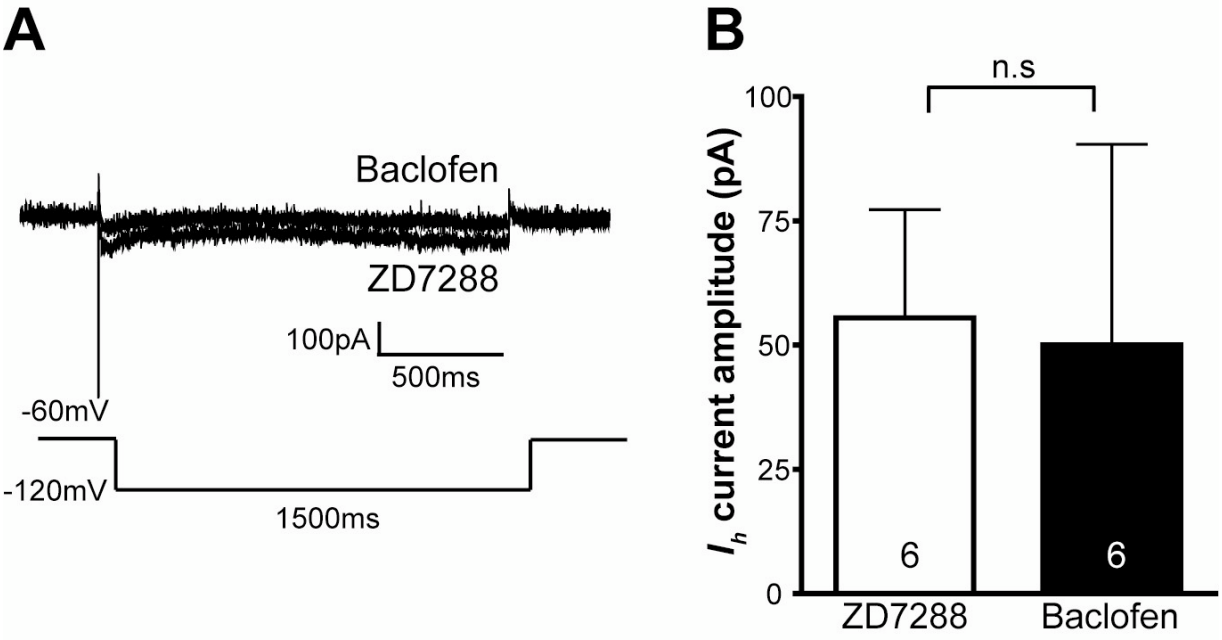


Figure 3.13: In the presence of I_h blocker ZD7288 baclofen has no effect on I_h. I_h current is evoked by a hyperpolarizing step from the holding potential of -60mV to -120mV as indicated. A: Representative traces of I_h in the continuous presence of 100µM ZD7288, and after $GABA_B$ receptor activation by 5 µM baclofen: B: Mean amplitude of I_h current in the presence of ZD7288 and after baclofen application: Data are expressed as mean±SEM. Numbers in the bar graphs indicate the number of the cells tested.

The biphasic effects of baclofen, described above, were surprising, because it is known that $GABA_B$ receptors inhibit basal activity of cAMP, which in turn would decrease, not increase I_h current amplitude. Taking into account the fact that studies suggest the exictance of other $GABA_B$ receptors, we proposed an idea that the enhancement of Ih mediated by $GABA_B$ receptors might be due to another $GABA_B$ receptor subtype, which in contrast to known $GABA_B$ receptor is positively coupled to cAMP. To directly test this hypothesis, we used mice with deletions in the $GABA_{B1}$ gene (lacking both $GABA_{B1a}$ and $GABA_{B1b}$ subunits). The neurons of pre-Bötzinger complex were held at -60mV, and I_h was evoked by giving 1,5s duration hyperpolarization step to -120mV. We have compared the data obtained from the wild type and $GABA_{B1a/b}^{-/-}$ knockout mice. The age of animals varied from P6 to P10.

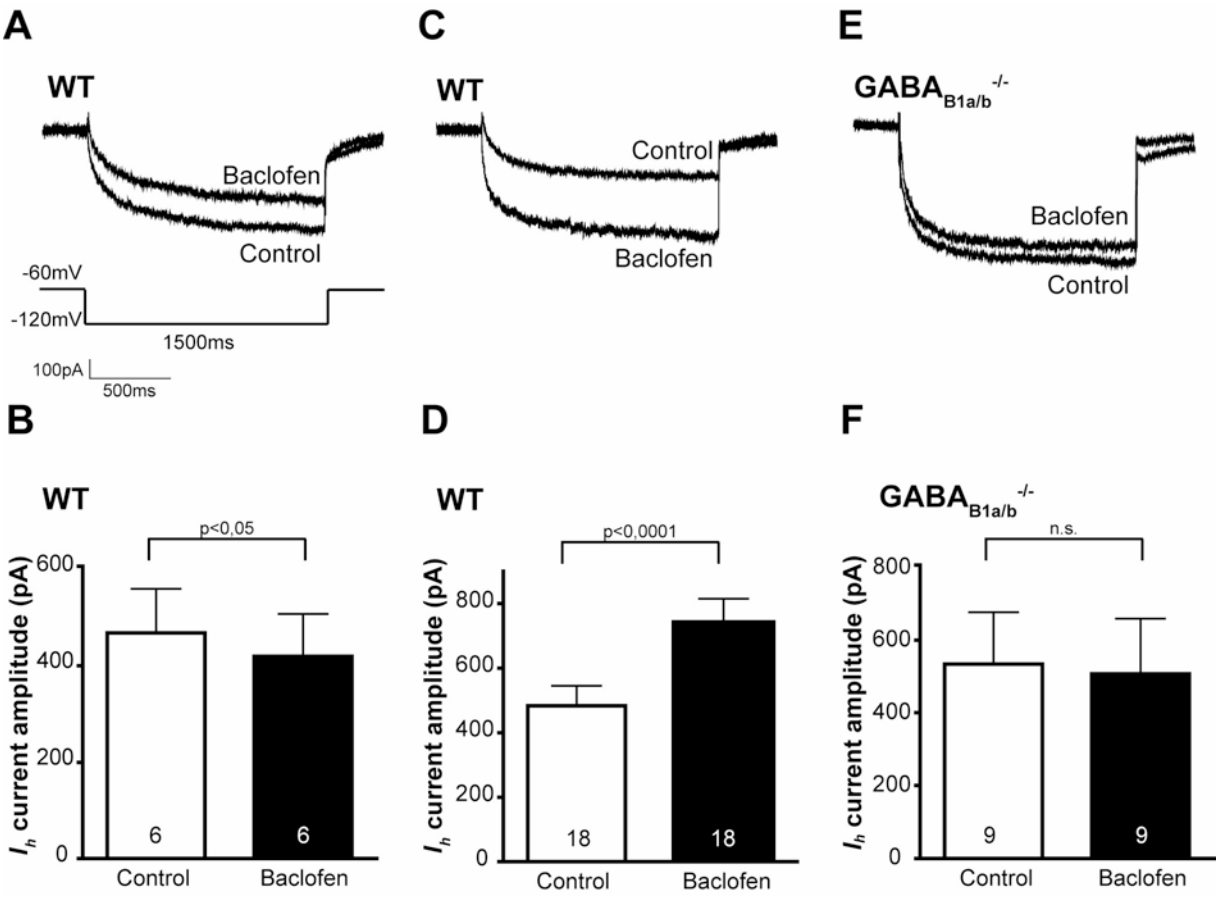


Figure 3.14: I_h current measurement in $GABA_{B1a/b}$ knockout mice. I_h was evoked in the neurons of pre-Bötzinger complex by applying hyperpolarizing step from the holding potential of -60mV to -120mV. A: Representative traces of a cell in wild type (WT) mouse that responded to baclofen with decease. B: Averaged I_h current amplitude in WT mouse with decrease. C: Representative traces of a cell in WT mouse with an increased response to baclofen. D: Average Ih current amplitude in the group of cells with an increased answer. E: Representative traces of a cell in $GABA_{B1}$ knockout mouse in control and after baclofen application. F: Averaged Ih current amplitude in KO mice in control and after baclofen application. Data are expressed as Means±SEM. The age of animals varied from 6 till 10 days. Numbers in the bar graphs indicate the number of the neurons tested.

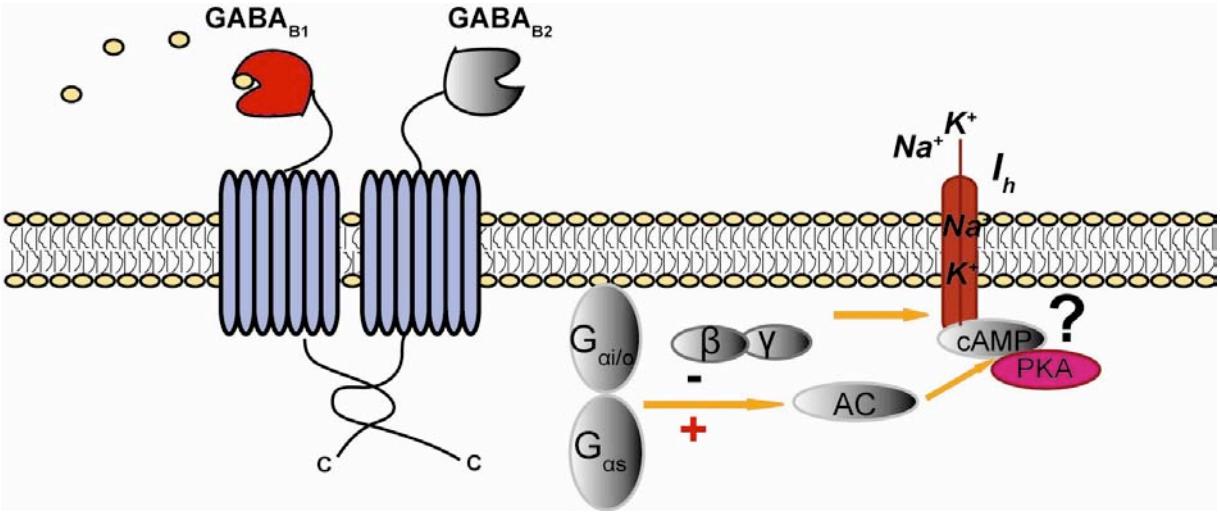
However, deletion of $GABA_{B1}$ gene revealed a complete absence of $GABA_B$ response in $GABA_{B1a/b}^{-/-}$ knockout mice. In contrast to $GABA_{B1a/b}^{-/-}$ knockout mice, in wild type mice baclofen application had again biphasic effects on I_h. 25% of the recorded neurons in wild type mice showed a reduction of I_h current amplitude, whereas 75% of the recoreded neurons showed an enhancement of I_h current amplitude. A, C and E in Figure 3.14 show representative traces of I_h current in wild type and $GABA_{B1a/b}^{-/-}$ knockout mice. As is seen from the traces, baclofen administration at the

concentration of 5µM causes decrease in one neuron in wild type mouse (A), increase in another neuron in wild type mouse (C) and no change in I_h current amplitude in $GABA_{B1a/b}^{-/-}$ knockout mouse. Averaged I_h current amplitude in the group of neurons that responded with decrease to baclofen in wild type mice, was 462,5± 91,27 pA and 414,2±92,71pA prior to baclofen (n=6, p<0,05, Figure 3.14B). In another group of neurons, with an increased response, in WT mice baclofen increased the averaged I_h current amplitude from 483,3± 61,69pA (n=18) to 738,9± 76,29pA (n=18, p<0,0001; Figure 3.14D). In $GABA_{B1a/b}^{-/-}$ knockout mice averaged I_h current amplitude was 527,2±142,4pA (n=9) and 507,2±144,8pA after baclofen application (n=9, n.s, Figure 3.14F).

3.5 Mechanism of $GABA_B$ modulation of I_h

To shed light on the mechanism of $GABA_B$ modulation of I_h current we next performed detailed investigation of cAMP pathway by using specific blockers.

3.5.1 The role of PKA in $GABA_B$ mediated modulation of I_h



Schematic representation of $GABA_B$ heterodimer and cAMP signaling pathway. In these experiments the role of PKA in $GABA_B$ mediated I_h modulation was investigated.

One of the candidates that might be involved in modulatory effect of baclofen on I_h is PKA, since in many cells I_h current had been found to be dependent on channel phosphorylation (Accili et al., 1997; Vargas & Lucero, 2002). To directly study if phosphorylation of I_h channel is responsible of $GABA_B$ mediated modulation of I_h, we tested the effects of baclofen in the presence of PKA inhibitor. In 34% of the recorded neurons baclofen in the presence of Rp-cAMP, which is a membrane permeable inhibitor of PKA did not have any significant effect on I_h. Figure 3.15A shows representative traces of a neuron recorded in the pre-Bötzinger complex from the holding potential of -60mV to -120mV. Bath application of 200μM Rp-cAMP did not decrease the amplitude of I_h, neither did 5μM baclofen application. On average, as is shown in Figure 3.15B, I_h current amplitude was 315,0±98,51pA (n=7) in control and 230,7±51,47pA (n=7) after Rp-cAMP application (n.s). After baclofen application I_h amplitude was unchanged: 237,9±49,62pA (n=7, n.s). In 66% of the recorded neurons, baclofen significantly increased I_h current amplitude in the presence of Rp-cAMP. In the Figure 3.15C are shown representative traces of I_h current in control condition, after 30 min. superfusion of 200μM Rp-cAMP and after application of 5μM baclofen application. However, this population of the neurons in contrast to the neuron population described above responded differently to PKA inhibitor application. The latter caused a dramatic reduction of I_h current amplitude, and when 5 μM baclofen was present in the bath I_h current was increased more than control. On average, as is shown in the Figure 3.15D, Rp-cAMP caused an inhibition of I_h current from 410,7±46,34pA (n=14) to 259,3±33,07pA (n=14). Baclofen caused almost two fold increase of I_h to 511,8±77,38pA (n=14).

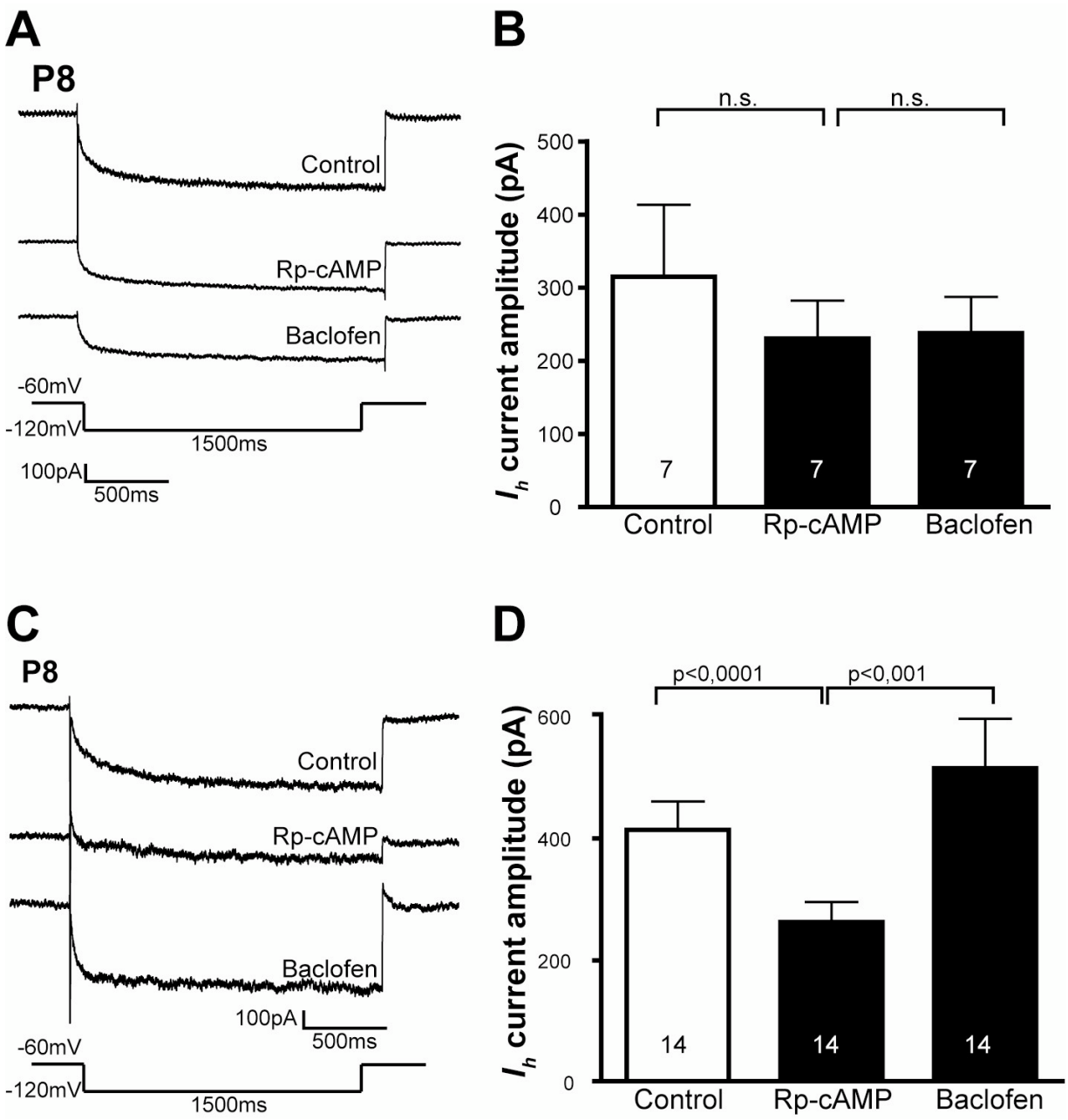
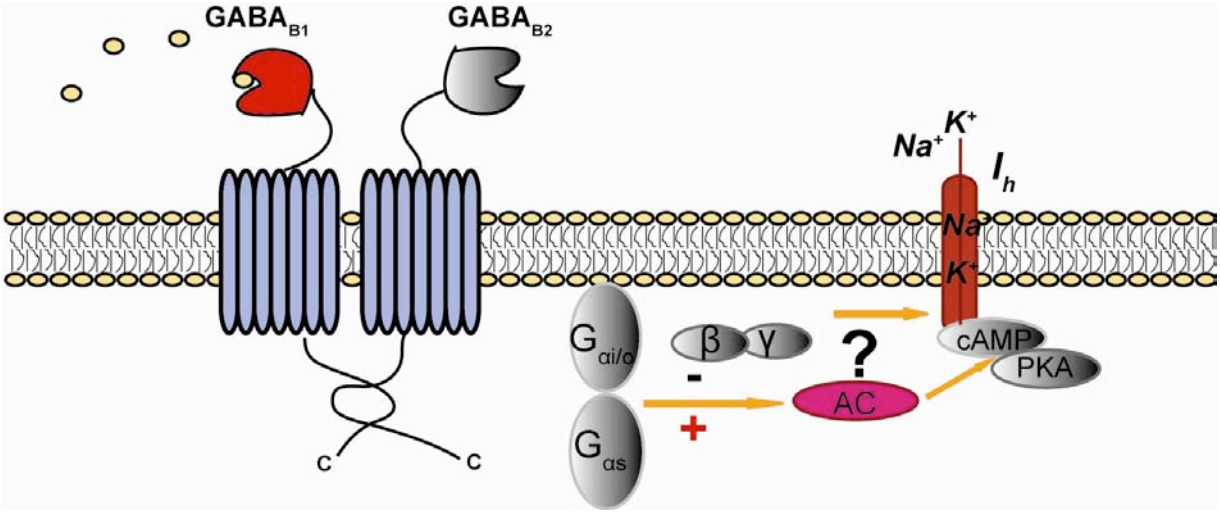


Figure 3.15: The effect of baclofen on I_h in the presence of PKA inhibitor. I_h was evoked in the neurons of pre-Bötzinger complex by giving a single hyperpolarization step from the holding potential of -60mV to -120mV. A: Representative traces of a nneuron at the age of P8 recorded in the absence of any drug (control), after 30 min perfusion of 200µM Rp-cAMP (Rp-cAMP) and after 5 µM baclofen application (baclofen) (group of neurons that did not respond to baclofen). B: Averaged mean I_h current amplitude of the same neuron group. C: Representative traces of a recorded neuron at the age of P8 in control, after 30min perfusion of 200µM Rp-cAMP (Rp-cAMP) and after 5 µM baclofen application (baclofen) (group of neurons that responded to baclofen with an increase of I_h current amplitude). D: Averaged mean I_h current amplitude of the same group of neurons. Data are shown as Means±SEM. Statistics was done by paired student's *t-test.* Numbers in the bar graphs indicate the number of the neurons tested.

3.5.2 Effect of adenylyl cyclase inhibitor in $GABA_B$ mediated modulation of I_h



Schematic representation of $GABA_B$ heterodimer and cAMP signaling pathway. In these experiments the role of adenylyl cyclases (AC) in $GABA_B$ mediated I_h modulation was investigated.

Next, we investigated whether *SQ 22536*, a broad-spectrum adenylyl cyclase inhibitor, would inhibit the $GABA_B$ receptor mediated effects on I_h. Recordings were performed in neurons of pre-Bötzinger complex in P5-P8 mice. 40 % of the recorded neurons, in the presence of *SQ 22536,* responded to baclofen application with no change in I_h current amplitude. In Figure 3.16A are shown I_h current traces in control, and after subsequent application of 500µM *SQ 22536* (I_h current was recorded after 30 min superfusion with *SQ 22536*) and 5µM baclofen, respectively. *SQ 22536* itself did not change I_h current amplitude in this group of neurons. The averaged mean I_h current amplitude was 470,0±73,16pA in control (n=6), 424,2±62,81pA after *SQ,22536* application (n=6), and 400,8±65,04pA after baclofen application (n=6). Statistical analysis showed no significance between the analyzed groups. However, in the 60% of the recorded neurons, baclofen application in the presence of *SQ 22536* enhanced I_h current amplitude. In Figure 3.16C are shown representative traces of I_h current in control, and after subsequent application of 500µM *SQ 22536* and 5µM

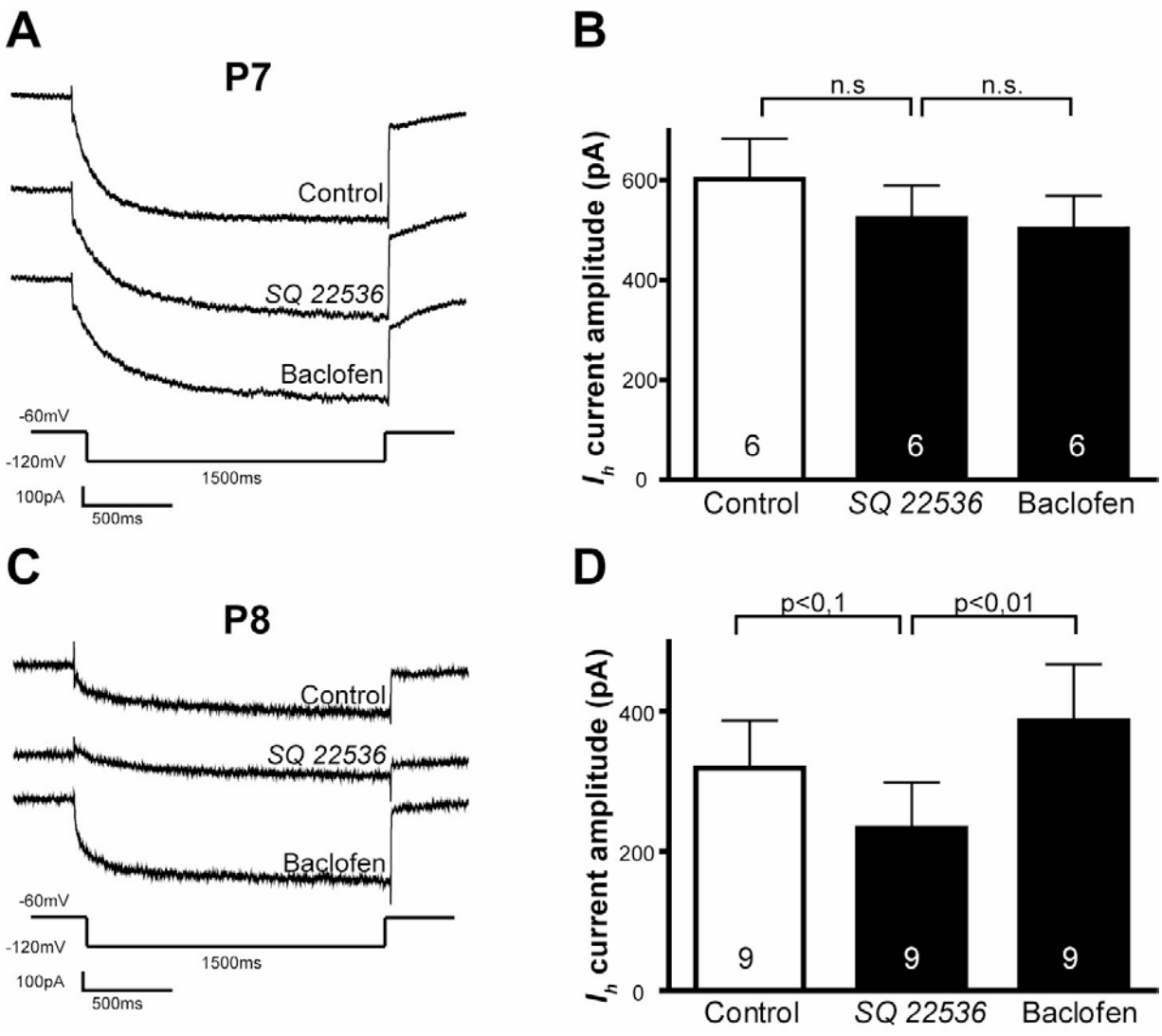
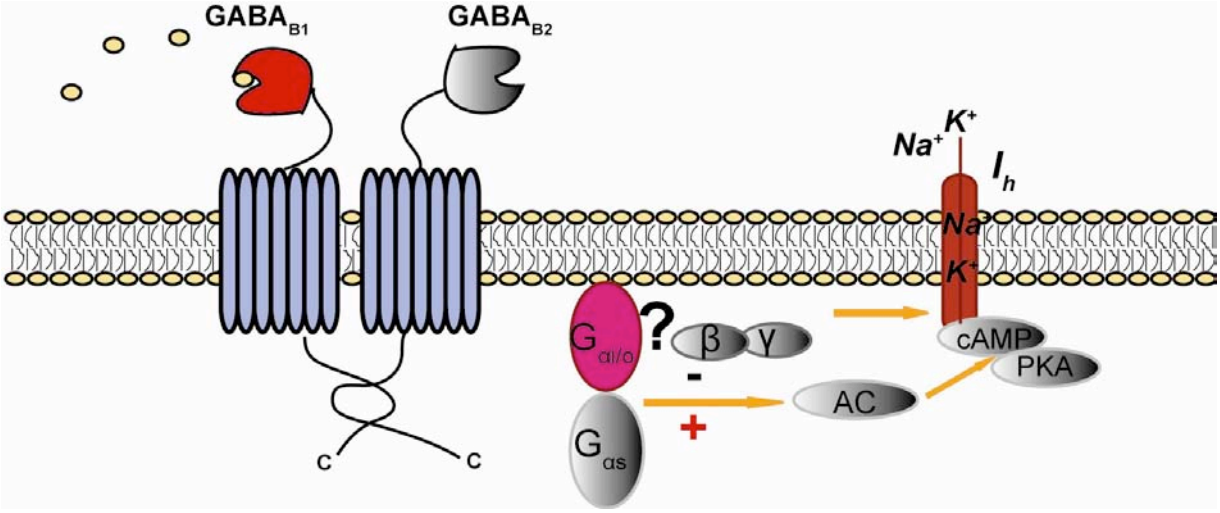


Figure 3.16: Effect of $GABA_B$ receptors on I_h in the presence of adenylyl cyclase inhibitor: I_h current was evoked in the pre-Bötzinger complex by giving 1,5s hyperpolarization step from the holding potential of -60mM to -120mV. A: Typical I_h current traces of a recorded neuron in control, and after subsequent application of 500µM *SQ 22536* and 5µM baclofen, respectively (in the group of neurons that did not respond to baclofen). B: I_h current mean amplitude of the appropriate group of neurons. C: Typical I_h current traces of a recorded neuron in control, and after subsequent application of 500µM *SQ 22536* and 5µM baclofen, respectively (in the group of neurons that responded to baclofen with an increase of I_h). D: I_h current mean amplitude of the appropriate group of neurons. Statistics was done by Student's paired t-test. Data are expressed as Means±SEM. Numbers in the bar graphs indicate the number of the neurons tested.

baclofen, respectively. I_h current was changed not only by baclofen application, but also by *SQ 22536*, the effect that we did not observe in the group of neurons

mentioned above. In Figure 3.16D are summarized the averaged mean of I_h current amplitudes. Application of *SQ 22536* decreased I_h current amplitude from 318,9± 67,77pA (n=9) to 232,8± 65,53 pA (n=9, p<0,1), and application of baclofen caused a significant increase of I_h current amplitude to more than control, 386,1±81,09 pA (n=9, p<0,01). Our results show, that when adenylyl caclase is inhibited by *SQ 22536* baclofen in one hand has no effect on I_h, on the other hand, baclofen has effect on I_h, which suggests that there are adenyly cyclase dependent and independent pathways.

3.5.3 Involvement of PTX-sensitive G proteins in $GABA_B$ mediated modulation of I_h



Schematic representation of $GABA_B$ heterodimer and cAMP signaling pathway. In these experiments the role of PTX-senstitive G-proteins in $GABA_B$ mediated I_h modulation was investigated.

It is known that some G proteins, Gi, Go, and transducin are ADP-ribosylated by pertusis toxin (PTX) and loose their ability to become activated (Ui, 1984). To directly test whether $GABA_B$ mediated modulation of I_h would be abolished by PTX, we performed experiments in which PTX was included in the pipette solution. I_h current was measured in mouse pre-Bötzinger complex (P7) after 30 min. of rupturing cell membrane by giving a hyperpolarizing step from the holding potential of -60mV to -120mV.

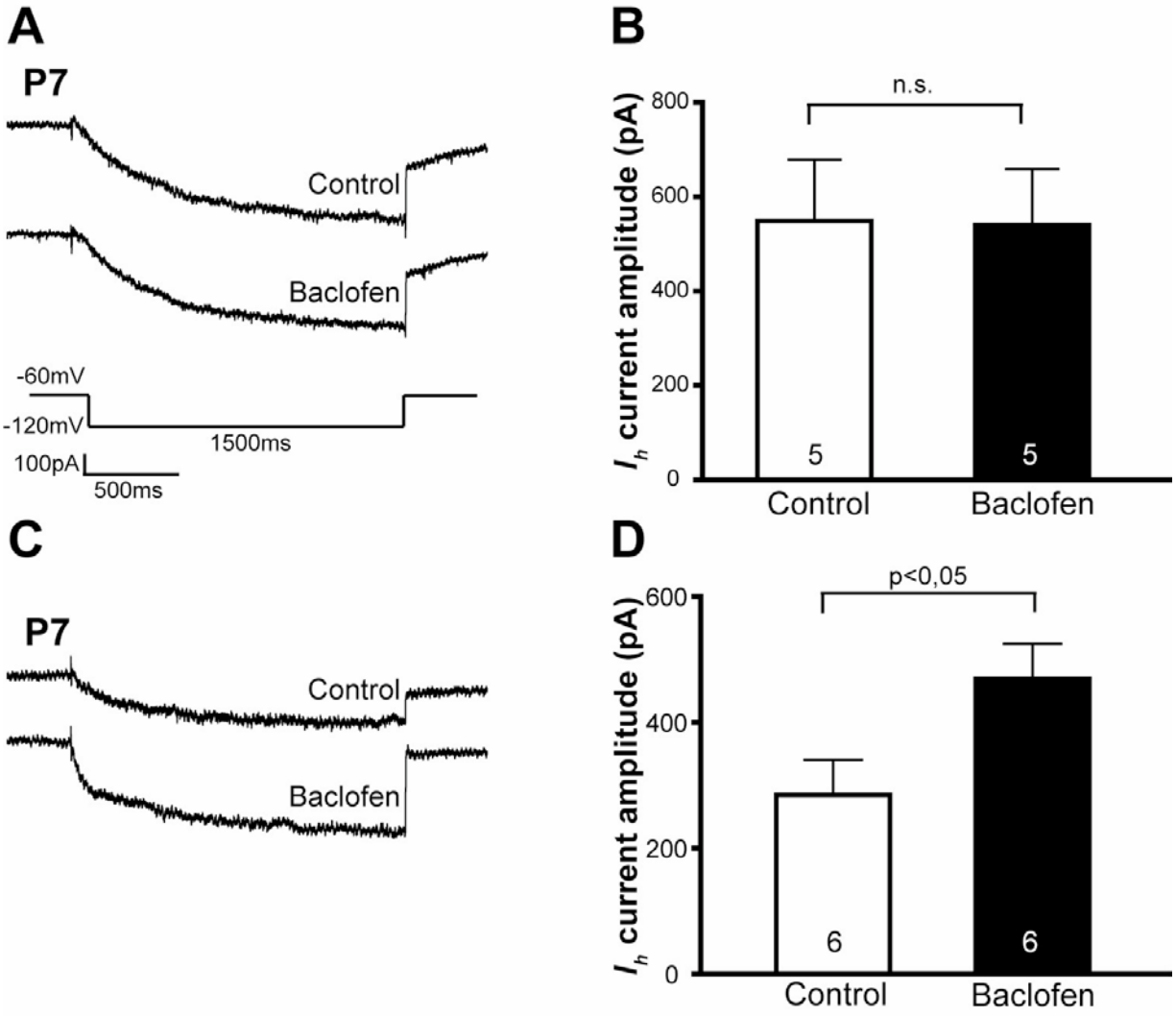
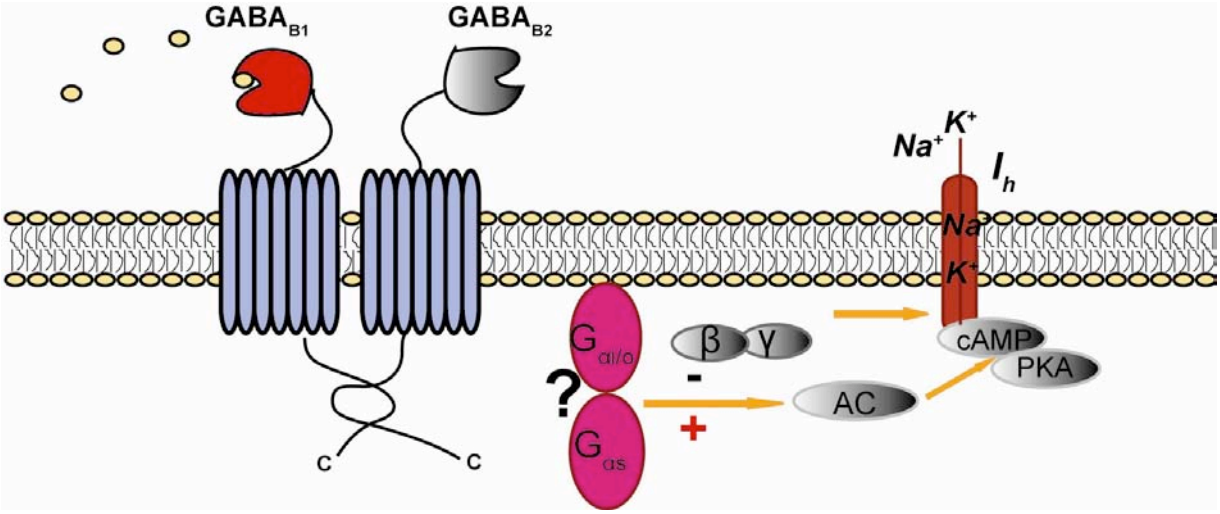


Figure 3.17: Modulation of I_h current by $GABA_B$ receptors is mediated via PTX-sensitive and insensitive patways. PTX was included in the pipette solution. I_h current was evoked in the pre-Bötzinger complex by giving 1,5s hyperpolarization step from the holding potential of -60mM to -120mV, as indicated. A: Representative I_h current traces of a recorded neuron. B: I_h current mean amplitude before and after baclofen application. C: Typical I_h current traces of a recorded neuron: D: averaged I_h current amplitude before and after baclofen application. Significance was done by Student's paired t-test. Data are expressed as Means±SEM. Numbers in the bar graphs indicate the number of the neurons tested.

Afterwards, baclofen was bath applied at the concentration of 5µM. In our experiments the inhibitory effect of baclofen was totally abolished by PTX, whereas the increasing effect was still there. In detail, 45 % of the recorded neurons did not respond to baclofen. The representative I_h current traces are illustrated in Figure 3.17A. In this group of the neurons, mean I_h current amplitude was 549,0±129,5pA

and 540,0±114,0pA (n=5) after baclofen application (Figure 3.17B). Furthermore, in 55% of the neurons that did respond, baclofen application increased I_h current amplitude from 285,0±55,62pA to 469,2±55,71pA (n=6, p<0,05; Figure 3.17D).
These data enabled us to conclude that on one hand, the baclofen induced decrease of I_h current was mediated via activation of PTX sensitive G proteins, and on the other hand baclofen induced increase of I_h current was mediated via PTX insensitive pathway.

3.5.4 Effects of dialysis of G-protein antibodies on $GABA_B$-mediated modulation of I_h



Schematic representation of $GABA_B$ heterodimer and cAMP signaling pathway. In these experiments the role of PTX-senstitive $G\alpha_{i/o}$- and $G\alpha_s$-proteins in $GABA_B$ mediated I_h modulation was investigated.

In these series of experiments, we tested the effect of baclofen on I_h during dialysis of PBC neurons with G-protein antibodies specific for α subunits of G_{i3} and $G_{\alpha s}$ to determine which subtype of G-proteins is involved in the coupling of $GABA_B$ receptors to I_h channels. Responses of a neuron were examined at an early and a late stage of recording so that each neuron served as its own control. Intracellular administration of anti- G_{i3} did not attenuate baclofen mediated enhancement of I_h current (Figure 3.18A,B). Representative traces, presented in Figure 3.18A, illustrate that 5 µM

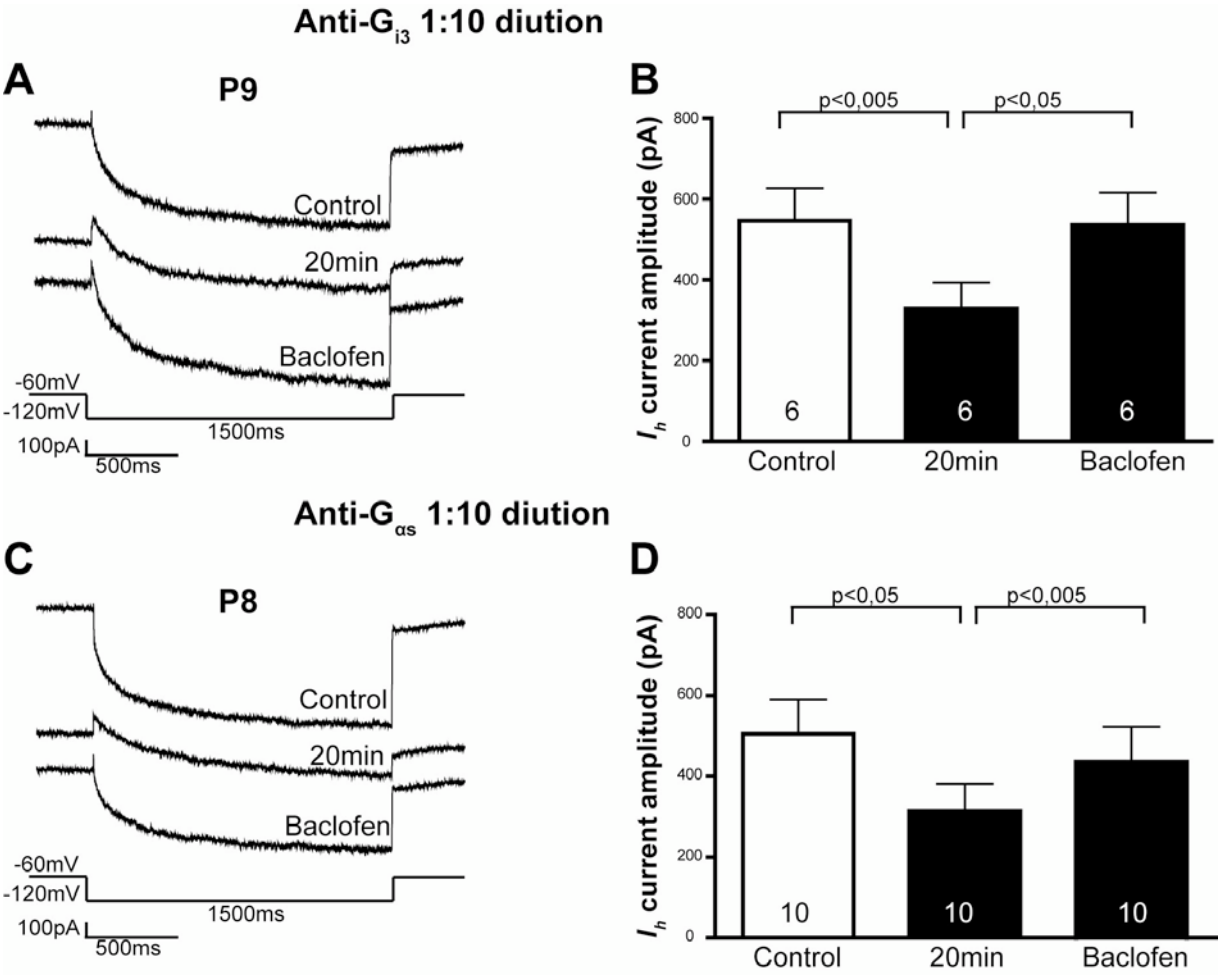
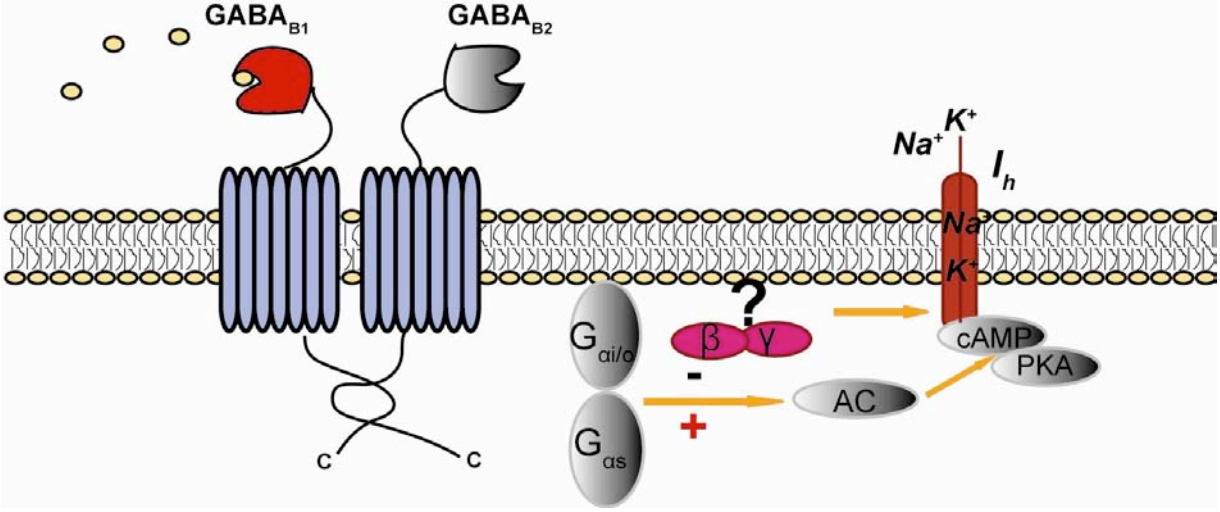


Figure 3.18: The response to baclofen in neurons dialyzed with specific G_{i3}- and $G_{αs}$-protein antibodies. Antibodies were included in pipette solution at 1:10 dillution. I_h current was evoked in the pre-Bötzinger complex by giving hyperpolarizing step from the holding potential of -60mV to -120mV. I_h current was measured immediately after rupturing cell membrane (control), after 20min dialysis of an antibody and after baclofen application. A: Typical I_h current traces of a neuron with intracellular administration of G_{i3} antibody. B: I_h current mean amplitude with intracellular administration of G_{i3} antibody. C: Typical I_h current traces of a recorded neuron with intracellular administration of $G_{αs}$ antibody: D: Averaged I_h current amplitude with intracellular administration of G_{i3} antibody. Significance was done by Student's paired t-test. Data are expressed as Means±SEM. The numbers in bar graphs indicate the number of the neurons tested.

baclofen application after 20 min intracellular administration of G_{i3} antibody was able to significantly enhance I_h current amplitude. While averaged I_h current amplitude was 545,7±80,94pA (n=6), whereas 20min dialysis of the neurons with anti-G_{i3} antibody significantly reduced I_h current amplitude to 328,3± 64,66pA (n=6, p<0,005), and baclofen increased I_h current amplitude to 535,0± 80,86pA (n=6, p<0,05). Strikingly,

baclofen application in the presence of intracellular administration of anti-$G_{\alpha s}$-protein antibody had similar effects on I_h. As is seen from the representative traces of a neuron, shown in Figure 3.18C, the enhancing effect of baclofen on I_h was not attenuated after 20min dialysis of on a neuron with anti-$G_{\alpha s}$-protein antibody. Overall, the mean of I_h current amplitude was 505,0±85,26pA (n=10), after 20 min it was decreased to 314,0±67,75pA (n=10, p<0,05, Figure 3.18D), and baclofen caused an increase to 436,0±86,58pA. Taken together, these results did not really show whether α subunits of G-proteins are not involved in $GABA_B$ mediated modulation of I_h current or not.

3.5.5 Involvement of $G_{\beta\gamma}$ subunits of G-proteins in $GABA_B$ mediated modulation I_h current



Schematic representation of $GABA_B$ heterodimer and cAMP signaling pathway. In these experiments the role of $G_{\beta\gamma}$ subunits of G-proteins in $GABA_B$ mediated I_h modulation was investigated.

In the final series of experiments, we tested the effect of $G_{\beta\gamma}$ subunits in $GABA_B$ mediated modulation of I_h currents. In this regard, dialysis of neurons with 200µM $SP_{\beta\gamma}$, an inhibitory peptide that interferes with binding of $G_{\beta\gamma}$ subunits to several targets (Ma et al., 1997), prevented the modulation of I_h currents by baclofen (Figure

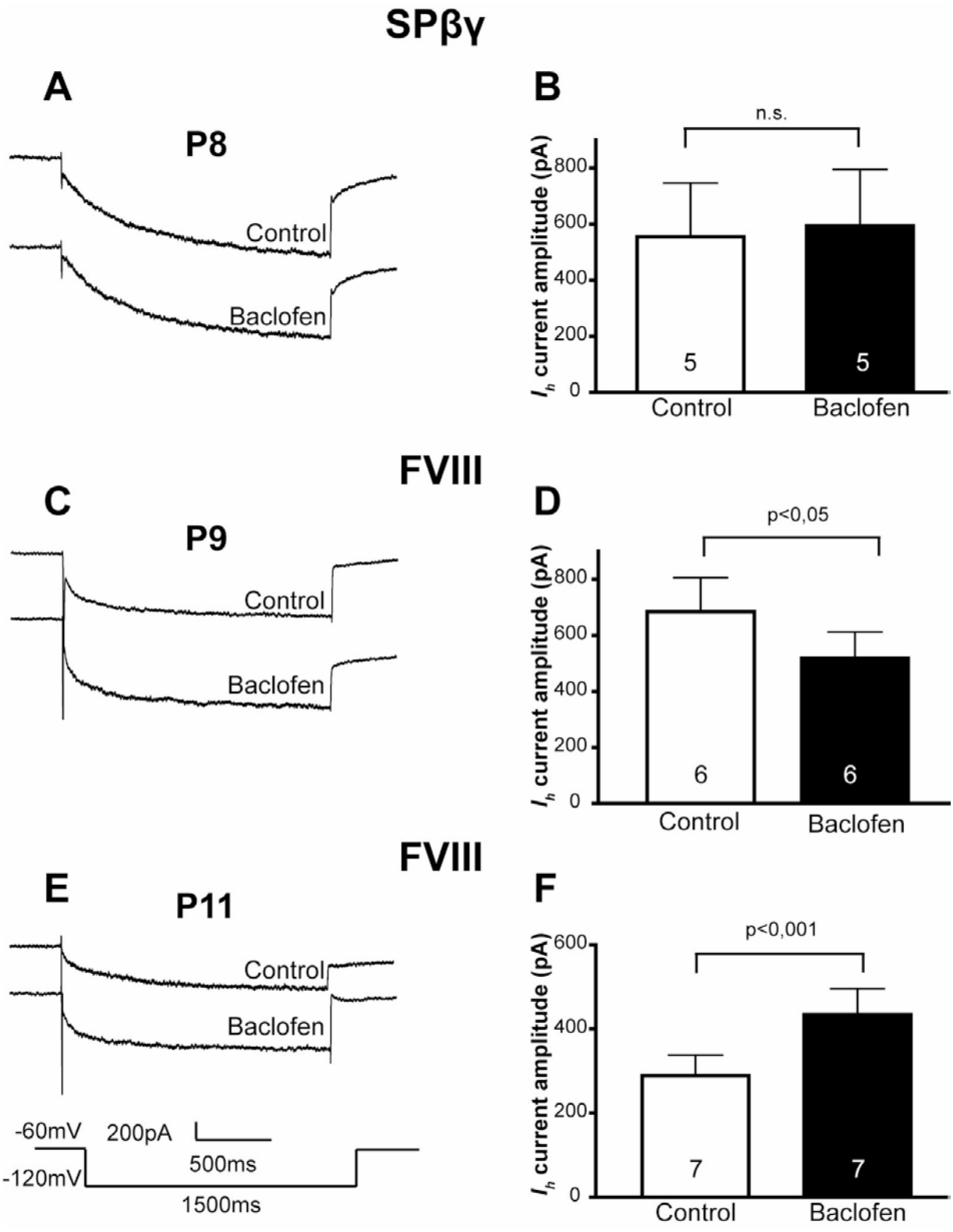

SPβγ
A
P8
Control
Baclofen
B
n.s.
I_h current amplitude (pA)
800
600
400
200
0
5
5
Control
Baclofen
FVIII
C
P9
Control
Baclofen
D
p<0,05
I_h current amplitude (pA)
800
600
400
200
0
6
6
Control
Baclofen
FVIII
E
P11
Control
Baclofen
-60mV
200pA
500ms
-120mV
1500ms
F
p<0,001
I_h current amplitude (pA)
600
400
200
0
7
7
Control
Baclofen

Figure 3.19: $G_{\beta\gamma}$ is required for the $GABA_B$ mediated modulation of I_h currents. I_h current was evoked in the pre-Bötzinger complex by giving a hyperpolarizing step from the holding potential of -60mV to -120mV. A: Typical I_h current traces of a recorded neuron dialyzed with $SP_{\beta\gamma}$, the $G_{\beta\gamma}$ inhibitory peptide. B: I_h current mean amplitude. C: Typical I_h current traces of a recorded neuron dialyzed with FVIII, an inactive control of $G_{\beta\gamma}$: D: Averaged I_h current amplitude. E: Typical I_h current traces of a recorded neuron dialyzed with FVIII, an inactive control of $G_{\beta\gamma}$: F: Averaged I_h current amplitude. Significance was done by Student's paired t-test. Data are expressed as Means±SEM. The numbers in bar graphs indicate the number of the neurons tested.

3.19A), whereas FVIII, which is an inactive peptide, at the concentration of 200µM had no effect (Figure 3.19C). Overall, when $SP_{\beta\gamma}$ was in pipette solution the mean amplitude of I_h current was 555,0±191,3pA, and 594,0±200,8pA after baclofen application. As it was expected, when FVIII was included in pipette solution, there were biphasic effects prior to baclofen application: decreasing and increasing effects. The mean of I_h current amplitude in the first group (decreasing) was 683,8±121,4pA, and 517,5±94,41pA after baclofen application (n=6, $p<0,05$, Figure 3.19C,D). In the second group (increasing) baclofen application caused an increase of I_h current amplitude from 288,6± 49,31pA to 434,3± 61,21pA (n=7, $p<0,001$, Figure 3.19E,F).
If $GABA_B$ mediated effects on I_h occurred via $G_{\beta\gamma}$-dependent mechanism as described above, we thought that, after intracellular perfusion with $G_{\beta\gamma}$ subunits, the response of the neurons to $GABA_B$ receptor activation might be enhanced. However, the effect of baclofen on I_h was not enhanced, when the neurons were intracellularly perfused with purified bovine brain $G_{\beta\gamma}$ subunit (20nM, Figure 3.20). There were two kinds of responses prior to baclofen application: decreasing and increasing response. In the first case, baclofen decreased I_h current amplitude from 662,0± 91,55pA to 509,0± 94,23pA (n=5, $p<0,05$). In the second case, baclofen caused an increase of I_h current amplitude from 317,0± 38,29pA to 455,0± 54,29pA (n=5, $p<0,05$).

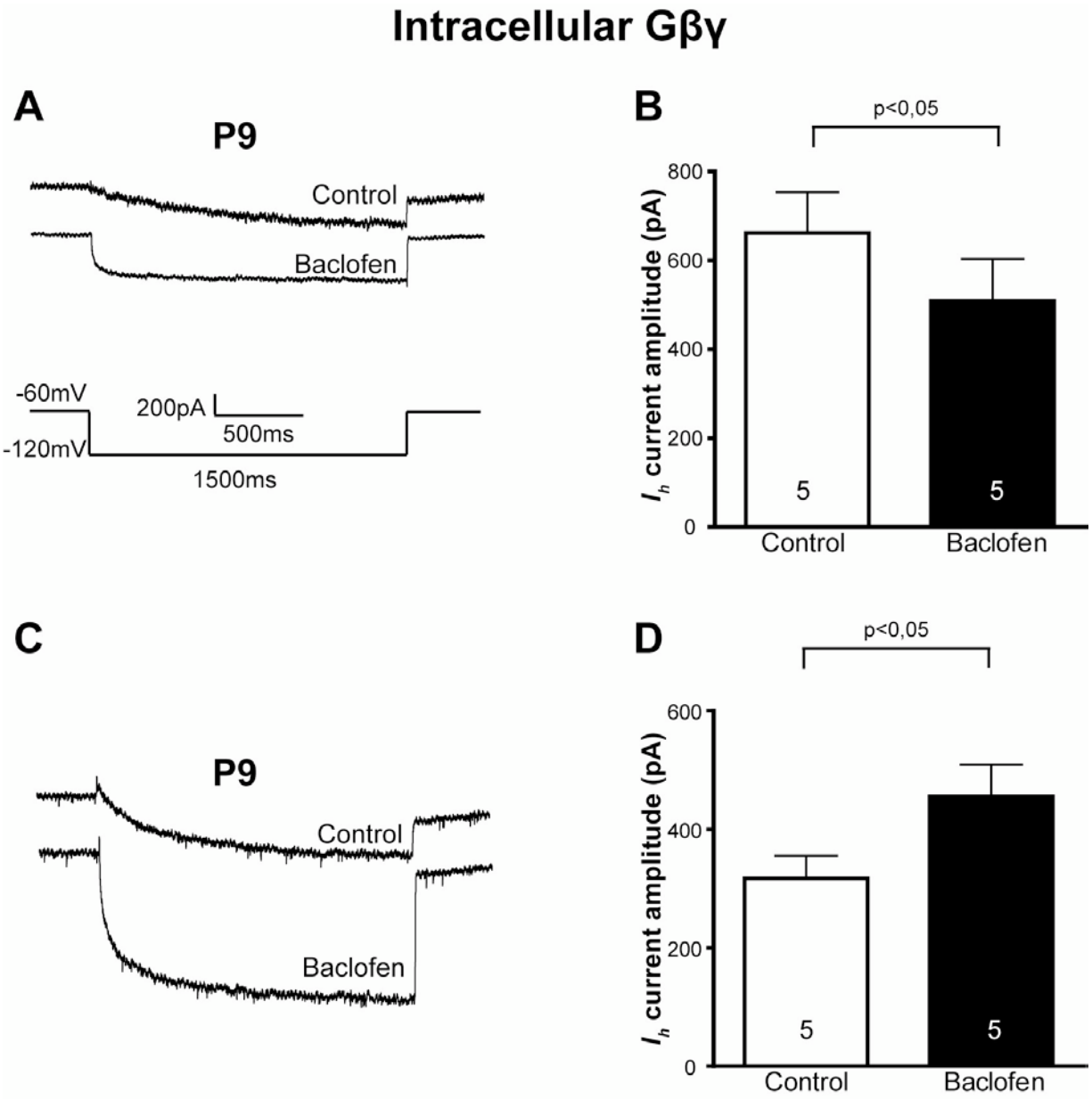


Figure 3.20: Intracellular perfusion of $G_{\beta\gamma}$ subunits did not potentate $GABA_B$ mediated modulation of I_h. I_h current was evoked in the pre-Bötzinger complex by giving a hyperpolarizing step from the holding potential of -60mV to -120mV. A: Typical I_h current traces of a recorded neuron (that responded to baclofen application with a decrease on I_h) dialyzed with $G_{\beta\gamma}$ subunit B: I_h current mean amplitude. C: Typical I_h current traces of a recorded neuron (that responded to baclofen application with a decrease on I_h) dialyzed with $G_{\beta\gamma}$ subunit: D: Averaged I_h current amplitude. Significance was done by Student's paired t-test. Data are expressed as Means±SEM. The numbers in bar graphs indicate the number of the neurons tested.

Interestingly, when $G_{\beta\gamma}$ subunit was included in pipette solution, baclofen caused a significant increase of I_{inst}-current (non-I_h). To note, I_{inst}-current is a component of a current evoked by applying hyperpolarization voltage step from -60mV to -120mV. I_{inst} was measured immediately following the capacitive transient (for more detail see 3.3.1 part). In Figure 3.21 are summarized the data of I_{inst}-current in different experiments when different drugs were applied either intracellular or extracellular. Baclofen induced a significant increase of I_{inst} only when intracellular $G_{\beta\gamma}$ subunit was present. This observation might be due to activation of K^+-currents, since it is known that $GABA_B$ activates K^+-current via $G_{\beta\gamma}$ subunits.

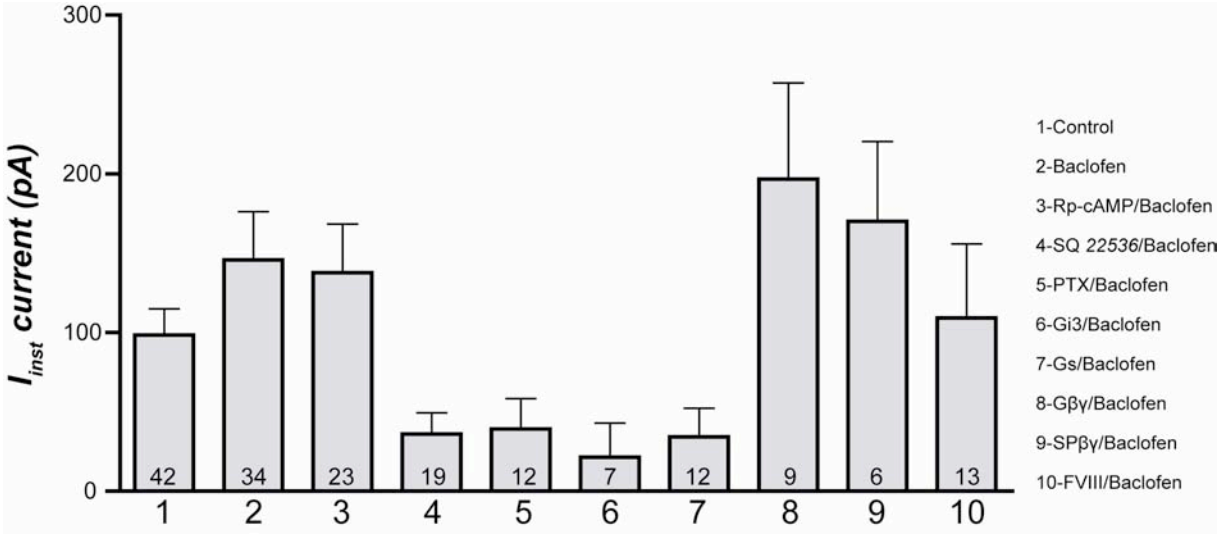


Figure 3.21: Quantification of I_{inst} current. I_{inst} current was a component of a current evoked by hyperpolarizing voltage step from a holding potential of -60mV to -120mV. Instantaneous current was measured at the end of capacitive transient. Bar graphs represent I_{inst} current. 1st bar graph represents a control case (no drug). 2-bar graph represents the response of the neurons to baclofen. 3- and 4-bar graphs represent the responses to baclofen when extracellular Rp-cAMP and SQ 22536 were present, respectively. 5-, 6-, 7-, 8-, 9,- and 10-bar graphs represent the responses to baclofen when PTX, Gi3, Gs, $G_{\beta\gamma}$, $SP_{\beta\gamma}$ and FVIII were present ntracellularly in the pipette solution. Students unpaired t-test showed significance only between 1 and 8 bar graphs ($p<0, 05$). The numbers in bar graphs represent tested neurons for each experiment.

4. Discussion

$GABA_B$ receptors belong to a family of metabotropic receptors mediating slow synaptic transmission in central nervous system. It is known that activation of $GABA_B$ receptors inhibits adenylyl cyclase via the $G\alpha_{i/o}$ subunits of the activated G-protein in adult animals (Hill, 1985). However, little is known about the physiological consequences of inhibiting adenylyl cyclase activity via $GABA_B$ receptors during postnatal development. In this regard, pacemaker channels, or in other term hyperoloarization activated cation channels (I_h), are perfect effector system, because they had been shown to be directly modulated by cAMP.

In the present study we first of all extended the analysis of the function of $GABA_B$ receptors by using $GABA_{B1}$ null mutant mice. Here we found that 1) the deletion of $GABA_{B1}$ does not cause any failure in respiration, but, indeed, it causes dramatic impairment of synaptic transmission, 2) $GABA_B$ receptors might be involved in sensing extracellular pH. We then explored the signaling of $GABA_B$ receptors and their regulation of I_h channels in pre-Bötzinger complex during postnatal development. We showed that 1) I_h current densitiy and the numbers of the neurons having I_h are increasing during postnatal development, 2) $GABA_B$ receptors modulate I_h channels in relatively older mice (P5-P11), 3), we present a novel, cAMP dependent and independent, mechanism by which $GABA_B$ receptors modulate I_h.

4.1 Funcional $GABA_B$ receptors are present in pre-Bötzinger complex of neonatal mice

$GABA_B$ receptors have been shown to be expressed (Lopez-Bendito et al., 2002; Ritter et al., 2005) and functional (Zhang et al., 2002) at early stages of development. Before the maturation of chloride extrusion mechanisms, the function of $GABA_A$ receptors is excitatory (Ben-Ari, 2002). At this stage of development, $GABA_B$ receptors probably are the major inhibitory receptor in the CNS (Gaiarsa et al., 1995; McLean et al., 1996; Zhang et al., 2002), so it could be one of the reasons that they are expressed at high levels in neonatal animals. Immunofluorescence staining performed in this study, indeed, confirmed the previous observation that $GABA_B$

receptors are expressed in high levels in the pre-Bötzinger complex of neonatal mice (Figure 3.1). Furthermore, the functional analysis of $GABA_B$ receptors in neonatal mice was extended by using $GABA_{B1}$ null mutant mice, with ablations of $GABA_{B1a}$ and $GABA_{B1b.}$ The phenotype of the $GABA_{B1a/b}^{-/-}$ mice was mainly similar to that described previously (Prosser et al., 2001; Schuler et al., 2001). $GABA_{B1a/b}^{-/-}$ mice are generated in Balb/c background, they are viable, have normal lifespan and show regular breathing as demonstrated by whole-body pletismography, which indicates that the deletion of $GABA_{B1}$ receptors is not essential in respiration (Figure 3.2). By contrast, $GABA_{B1a/b}^{-/-}$ mice having different genetic background (C57B16/129SvJ) have shorter lifespan most likely due to generalized epileptic seizures (Prosser et al., 2001; Quèva et al., 2003). Previous studies have shown that deletion of $GABA_{B1}$ did not cause any histopathological alterations in $GABA_{B1a/b}^{-/-}$ mice (Prosser et al., 2001; Schuler et al., 2001; Quèva et al., 2003). Patch-clamp recordings obtained from the neurons of pre-Bötzinger complex, revealed a dramatic impairment of synaptic transmission in $GABA_{B1a/b}^{-/-}$ mice. While the frequency of spontaneous postsynaptic current (sPSC) was significantly reduced in $GABA_{B1a/b}^{-/-}$ mice compared to wild type littermates the amplitude of sPSC was increased in $GABA_{B1a/b}^{-/-}$ mice (Figure 3.3). A large body of work on native receptors suggests existence of distinct $GABA_B$ receptor subtypes (Bonanno & Raiteri, 1993; Gemignani et al., 1994; Cunningham & Enna, 1996; Deisz et al., 1997; Mohler & Fritschy, 1999; Yamada et al., 1999; Bowery et al., 2002). However, the cloned $GABA_B$ receptors do not reproduce the pharmacological heterogeneity of native $GABA_B$ receptors. Thus, to find out the pharmacological distinct $GABA_B$ receptor would be very important for drug development. In this study, we have measured sIPSC after baclofen application and compared the data obtained from wild type and $GABA_{B1a/b}^{-/-}$ mice. In contrast to wild type mice, where baclofen inhibited the frequency and decreased the amplitude of sIPSC, it affected neither the frequency nor the amplitude of sIPSC, which revealed a loss of functional $GABA_B$ receptors in $GABA_{B1a/b}^{-/-}$ mice (Figure 3.4). These results are in line with other observations, where it was shown that $GABA_{B1a/b}^{-/-}$ mice lack detectable $GABA_B$ responses in all biochemical, electrophysiological and behavioral paradigms studied (Prosser et al., 2001; Quèva et al., 2003; Schuler et al., 2001). This demonstrates that

most probably all $GABA_B$ receptors in the brain incorporate $GABA_{B1}$ subunit.

4.2 The essential role of $GABA_B$ receptors in a network sensing extracellular pH

Central respiratory chemoreception is the mechanism by which the brain senses changes in CO_2 and/or pH to regulate the rate and depth of breathing (Feldman et al., 2003). Despite significant progress in identification of candidate brainstem regions involved in respiratory chemoreception (Dean et al., 1990; Coates and Nattie, 1993; Huang et al., 1997) and of candidate proteins that can impart an intrinsic neuronal pH sensitivity, the neuronal and molecular substrates for central respiratory chemosensitivity remain largely undefined (Bayliss et al., 2001; Jiang et al., 2001; Putnam et al., 2004). In this part of study, we have hypothesized that $GABA_B$ receptors could also play a role in chemoreception. For this purpose, by using $GABA_{B1}$ null mutant mice, we measured sPSC and compared the data obtained from wild type and $GABA_{B1a/b}^{-/-}$ mice in control condition (extracellular pH 7,4) and in lower pH (extracellular pH 7,2). In contrast to wild type mice, where the frequency and amplitude of sPSC was significantly decreased in lower extracellular pH, in $GABA_{B1a/b}^{-/-}$ mice the frequency was unaffected, while the amplitude was significantly reduced, indicating that $GABA_{B1}$ is important for this response (Figure 3.5). Whether this observation reflects the direct involvement of $GABA_B$ receptors in chemoreception, or if this depends on secondary alterations, remains to be studied. Nonetheless, our results suggest a putative role of $GABA_B$ receptors in a network sensing extracellular pH, which may play a significant role in central respiratory chemoreception.

In a final set of experiments, we studied whether baclofen would have any “effect” on synaptic transmission in $GABA_{B1}$ null mutant mice when the extracellular pH is low. The measurements have been performed in neurons of pre-Bötzinger complex in lower extracellular pH (7,2), and $GABA_B$ receptor agonist baclofen and antagonist CGP55845A were applied subsequently. In these experiments, baclofen in wild type mice inhibited dramatically the frequency of sPSC, leaving the amplitude unaffected (Figure 3.6). Strikingly, baclofen caused a decrease in both frequency and

amplitude of sPSC in $GABA_{B1a/b}^{-/-}$ mice. However, in contrast to wild type mice, where CGP55845A was able to antagonize the effect of baclofen, in $GABA_{B1a/b}^{-/-}$ mice CGP55845A was not effective to antagonize neither the decrease of frequency nor the amplitude of sPSC. Considering the fact that CGP55845A was ineffective in $GABA_{B1a/b}^{-/-}$ mice, although unlikely, it cannot be excluded that the effects of baclofen in synaptic transmission in lower extracellular pH may not be exclusively mediated by $GABA_B$ receptors. In other studies some G-protein dependent $GABA_B$ responses have been observed in the $GABA_{B2}$ deficient, but not in the $GABA_{B1}$-deficient mice (Gassmann et al., 2004). These residual $GABA_B$ responses were mediated by $GABA_{B1}$ and caused by the inhibition of a constitutively active K^+-channel, (Bettler & Tiao, 2006). However, it remains unclear whether the observed atypical $GABA_B$ responses are of physiological relevance or represent a cloning artifact.

4.3 Identification and developmental changes of I_h current in pre-Bötzinger complex

Ionic currents similar to *Ih* were found in diverse cell groups in various species (DiFranceso 1993; Pape 1996; Frère 2004,). In this study we have characterized *Ih* in pre- Bötzinger complex of neonatal mice, and have presented evidence that this current is similar to that observed in other cell types. The unique property of I_h, i.e. activation upon hyperpolarization beyond resting membrane potential, made it possible to isolate I_h fromother membrane currents. Current, evoked by hyperpolarizing test pulse from the holding potential of -60mV, showed an instantaneous (non- I_h) and a time dependant current component (I_h) (Figure 3.7). Consistent with other studies, I_h current was sensitive to extracellular ZD7288, the known antagonist of I_h channels (Figure 3.8). Instantaneous components have been recently reported for both recombinant (Macri & Accili, 2004; Proenza & Yellen, 2006) and native I_h channels (Day *et al.* 2005; Rodrigues & Oertel, 2006). However, in recombinant channels the instantaneous component appears to be sensitive to ZD 7288 as well (Macri & Accili, 2004).

Developmental changes associated with I_h currents have not yet been assessed in pre-Bötzinger complex. To address this question, we have analyzed *Ih*

current in pre-Bötzinger complex during first two weeks of postnatal development (P0-P11). We have found that both I_h current amplitude and density increases significantly after 4 postnatal days (Figure 3.9). Moreover, detailed quantitative analysis showed a remarkable increase in the number of the cells having I_h current (23% in the age group of P0-P4 and 67% in the age group of P5-P11), which means that the expression of I_h channels is increasing during postnatal development. This observation was in line with studies, where I_h amplitude increases progressively during the first postnatal weeks, consistent with overall increased expression of the I_h channel isoforms (Bayliss et al., 1994; Surges et al., 2006). Overall, this data demonstrate that neurons in pre-Bötzinger complex, although not all, have I_h current, which is increasing significantly during postnatal development.

4.4 $GABA_B$ modulates I_h currents

A key property of neuronal pacemaker channels is their regulation by neurotransmitters and hormones acting through the second-messenger cAMP. Since $GABA_B$ receptors functionally couple to $G\alpha_{i/o}$-proteins, we would expect that activation of $GABA_B$ receptors would decrease the basal activity of adenyly cyclase in the neurons of pre-Bötzinger complex, and as a consequence, decrease the intracellular cAMP concentration, and ultimately, decrease I_h. To directly test this hypothesis, we measured I_h current after activation of $GABA_B$ receptors. This was performed during first two weeks of postnatal development. Our results demonstrate that $GABA_B$ receptors modulate I_h current in older (age group of P5-P11), but not in younger mice (age group of P0-P4, Figure 3.10). The absence of modulatory effect of $GABA_B$ receptors in the age group of P0-P4 is most probably due to low expression of HCN channels.

In older mice (P4-P11) we observed biphasic effects to baclofen, which were opposite to each other. In majority of the neurons (60%) baclofen caused an increase, while in 40% of the recorded neurons baclofen caused a decrease in I_h current amplitude and density (Figure 3.11 and Figure 3.12). This would mean that a basal cAMP level can be up- and downregulated in pre-Bötzinger complex, under influence of $GABA_B$ receptors, which in turn up- and downregulates I_h channels. Reports had

been shown that coactivation of $GABA_B$ and β-adrenergic receptors in thalamocortical neurons leads to upregulation of I_h current, which was not observed when only $GABA_B$ receptors were activated (Frère and Luthi, 2004). Authors suggested possible involvement of synaptic $GABA_B$ receptors that couple positively to cAMP synthesis induced by β-adrenergic receptors. Other studies have shown that $GABA_B$ receptors can also activate LVA Ca^{2+}-channles in pre-Bötzinger complex, which seemed to be developmentally regulated, since the enhancment was observed only the first few days of postnatal development (Zhang et al., 1999).

Since many reports on native receptors suggest existence of distinct $GABA_B$ receptor subtypes (Bonanno & Raiteri, 1993; Gemignani et al., 1994; Cunningham & Enna, 1996; Deisz et al., 1997; Mohler & Fritschy, 1999; Yamada et al., 1999; Bowery et al., 2002), we have hypothesized that there might be another $GABA_B$ receptor subtype apart from the known $GABA_B$ receptor, which by contrast, is positively coupled to cAMP system. To directly test this hypothesis, we used $GABA_{B1}$ null mutant mice. However, in contrast to wild type mice, deletion of $GABA_{B1}$ revealed a complete absence of $GABA_B$ response in $GABA_{B1a/b}^{-/-}$ knockout mice (3.14). This data, in one hand, demonstrated that there is no other additional $GABA_{B1}$ subunit causing the enhancing effects of $GABA_B$ receptors, in the other hand, it did confirm that the effects of baclofen are mediated exclusively by $GABA_{B1}$ receptor.

4.5 The mechanism of I_h modulation by $GABA_B$

Metabotropic receptors, such as $GABA_B$ receptors, activate heteromeric (αβγ) G-proteins by catalyzing replacement by GTP of GDP bound to the α subunit, resulting in dissociation of α-GTP from βγ subunits. In most cases, α-GTP carries the signal to effectors, as in hormonal stimulation (Birnbaumer, 1990; Kaziro et al., 1991; Bourne et al., 1990; Freissmuth et al., 1989) and inhibition of (Wong et al., 1991, 1992) adenylyl cyclase by $G\alpha_s$ and $G\alpha_{i/o}$ respectively. In our study, the observed up- and downregulation of I_h channels by $GABA_B$ receptors would mean that cAMP synthesis is under the influence of two separate signaling pathways, which in turn either up- or downregulate I_h channel activity.

The experiments with PKA inhibitor (Rp-cAMP) demonstrate that cAMP

dependent phosphorilation, indeed is an important regulatory pathway of I_h channel acitivity mediated by $GABA_B$ receptors. In 34% of the recorded neurons the responses to baclofen were diminished in the presence of extracellular Rp-cAMP (Figure 3.15A, B). Ability of baclofen to modulate I_h current was not surprising, while in many other cell types I_h current had been shown to be dependent on channel phosphorylation by PKA (Accili et al., 1997; Vargas & Lucero, 2002). However, in 66% of the recorded neurons the enhancing effect of baclofen on I_h was not occluded in the same recording conditions (Figure 3.15C, D). These results indicate that the upregulation of I_h channels by $GABA_B$ receptors appears to happen at a point upstream of PKA. Accordingly, we were able to interfer the down-, but not upregulation of I_h channels by $GABA_B$ by using SQ22536, an inhibitor of adenylyl cyclase. In 40% of the recorded neurons the decreasing effect of baclofen on I_h current was absent in the presence of SQ22536 revealing that the downregulation of I_h is mediated by adenylyl cyclase pathway (Figure 3.16A, B). However, in 60% of the recorded neurons baclofen could still enhance I_h current in the presence of SQ22536, suggesting an adneyly cyclase independent signaling pathway (Figure 3.16C, D). Interestingly, we found that both Rp-cAMP and SQ22536 themselves significantly marked I_h current amplitude in almost 60% of the recorded neurons, which was not observed in 40% of the recorded neurons. One interpretation of these data could be that the neurons in pre-Bötzinger complex express different combinations of I_h channels, which in turn determines the sensitivity to cAMP.

In this study, by using intracellular pertussis toxin (PTX), we demonstrate that baclofen mediated downregulation of I_h channels involves PTX-sensitive G-proteins, while baclofen mediated upregulation does not. In 45% of the neurons intracellular dialysis with PTX attenuated baclofen mediated decrease of I_h currents (Figure 3.17). The ability of PTX to disrupt the coupling of inhibitory receptors to other ion channels has already been well documented (Gross et al., 1990). By contrast, in 55% of the recorded neurons PTX treatment did not occlude the enhancing effect of baclofen on I_h. The possible mechanism of modulation of I_h channels by $GABA_B$ receptors as shown in the experiments with PTX is the involvement of PTX-sensitive $G\alpha_{i/o}$ and PTX-insensitive $G_{\alpha s}$ proteins that down- and upregulate I_h channel activity,

respectively. In our experiments, baclofen mediated decrease of I_h current was attenuated in neurons dialyzed with the both anti-$G\alpha_{i3}$ and anti-$G\alpha_s$ antibodies, while the baclofen mediated enhancement of I_h current was not affected in the same experimental conditions (Figure 3.18). Therefore, the results of our experiments in neurons dialyzed with specific G-protein antibodies (anti $G\alpha_{i3}$ and $G\alpha_s$) in one hand might suggest that both the reduction and enhancement of I_h current by baclofen were not mediated by the tested G-proteins. On the other hand we can not exclude the possibility that both subunits of G-proteins are involved in the modulation, since it might be also possible that our inability to demonstrate any specific effect of either antibody on response to baclofen was a consequence of experimental protocol, i.e. antibodies were not dialyzed properly or the high concentration of the antibody has lead to unspecific effects. Another possibility is the low affinity of these antibodies for native $G\alpha_{i3}$ and $G\alpha_s$ proteins, which seems unlikely, since biochemical assays in our lab (data not published) by using the same G-protein antibodies (anti Gαi3 and Gαs) has revealed a coupling of $GABA_B$ receptors to both Gαs and Gαi proteins in younger mice.

The intracellular dialysis of SPβγ peptide (peptide against to Gβγ subunit) occluded the effects of baclofen revealing that both increasing and decreasing effects of baclofen on *Ih* occurs through Gβγ subunits (Figure 3.19). This effect seemed to be specific, because the inactive peptide (FVIII) could not occlude the both effects of baclofen on I_h. The reversal of $G_{\beta\gamma}$ modulation by the SPβγ peptide is an important clue and seems to be a primary requirement to the molecular basis of $GABA_B$ receptor mediated modulatory process on I_h. In addition, intracellular dialysis of purified bovine brain $G_{\beta\gamma}$ subunit did not enhance the effect of baclofen in I_h current itself, but did increase the I_{inst}, most probably due to activation of K^+-conductance (Figure 3.21).

Previous studies have shown that $G\alpha_{i/o}$-coupled receptors, such as μ-opioid receptors, unexpectedly couple positively to adenylyl cyclase, thus raising the intracellular cAMP concentration (Federman et al., 1992; Uezono et al., 1993; Kaneko et al., 1994; Birnbaum et al., 1995; Tsu et al., 1995; Ulens & Tytgat, 2001). This unexpected activation of adenylyl cyclase occurs through adenylyl cyclase type II, an

isotype that can be activated by $G_{\beta\gamma}$ subunits of any G protein-coupled receptors (thus including Gi/o-coupled receptors) provided that $G_{\alpha s}$ is present (Tang & Gilman, 1991). Interestingly, others have shown that $G\alpha_{i/o}$-coupled receptors can stimulate adenylyl cyclase in native cells as well (Andrade 1993; Gereau & Conn, 1994). These findings may account for the observation that $G\alpha_{i/o}$-coupled receptors not only fail to inhibit, but actually enhance, $G\alpha_s$-receptor responses, as demonstrated (Andrade 1993; Gereau & Conn, 1994; Ulens &Tytgat, 2001).

However, all these findings show an involvement of adenylyl cyclases, which at least partially we did not observe in our experiments. This is the first time to show that $GABA_B$ receptors are positively coupled to Ih channels by their own, without any additional co-factors. However, we can not exclude the possibility that this upregulation of I_h channels in PBC happens only in neonatal mice, because these experiments were performed during first two weeks of postanatl development. Nevertheless, our data offer new insights regarding to the nature of the G-protein-coupled signaling pathways by which $GABA_B$ receptors affect I_h channel activity. The upregulation signaling pathway appears to play a dominant role in coupling of $GABA_B$ receptors to neuronal I_h channels. Here we suggest a new hypothesis about distinct signaling pathways of $GABA_B$ receptors during postnatal development. Both pathways start with activation of $GABA_B$ receptors, which leads to dissociation of Gβγ subunits from Gα and trigger different signaling pathways. The upregulation occurs independently of cAMP pathway, while the downreulation occurs through adenylyl cyclase, cAMP, and PKA dependent pathway. Figure 4.1 presents a model of $GABA_B$ mediated two signaling pathways.

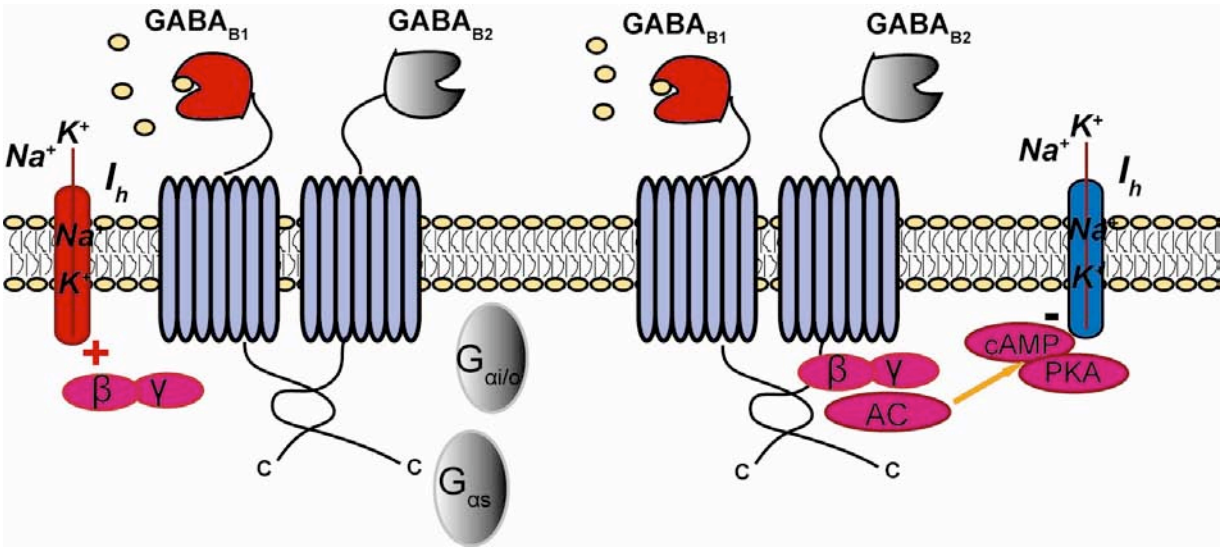


Figure 4.1: A model depicting the proposed regulation of I_h channels by $GABA_B$ receptors. The binding of agonist to $GABA_B$ receptors leads to dissociation of α-GTP from βγ subunits. In one modulatory pathway $GABA_B$ through Gβγ subunits leads to upregulation of I_h channels, independent on cAMP. In another modulatory pathway, Gβγ subunits lead to downregulation of I_h channels through adenylyl cyclase, cAMP and PKA.

4.5 Concluding remarks and future outlook

At this work we have investigated the function and signaling of $GABA_B$ receptors in pre-Bötzinger complex. Our data unambiguously showed the involvement of $GABA_B$ receptors in a neuronal network sensing extracellular pH. However, this observation does not really rule out whether $GABA_B$ receptors are directly involved in the network. In this regard, futher in vivo experiments need to be done.

Our experiments have revelaed that $GABA_B$ receptors modulate neuronal I_h channels in pre-Bötzinger complex. This modulation seemed to involve distinct signaling pathways. The next step would be to examine which HCN channel isoform(s) are the targets of $GABA_B$ receptors. For example, single-cell PCR and immunohistochemistry can be applied to verify the expression of particular HCN isoform(s) in pre-Bötzinger complex. From another side, more pharmacological experiments could be done. For example, sense and antisense approach could be used to study the involvement of different G-proteins in this modulation. Another line of experiment shoud be done to

see whether Gβγ subunits are directly involved in the modulation or involve other signaling molecules. Moroever, a possible involvement of PKA and PLC also could be tested.

5. Summary

The present project investigated the novel aspects of the function and downstream signaling of $GABA_B$ receptors in the developing brainstem respiratory network of mouse.

The plethysmography studies performed with $GABA_{B1}$ null mutant mice have shown that $GABA_B$ receptors are not important for respiration. Remarkably, the deletion of $GABA_{B1}$ causes an impairment of synaptic transmission in brainstem respiratory network. One of the crucial aspects in the present study was uncovering an important role of $GABA_B$ receptors as candidate proteins involved in the neuronal network of sensing extracellular pH, which may play a significant role in central respiratory chemoreception. In addition, upon $GABA_B$ agonist application null mutant mice showed atypical electrophysiological $GABA_B$ responses when the extracellular pH was more acidic (pH 7,2), which was not observed in normal extracellular pH value (pH 7,4). However, the present study does not rule out the existence of obligatory subunit of $GABA_B$ receptors, and it remains to be elucidated whether these responses have any physiological relevance.

Whole cell patch-clamp recordings have shown that neurons in brainstem respiratory network have I_h currents and that this current density together with the number of the cells having I_h current undergoes marked developmental changes during the first two postnatal weeks.

Our results demonstrate that there are two distinct signaling pathways by which $GABA_B$ receptors functionally couple to I_h channels. In one pathway, which appears to play a dominant role, $GABA_B$ receptor activation causes upregulation of I_h channels that requires the Gβγ subunit of activated G-proteins. Importantly this pathway does not depend on activity of adenylyl cyclases. By contrast, another pathway by which $GABA_B$ receptors downregulate I_h channels is dependent on adenyly cyclases, and consequently the cAMP and PKA activity. However, it was not possible to determine whether or not a distinct subunit of Gα, particularly $G\alpha_s$ and $G\alpha_{i/o}$ are specifically involved in coupling of $GABA_B$ receptors to I_h channels, although the experiments

involving PTX suggested the involvement of $G\alpha_{i/o}$ proteins in $GABA_B$ mediated downregulation of I_h channels.

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Zucker RS (1993). Calcium and transmitter release. *J Physiol Paris* **87**:25-36

8 Curriculum vitae

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EDUCATION

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9. Publication list

PUBLICATIONS

Dudanova I, Sedej S, Ahmad M, Masius H, **Sargsyan V**, Zhang W, Riedel D, Angenstein F, Schild D, Rupnik M, Missler M.
Important contribution of alpha-neurexins to Ca^{2+}-triggered exocytosis of secretory granules. J Neurosci. 2006 Oct 11; 26(41):10599-613.

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