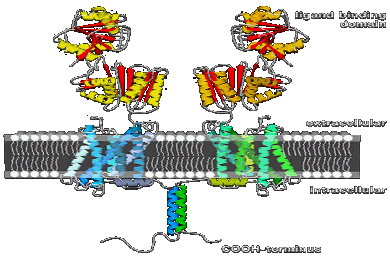
Friedrich Miescher Institute
Novartis Research Foundation
Basel, Switzerland

$GABA_B$ RECEPTOR-MEDIATED MODULATION OF SYNAPTIC PLASTICITY IN THE LATERAL AMYGDALA



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Hamdy Shaban

aus Alexandria, Aegypten

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auf Antrag von

Prof. Dr. Andreas Lüthi , Prof. Dr.Bernhard Bettler

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Dekan

Prof. Dr. Hans-Jakob Wirz

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1. SUMMARY

Fear conditioning, one of the most powerful and widely used methods to investigate the mechanisms of associative learning in animals, involves the pairing of an aversive stimulus such as a foot-shock (the unconditioned stimulus; US) with a neutral stimulus such as a tone (the conditioned stimulus; CS). The tone acquires aversive properties and, on subsequent exposure, will elicit a fear response. Behavioral and *in vivo* electrophysiological experiments indicate that NMDA receptor-mediated long-term potentiation (LTP) in the lateral amygdala (LA), a key structure for emotional learning, underlies the acquisition of Pavlovian fear conditioning.

Neuronal activity in the LA is tightly controlled by local inhibitory interneurons. Interneurons exert their inhibitory effect by releasing the neurotransmitter GABA acting on ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. There is accumulating evidence suggesting a role for $GABA_A$ and $GABA_B$ receptors in regulating amygdala-dependent fear and anxiety behavior. However, whereas the role of $GABA_A$ receptors for postsynaptic integration and gating of LTP induction is well documented, nothing is known about the role of $GABA_B$ receptors in the LA.

$GABA_B$Rs are G-protein-coupled receptors that are localized both pre- and postsynaptically. Postsynaptic $GABA_B$Rs are coupled to inwardly rectifying K^+ channels. Presynaptic $GABA_B$Rs inhibit neurotransmitter release by decreasing Ca^{2+} influx at both GABAergic terminals and glutamatergic terminals. Functional $GABA_B$ receptors are generally thought to be heterodimers containing $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits. The $GABA_{B(1)}$ subunit exists in two differentially expressed isoforms, $GABA_{B(1a)}$ and $GABA_{B(1b)}$, differing by the presence of two N-terminal “sushi” domains in the $GABA_{B(1a)}$ isoform.

In the main study of the present thesis, using a combined electrophysiological and genetic approach in mice, I found that presynaptic $GABA_B$ heteroreceptors on glutamatergic cortical afferents are predominantly comprised of $GABA_{B(1a)}$ subunits, and critically determine associative properties of presynaptic cortical LTP. In the absence of functional presynaptic $GABA_B$ heteroreceptors, an NMDA receptor-independent, non-associative

form of presynaptic LTP is unmasked. Strikingly, the loss of associativity of cortico-amygdala LTP is accompanied by a generalization of conditioned fear at the behavioral level. This indicates that the specificity of information processing in the LA can be set by activity-dependent presynaptic inhibition mediated by specific $GABA_B$ receptors.

In contrast to synaptic plasticity at cortico-amygdala afferents, I found that at thalamic afferents, $GABA_B$ receptors facilitate LTP induction by a postsynaptic mechanism. Moreover, this effect could be attributed to $GABA_{B(1b)}$ containing receptors. Thus, in the LA specific subtypes of pre- and postsynaptic $GABA_B$ receptors control induction pre- or postsynaptic LTP in an afferent-specific manner.

Taken together, the present findings indicate that $GABA_B$ receptors are playing a key role in controlling associative plasticity in the LA, and suggest that $GABA_B$ receptors could be a pharmacological target for treatment of psychiatric conditions like anxiety and post traumatic stress disorder.

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2. INTRODUCTION

2. Overview

In this introduction, I will go through the historical development and the early hypothesis about the formation of emotional memory. Later, I will outline the anatomical features of one of the key structures in fear memory formation, namely the amygdaloid nucleus, and its connectivity to other brain areas. Then I will discuss the different cellular mechanisms of synaptic plasticity implicated in fear memory formation. Finally, I will elucidate the important role of inhibition in the lateral amygdala.

2.1. Fear emotion and memory formation

Our memories are our identity. All information is stored in the brain by an unknown encoding mechanism. However, not all information is stored in the same intensity. The reason why some memories remain in our mind forever and others not, is embedded in the emotional information accompanying this memory formation. Whether it is a pleasant emotion or an aversive one, both reinforce memory formation in the brain. Here, I will focus only on the formation of fear memory on the brain. Fear associative learning in mammalian is organized into separate anatomically defined functional systems. The amygdala serves as the neuroanatomical hub of fear memory formation. Pathways that convey information about signals for biologically important events arrive at these hubs by circuitry that depends on stimulus modality and complexity. Within the amygdala, neural plasticity occurs because of convergence of these stimuli and the biologically important information they predict. This neural plasticity is the physical basis of associative memory formation

2.1.1. Early thoughts about emotion

Charles Darwin, in 1872, was the first who described that the expression of emotions in humans and animals is similar (Darwin 1872/1965). By comparing and analysing several

sketches and photographs of animals and people in different emotional states, he claimed that there are similarities in the expression of emotional state across species (Fig. 1). He also proposed that many emotional expressions in humans, such as tears when upset or baring the teeth when angry, are rudimentary patterns of action. Darwin`s second contribution was the proposal that a limited set of fundamental or 'basic' emotions are present across species and across cultures.

Weiskrantz was the first to show that bilateral lesions of the amygdala were sufficient to induce the orality, passivity, strange dietary behavior and increased exploratory tendencies of the Kluver–Bucy syndrome in monkeys (Weiskrantz 1956). The removal of the amygdala also permanently disrupted the social behavior of monkeys; usually resulting in a fall in social standing (Rosvold and Delgado 1956). This line of research established the significant role of the amygdala as one of the most important brain regions for emotion memory formation.

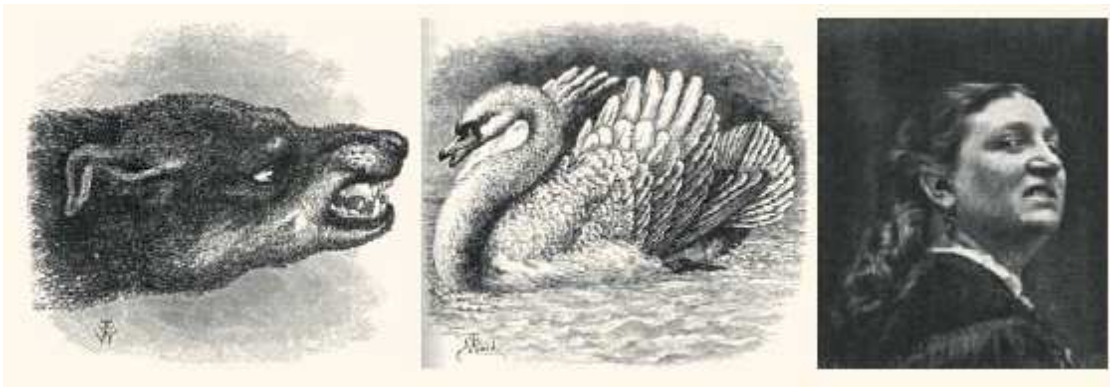

Figure 1) Drawings and photographs used by Darwin to illustrate cross-species similarities in emotion expression — in this case, anger/aggression. Adapted from (Dalgleish 2004)

2.1.2. Fear conditioning and the amygdala

Fear is one of the most crucial emotions for most animals and humans at least for survival. Animals and humans share similar mechanisms for fear learning, which seem to have been conserved throughout their evolution. Fear behavior could be simply observed in humans facial expressions. This is not the case in rodents; it is quite difficult to estimate the emotional state in mice by just observing their facial expressions. Pavlovian auditory fear conditioning, in which a neutral conditioned stimulus (CS) such as a tone is

paired with an unconditioned stimulus (US), typically a foot shock, results in long lasting changes in synaptic transmission in the lateral amygdala (LA) (LeDoux et al. 1984; Clugnet and LeDoux 1990). This behavioral paradigm was classically used to investigate the molecular mechanism underlying fear learning. The fear conditioning paradigm provides an applicable experimental model to study fear learning. Thus, emotional significances are attached to an initially biologically insignificant CS (tone) when such neutral stimulus is paired with an aversive US (foot shock) (Fig.2A). When these associations between CS and US are learned, an animal responds to the CS with a stereotypical defensive behavioral response, including freezing, increased heart rate, or startle (LeDoux 2000; Medina et al. 2002; Maren 2003). The CS can be unimodal, involving only a single sensory modality such as a sound, light, smell, or touch. Alternatively, it can be multimodal, involving several sensory modalities such as the context (i.e. the environment associated with the CS).

A study by Bechara and colleagues (Bechara et al. 1995) described a patient with bilateral amygdala damage who failed to be fear-conditioned to aversive stimuli, but who could nevertheless report the facts about the conditioning experience. By contrast, another patient with hippocampal damage successfully acquired a conditioned fear response but had no explicit memory of the conditioning context — indicating that contextual information depends on the hippocampus. Functional magnetic resonance imaging (fMRI) in humans showed that upon exposure to a fearful facial expression, the amygdala was highly activated (Phillips et al. 1997; Glascher et al. 2004).

Neural circuitries of fear conditioning were intensively investigated using lesion or selective inactivation of brain structures combined with behavioral observation. All these studies indicate that the amygdala is a key player in establishing the fear memory (Weiskrantz 1956; Armony et al. 1995; LeDoux 2000; LeDoux 2003). Anatomical tracing studies combined with single unit recordings in experimental animals suggest that LA is a site of convergence of somatosensory input conveying US and afferent inputs conveying CS of different sensory modalities (Pitkanen et al. 1997), where the association of learned information about CS and US apparently occurs during fear conditioning (Fanselow and LeDoux 1999). The neural circuitry of auditory fear conditioning, which uses tone as the CS, as well as its cellular and molecular mechanisms

are particularly well understood (LeDoux 2000; Maren 2000). The sound CS invade the LA by way of two main pathways: the thalamic input, consisting of a direct thalamo-amygdala projection, originates in the medial geniculate nucleus (MGm) and in the posterior intralaminar nucleus (PIN) of the thalamus; and the indirect cortico-amygdala pathway, which extends from the auditory thalamus to the auditory cortex (TE3 area) and includes further projections that relay the auditory information from the cortex to the LA (Maren et al. 2001). It was demonstrated that at least one of these two pathways is essential for fear memory (Romanski and LeDoux 1992). (Fig. 2B)

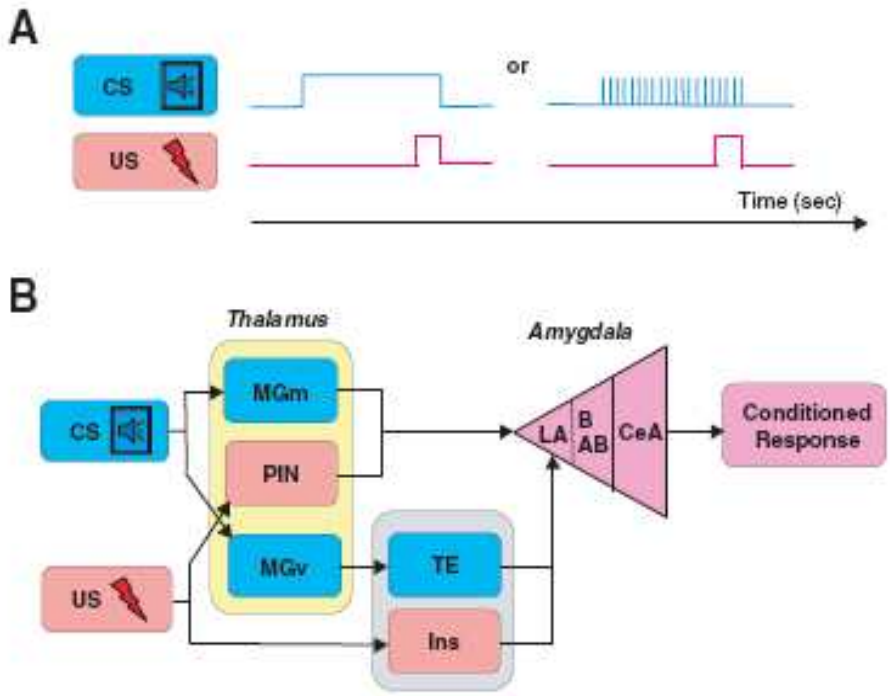

Figure 2) Auditory fear-conditioning. *A*, Scheme of the experiment during which a neutral tone (continuous or a series of short tones) is presented to an animal for several seconds, co-terminating with a foot shock. *B*, Neuronal circuitry involved in auditory fear conditioning. The amygdala nuclei can be roughly divided into two subsystems. These include the lateral (LA), basal (B), and accessory basal (AB) nuclei, which together form the basolateral complex, and the central nucleus (CeA). The basolateral amygdala, specifically the lateral nucleus, receives and integrates sensory information from a variety of sources. These include the medial and ventral divisions of the thalamic medial geniculate nucleus (MGm and MGv, auditory), primary auditory cortex (TE), the insular cortex (Ins), and the thalamic posterior intralaminar nucleus (PIN, somatosensory). Thus, the LA is a locus of sensory convergence and a site of the CS-US (conditioned stimulus–unconditioned stimulus) association within the amygdala. The information is then sent to the CeA, which through the divergent projections to the hypothalamus and brainstem areas mediates fear responses such as freezing and potentiatedacoustic startle. (Adapted from (Dityatev and Bolshakov 2005))

2.1.3. The Amygdaloid complex: Structure and connectivity

The amygdala (Latin, *almond*, from Greek amugdal) is an almond-shaped structure located within the temporal lobe and composed of ~13 nuclei. There are many different classifications and nomenclatures of these nuclei and sub-nuclei. I will use the most widely accepted nomenclature. The basolateral amygdala (BLA), comprises the lateral nucleus (LA), the basal nucleus (BL), and the accessory basal nucleus (AB), which is also known as the basomedial nucleus (Fig. 3). The central nucleus (CE), which is the output sub-nucleus of the amygdala, is separated from the BLA by clusters of GABAergic neurons, the intercalated cells (ITC) (Nitecka and Ben-Ari 1987; McDonald and Augustine 1993). ITC neurons receive inputs from the lateral and basal nuclei and project to the central medial nucleus. (Millhouse 1986; Pare and Smith 1993; Royer et al. 1999; Pare et al. 2003). In contrary to the hippocampus, the amygdala shows heterogeneity in structure with a non-layered anatomy (Fig. 3). The dorsolateral subnucleus is the primary input to the fear-conditioning circuitry. This was shown in a number of studies using anatomical tracing techniques and in vivo electrophysiological recordings (Romanski and LeDoux 1992; Pitkanen et al. 1997; LeDoux 2000). Thus, the dorsolateral division of the LA is the site in the amygdala with the shortest latency of auditory-evoked responses, indicating that this division receives the earliest information about auditory stimuli (LeDoux 2000). After the information is processed in the LA, the signal is transferred to other sub-nuclei of the amygdala, like the basomedial nucleus, which also receives incoming inputs from the hippocampus with encoded contextual information. BLA also receives projection from nociceptive receptors via brain stem. The output nucleus CE projects in turn to areas in the brain stem that control the autonomic system (heart rate), somatic motor centres (freezing), and endocrine system (stress hormone). All these systems are implicated in the expression of fear (LeDoux 2000; Maren 2001) (Fig. 3). The architectonic organization and connectivity of the amygdala have been extensively reviewed (De Olmos and Hardy H 1985; Alheid Gf and De Olmos J 1995; McDonald 1998; Pitkanen 2000).

Tract tracing studies have revealed that amygdala nuclei have extensive intranuclear and internuclear connectivities (Krettek and Price 1978; Pitkanen 2000). These studies indicate that sensory information enters the amygdala through the basolateral nuclei, is processed locally, and then follows a predominantly lateral to medial progression to the centromedial nuclei which act as an output station (Rainnie et al. 1993). The LA sends extensive projections to the basal and accessory basal nuclei and the capsular part of the central nucleus (Pitkanen et al. 1995; Smith and Dudek 1996). There are extensive connections within and between the different nuclei of the amygdaloid complex. These connections indicate that there is extensive local processing of information entering the amygdala before it leads to the appropriate behavioral outcomes. These intranuclear and internuclear connections have mostly been studied using anatomical tract tracing techniques, coupled in some cases with electron microscopic examination of the synaptic specializations. However, physiological studies indicate that amygdala nuclei contain many types of cells that cannot be readily distinguished on anatomical grounds alone (Millhouse and DeOlmos 1983; Washburn and Moises 1992; Sah et al. 2003). Furthermore, reconstructed neurons in the lateral and basal nuclei show large dendritic trees. Neurons that have cell bodies in a particular nuclear subdivision (e.g., the dorsolateral subdivision of the lateral nucleus) may well have dendrites that extend into the next division (e.g., the medial subdivision of the lateral amygdala) (Rainnie et al. 1993; Pare and Gaudreau 1996; Faber et al. 2001). This implies that inputs that anatomically terminate in a particular subdivision of these nuclei may well innervate neurons whose cell bodies are in a different subdivision. Thus, the physiological impact of these local connections and their implications for information processing remain elusive.

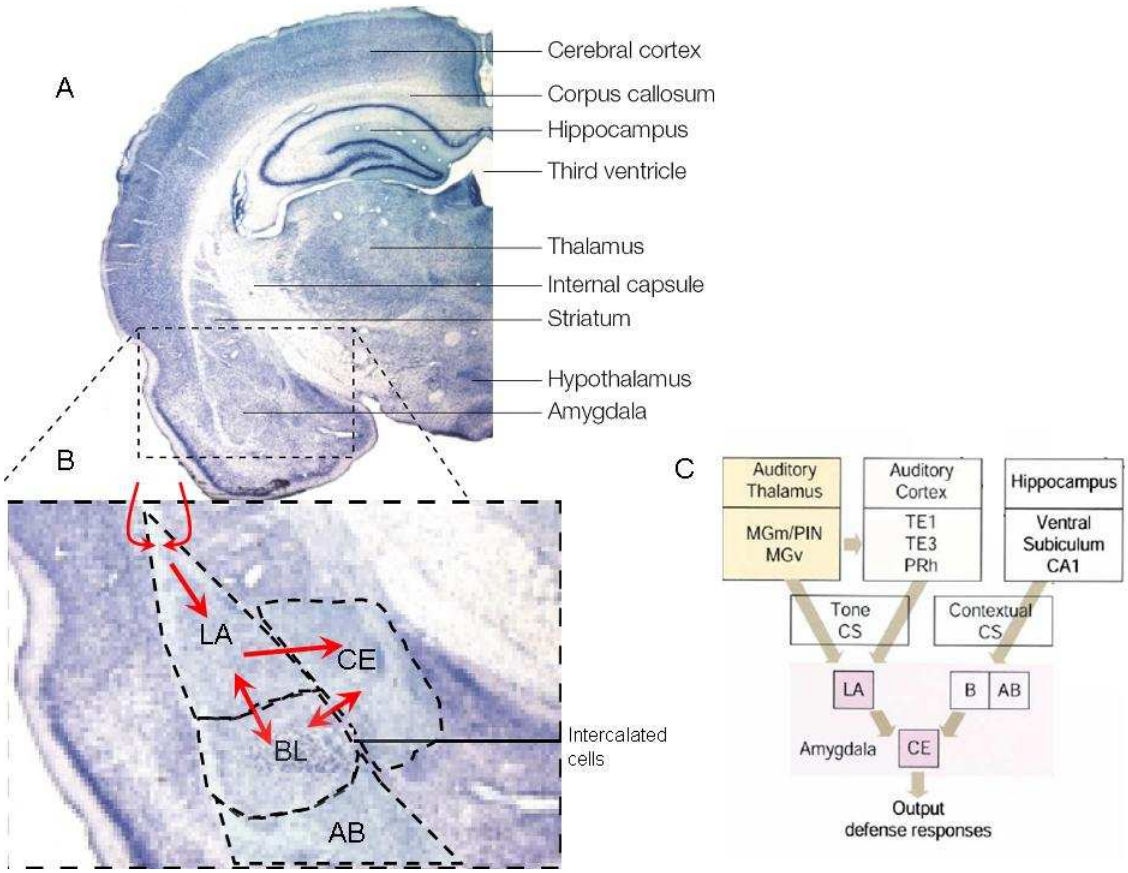

Figure 3.) Amygdala structure and connectivity A, An example of the amygdaloid region as it appears in acutely prepared coronal sections **B**, The area of the amygdala is enlarged to show the three main subdivisions of LA , BL, CE. The cs and us stimuli converge on single cells in the LA. From LA stimuli signal conveyed to CE and BL. Reciprocal connections connect BL with LA, and BL with CE. GABAerig intercalated cells separate between CE and BLA. **C**, Major areas that send auditory and contextual information to the amygdala obtained from tract-tracing studies. (Adapted from (Medina et al. 2002; Shumyatsky et al. 2002))

2.1.4. Fear conditioning and synaptic plasticity

The most extensively studied and best-characterized sensory pathway is a direct projection from the medial geniculate nucleus of the thalamus to the dorsal portion of the lateral nucleus of the amygdala (LeDoux and Farb 1991). This pathway transmits auditory information CS to the amygdala, which is accompanied by the US. When the CS requires greater processing, polysynaptic projection to amygdala become necessary and the amygdala receives CS information from the cortex. For example, the apparatus or context cues present at the time of shock reach the BLA via the ventral angular bundle after processing by the hippocampus and entorhinal cortex (LeDoux et al. 1991; Maren

and Fanselow 1995) and also reach the lateral amygdala from the perirhinal and postrhinal cortex (Amaral and Insausti 1992).

Fear not only produces behavior, it also changes the synaptic strength at synapses mediating the CS and US information. It was shown that fear conditioning induces long-term potentiation (LTP) in the lateral nucleus of the amygdala (LA) (Clugnet and LeDoux 1990; McKernan and Shinnick-Gallagher 1997), in the glutamatergic synapses (i.e., utilizing glutamate as neurotransmitter) (LeDoux 1993). This LTP was associative, in that it required concurrent pre- and postsynaptic activity, and it was synapse specific (Weisskopf et al. 1999).

One candidate mechanism for these changes is LTP of excitatory synaptic transmission. LTP can be induced in the major sensory input pathways to LA both in vivo (Rogan and LeDoux 1995; Doyere et al. 2003) and in vitro (Chapman et al. 1990; Huang and Kandel 1998; Weisskopf et al. 1999). Moreover, fear conditioning and LTP share similar biochemical mechanisms (Huang and Kandel 1998; Schafe and LeDoux 2000; Bauer et al. 2002; Rodrigues et al. 2004)

The group of LeDoux showed that electrical stimulation of auditory input in the medial geniculate to lateral nucleus synapses induces LTP (Clugnet and LeDoux 1990). The individual cells of LA region respond to tones that might serve as an auditory CS and shocks that might serve as a US (Romanski and LeDoux 1992). Furthermore, LTP induction in this pathway produced by electrical stimulation increases the amygdala response to a tone (Rogan et al. 1997). Another study showed that after fear conditioning, cells within the amygdala show increased firing to the CS, suggesting that the CS input has been potentiated following conditioning (Quirk et al. 1997). Finally, (McKernan and Shinnick-Gallagher 1997) compared brain slices containing the auditory pathway from the auditory thalamus to the lateral nucleus taken from fear-conditioned and control animals and they found long-lasting increase in the synaptic efficacy of this pathway in the fear-conditioned animals. Fear-conditioned animals showed a presynaptic facilitation of AMPA-receptor-mediated transmission, directly measured in vitro with whole-cell recordings in lateral amygdala neurons (McKernan and Shinnick-Gallagher 1997). These

findings represent the first in vitro measures of synaptic plasticity resulting from emotional learning by whole animals.

It was postulated that activity of N-Methyl-D-Aspartate (NMDA) receptor plays an important role in the formation of contextual fear learning as NMDAR antagonists injected into the hippocampus or genetic deletion of NMDA receptors from the CA1 region of the hippocampus interfere with contextual fear conditioning (Young et al. 1994; Shimizu et al. 2000). Indeed, genetic manipulations that enhance NMDA receptor function can enhance contextual fear learning (Tang et al. 1999). During fear conditioning, theta rhythm activity generated by a tone, paired with shock, synchronizes in the hippocampus and the amygdala (Seidenbecher et al. 2003). Thus, it is clear that fear conditioning represents a strong interaction between the structures that encode the emotional, signalling, and contextual aspects of the learning.

CS and US convergence in the LA leads to potentiation of the glutamatergic synapses activated by the CS, and this change must be occurring within the pre- and or postsynaptic neuron, or both (Quirk et al. 1995; Quirk et al. 1997). Presynaptic changes could take the form of greater neurotransmitter release per action potential arriving at the relevant synaptic terminals. Postsynaptic changes typically take the form of changes that make the postsynaptic cell more responsive to the same amount of neurotransmitter release. This could happen by insertion of more of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors that mediate the majority of excitatory glutamatergic transmission (Isaac et al. 1995; Liao et al. 1995). Finally, some forms of plasticity result in increased synaptic contacts through the growth of new dendritic spines (Muller et al. 2002).

2.2. Synaptic plasticity

It is thought that memory formation is associated with synaptic weight change; by either the strengthening of the synaptic transmission or the decrease of the synaptic weight transmission. Synaptic plasticity could be classified, according to its duration, into:

1- Short-term synaptic plasticity; changes happen as potentiation (STP) or depression (STD). They last from hundreds of milliseconds to a few minutes.

2- Long-term plasticity; changes which last from hours to weeks either as enhancement of synaptic strength, long-term potentiation (LTP) or as depression (LTD).

3- Late long-term plasticity; it includes persistent change within the synapses (synapses remodeling) that thought to be a form of consolidation of memory.

In this chapter, I will explain the different forms of short- and long-term plasticity without going into details of synapses remodeling.

2.2.1. Short-Term Plasticity

According to their duration and kinetics, short-term enhancements are defined as facilitation, augmentation or post tetanic potentiation. Short-term synaptic enhancement are usually attributed to effects of a residual elevation in presynaptic Ca^{2+}, acting on one or more molecular targets that appear to be distinct from the secretory trigger responsible for fast exocytosis and phasic release of transmitter to single action potentials (Fisher et al. 1997; Zucker and Regehr 2002). Depression is usually attributed to depletion of a readily releasable pool of vesicles, which follow a period of elevated activity. Short-term depression (STD) was shown to be induced in giant motoneuron of crayfish with low frequency stimulation, 5-20 Hz, (Czternasty and Bruner 1975). Depression can also arise from feedback activation of presynaptic receptors by the release of retrograde messenger (e.g.NO; Endocannabinoid) (Zucker 1993; Rouach and Nicoll 2003). Many presynaptic terminals in the mammalian CNS possess high-affinity metabotropic receptors (i.e. G-protein coupled receptors) that can be activated by neurotransmitters such as GABA, glutamate or adenosine. Moreover, many studies have demonstrated that glia may be involved in some forms of short-term plasticity (Araque et al. 2001; Haydon 2001). They have an established role in the clearance of the neurotransmitter and may participate in synaptic plasticity by controlling the speed and extent of such clearance (Danbolt 2001).

Regulation of short-term synaptic plasticity begins with the propagation of action potential (AP) to the presynapses. Consequently, this leads to a depolarization of the presynaptic terminals and activation. Of voltage gated Ca^{2+} channel. The following Ca^{2+} influx drives exocytosis and neurotransmitter release.

Excitatory synapses release glutamate neurotransmitter. Glutamate is typically referred to as an excitatory neurotransmitter, which activates ion channels receptors (ionotropic) and G-protein coupled receptors (metabotropic). There are three major subclasses of ionotropic receptors; AMPA, NMDA and kainate receptors. Metabotropic glutamate receptors are also located at the presynaptic site, as autoreceptors (i.e., at the same synapses) modulating glutamate release by decreasing Ca^{2+} influx into the presynapses. Activation of postsynaptic glutamate receptors triggers an excitatory postsynaptic potential (EPSP). This EPSP leads to a membrane potential change caused by current flow through postsynaptic receptors that tends to move the membrane potential toward the action-potential threshold.

Under physiological conditions presynaptic Ca^{2+} is regulated by a different key players like mitochondria, Ca^{2+} ATPase (ATP dephosphorylation enzyme), metabotropic glutamate receptors (mGlu) and metabotropic GABA receptors ($GABA_B$). All these play important role in regulating the residual Ca^{2+} (for review see (Zucker and Regehr 2002) (Fig. 4).

NMDA receptors are usually co-localized with AMPA receptors, but are not significantly activated at negative resting membrane potentials. This is because magnesium ions (Mg^+) in the extracellular solution block the NMDA channel pore at negative membrane potentials. Only upon significant depolarization of the postsynaptic membrane Mg^+ ions are expelled from the pore, allowing Ca^{2+} and sodium ions influx. The Mg^+ blockade of NMDA receptor channels imparts a voltage dependence as well as a transmitter dependence to channel opening.

The properties of postsynaptic receptors can also contribute to short-term plasticity. Desensitization of postsynaptic receptors, in which exposure to neurotransmitter results in receptors entering a non-responsive state, can reduce synaptic responses during repeated activation (Jones and Westbrook 1996; Sun et al. 2002; Zucker and Regehr 2002)

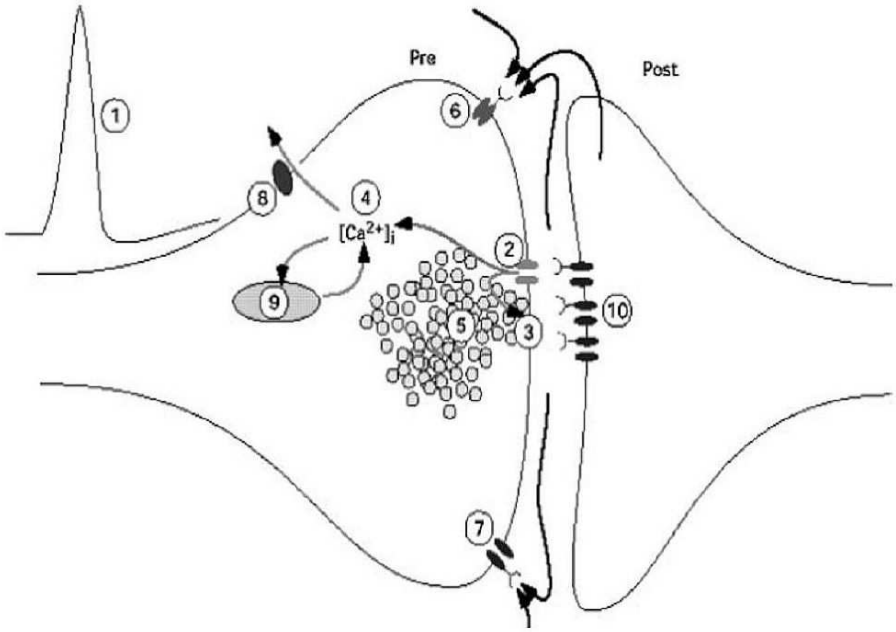

Figure 4) Sites of regulation of short-term synaptic plasticity. (1) AP waveform, (2) Ca^{2+} channel activation, (3) facilitation trigger and the readily releasable pool, (4) residual [Ca2+]i, (5) reserve pool, (6) metabotropic autoreceptors, (7) ionotropic autoreceptors, (8) Ca^{2+}-ATPase, regulating residual $[Ca^{2+}]$i in augmentation, (9) mitochondrial regulation of residual $[Ca^{2+}]$i in PTP, (10) postsynaptic receptor desensitization.(Zucker and Regehr 2002)

2.2.2. Long-term plasticity

Long-term plasticity changes take place either as an enhancement of synaptic strength, LTP, or a reduction, namely long-term depression (LTD), which can be homosynaptic (same synapses) (Wagner and Alger 1996) or heterosynaptic(different synaptic inputs) (Chen 2001). The long-lasting form of synaptic potentiation was first discovered in the hippocampus (Bliss and Lomo 1973) and can be induced when glutamate receptor activity at initially "weak" synapses is tetanized with high frequency stimulation. LTP has been observed in the three major excitatory synapses in the trisynaptic circuit of the hippocampus. In the hippocampus, the circuit is quite clear between several distinct areas: Cornu Ammonus (CA), such as CA-1 and CA-3, Dentate Gyrus (DG), Entorhinal cortex (ento) (Fig. 5). In this circuit, the perforant pathway projects from the pyramidal cells of the entorhinal area to the granule cells of the dentate gyrus. The mossy fiber pathway projects from the granule cells of the DG to the CA3 pyramidal cells; and the Schaffer collateral pathway projects from the CA3 pyramidal cells to the CA1 pyramidal cells (Insausti 1993). Hippocampal LTP can also be distinguished on the basis of their

dependence on NMDA receptors (Bliss and Collingridge 1993; Nicoll and Malenka 1995), Both dentate LTP and CA1 LTP are initiated postsynaptically by the activation of NMDA receptors (Malenka and Bear 2004). It is quite certain that CA3 LTP is independent of NMDA receptors and is thought to be initiated presynaptically (Zalutsky and Nicoll 1990).

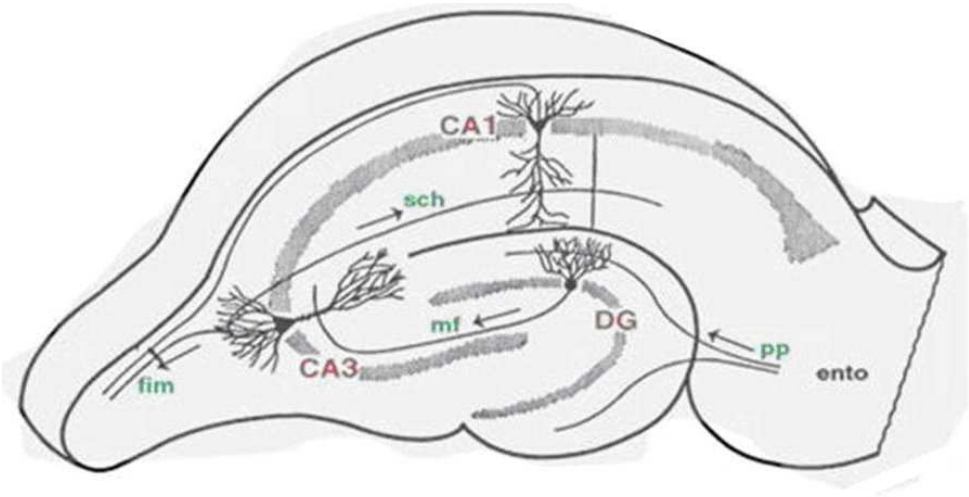

Figure 5) Anatomy and synaptic connections of the hippocampus.illustrating the trisynaptic circuit The perforant pathway (pp), from the entorhinal cortex (ent) to granule cells of dentate gyrus (DG). The mossy fibre pathway (mf), from DG to CA3 pyramidal cells. The Schaffer collateral pathway (sc), from CA3 pyramidal cells to CA1 pyramidal cells.

2.2.3. Presynaptic long-term potentiation: hippocampal mossy fiber LTP

Hippocampal CA3 pyramidal neurons display two different forms of LTP at two types of synaptic inputs from both the associational–commisural fibers and the mossy fiber pathway. It has been shown that although the induction of the associational–commisural pathway required the postsynaptic activation of NMDA receptors, membrane depolarization and calcium elevation, mossy fiber LTP did not require any of those effects to be induced (Zalutsky and Nicoll 1990). In addition, by studying paired-pulse facilitation (PPF) in mossy fibers, they showed that mossy fiber LTP is associated with a decrease in PPF (Zalutsky and Nicoll 1990). PPF is a form of synaptic plasticity observed in many synapses when two action potentials arrive at a presynaptic terminal separated by a short time interval (ten to hundred milliseconds) and characterized by a higher neurotransmitter release upon the arrival of the second action potential compared to the first one. It is accepted that synapses with a low probability of release (Pr) normally

present PPF, while synapses with a high Pr tend instead to have paired-pulse depression (Regehr and Mintz 1994). Mossy fiber LTP appears to involve a protein kinase type A (PKA) activation leading to long-lasting modulation of the presynaptic release machinery. These leads to an increased probability of transmitter release as well as presumably the recruitment of new or previously silent release sites (Tong et al. 1996) (Fig. 6). Like NMDAR-dependent LTP, new protein synthesis seems to be required for the late maintaining of mossy fiber LTP (Huang et al. 1994; Calixto et al. 2003). Although it is generally accepted that mossy fiber LTP expression is presynaptic, there are controversies regarding whether the induction is pre- or post-synaptic. In addition, the presynaptic protein adaptors Rab3A and RIM1α proteins play central role in this process, but much remain unknown about how they are modulated by PKA or perhaps other intracellular signaling cascades.

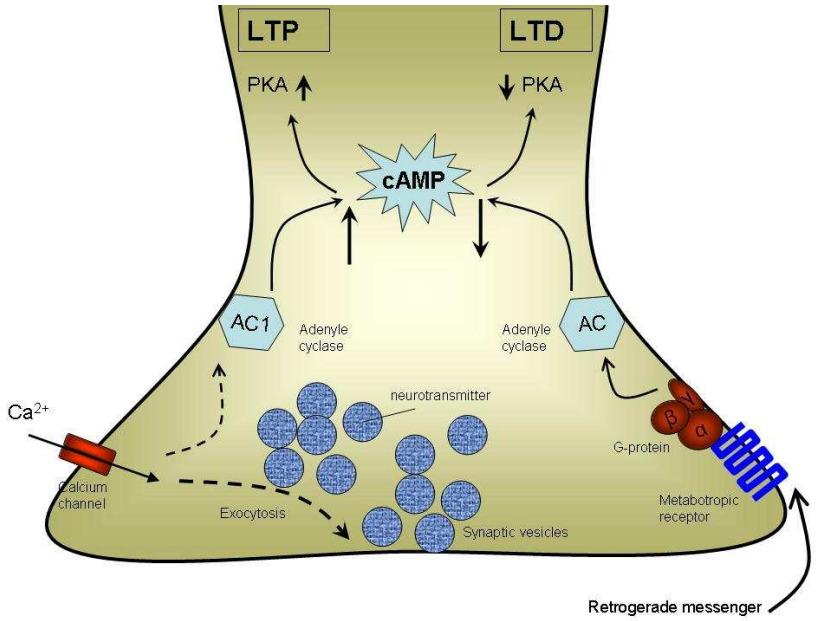

Figure 6). Presynaptic Long-term plasticity mechanism Diagram of the putative signal transduction cascades mediating presynaptic mossy fiber LTP and LTD (see text) (adapted from (Tzounopoulos et al. 1998))

2.2.4. Postsynaptic signal cascade of LTP induction and expression

LTP in CA1, which is widely accepted to be postsynaptically triggered, is also NMDAR-dependent. Activation of NMDA receptors leads to calcium influx and subsequent activation of PKC, CaMKII, and tyrosine kinases. (Collingridge et al. 1983; Malinow and Miller 1986; Malenka and Nicoll 1993). Similarly to LTP in CA1, many studies report

NMDAR-dependent LTP in the principal cells at the LA input afferents (Maren and Fanselow 1995; Huang and Kandel 1998; Weisskopf et al. 1999). Postsynaptic Ca^{2+} influx is not only mediated by NMDA receptor but also by voltage-dependent Ca^{2+} channels (VDCC) of the L-type. During induction of LTP, this Ca^{2+} influx leads to activation of different Ca^{2+}/calmodulin-dependent protein kinases subtypes (CaMKII and CaMKIV). In addition, metabotropic glutamate receptors (mGluR) and or β-adrenergic receptors (βAR) stimulate protein kinase A (PKA) via activation of adenylyl cyclase and production of cAMP. PKA, CaMKII and mitogen-activated protein kinase (MAPK) may induce numerous changes in activity of neurotransmitter receptors and ion channels properties. PKA, CaMKII, and MAPK may also signal via phosphorylation of the transcription factor, cAMP response element- binding protein (CREB), that activates the transcription and protein synthesis which is necessary for long-term synaptic modifications and synapses formation (Zalutsky and Nicoll 1990; Malenka and Nicoll 1993; Malenka and Bear 2004; Dityatev and Bolshakov 2005) (Fig. 7). The new proteins can be AMPA receptors that may be inserted into existing synapses (Malinow and Malenka 2002; Lüthi et al. 2004). Ras-related GTPases of the Rho family, such as RhoA and RhoB, are well-characterised mediators of morphological change in peripheral tissues via their effects on the actin cytoskeleton (Meng et al. 2003; O'Kane et al. 2003; O'Kane et al. 2004)

2.2.5. The Hebb rule of synaptic plasticity

Donald Hebb (1949) proposed that the efficacy of the synaptic transmission would be increased with the co-activation of pre- and post synaptic elements. This form of increase in synaptic efficacy could reflect the basis of learning. This suggestion was later enforced by the discovery of NMDAR-dependent LTP which needs the coincident activation of NMDA receptor by presynaptic EPSP and postsynaptic depolarization to remove the Mg^{2+} blockade in order to facilitate the induction of LTP. NMDA coincident activation was an attractive model for Pavlovian conditioning because a CS-generated glutamatergic input that at first weakly activates a synapse will be potentiated if the US causes the cell to fire within a temporally limited window. Thus, the cells that participate in this plasticity must receive both CS and US inputs.

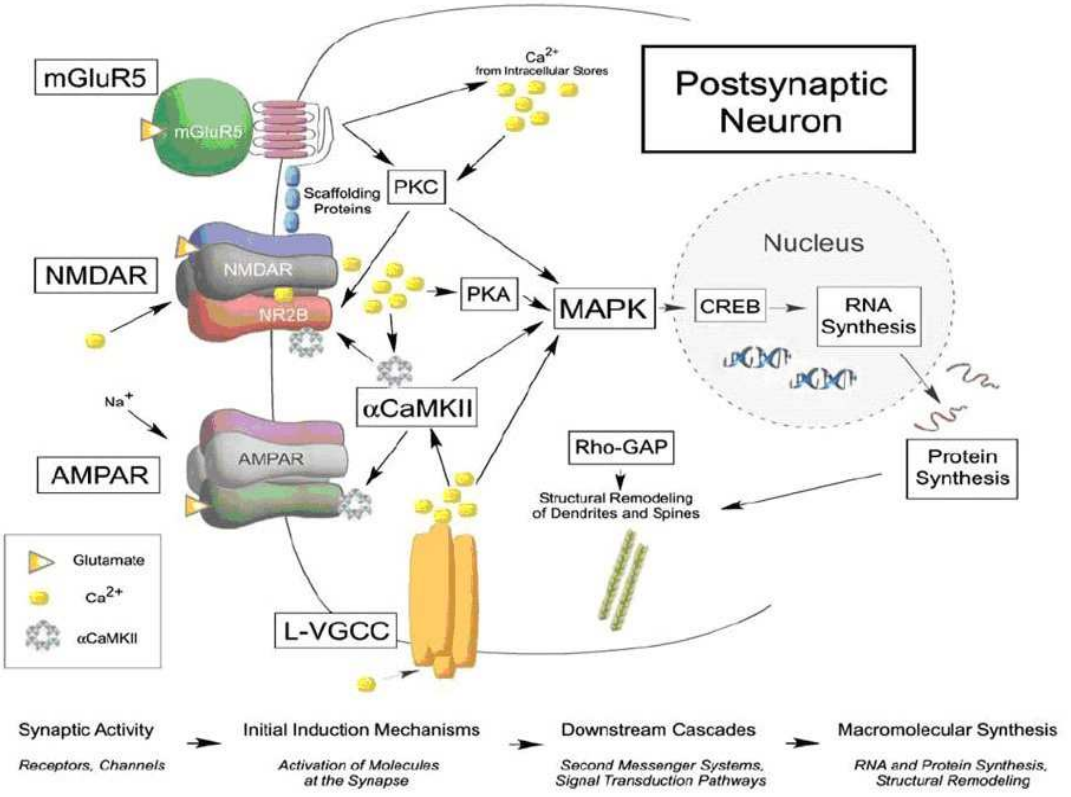

Figure 7) Molecular mechanisms underlying the acquisition and consolidation of emotional learning and memory in the lateral amygdala (Rodrigues et al. 2004) (see text above)

2.2.6. LTD, a different form of synaptic plasticity

Now it is clear that "LTP" and "LTD" are not unitary phenomena. Their mechanisms vary depending on the synapses and circuits in which they operate. In the earliest reports about LTD (Lynch et al. 1977) it was shown that in CA1 region of the hippocampus in vitro, long-term potentiation inducing stimuli delivered to one pathway resulted in a reversible depression in the non tetanized pathway. The same heterosynaptic LTD was also demonstrated in the dentate gyrus in vivo (Levy and Steward 1979). The phenomena was established later on by studies conducted by many investigators and classified into homosynaptic LTD (i.e. depression only in the pathway receiving the induction protocol) and heterosynaptic LTD. An induction protocol (600-900 stimuli, 1 Hz) was used to induce LTD of basal transmission in the hippocampal CA1 region in vitro (Mulkey and Malenka 1992). The mechanism underlying the induction of LTD was shown to be dependent on Ca^{2+} influx through NMDAR, mGluR, VDCCs (Mulkey and Malenka 1992; Nishiyama et al. 2000; Kemp and Bashir 2001), and the release of Ca^{2+} from the

intracellular stores induced by IP3 (Reyes and Stanton 1996; Miyata et al. 2000). The released intracellular calcium binds to calmodulin forming a complex, which activates calcineurin, protein phosphatase 2B (PP2B). Calcineurin then dephosphorylates and hence inactivates inhibitor 1. This removes the braking effect of inhibitor 1 on protein phosphatase 1 (PP1) allowing PP1 to become active, and dephosphorylates its substrates. This dephosphorylation process include AMPA receptors and CaMKII, which consequently decrease basal neurotransmission by removal of AMPA receptors or protein degradation (Lüthi et al. 1999; Kemp and Bashir 2001; Malenka and Bear 2004)(Fig. 8).

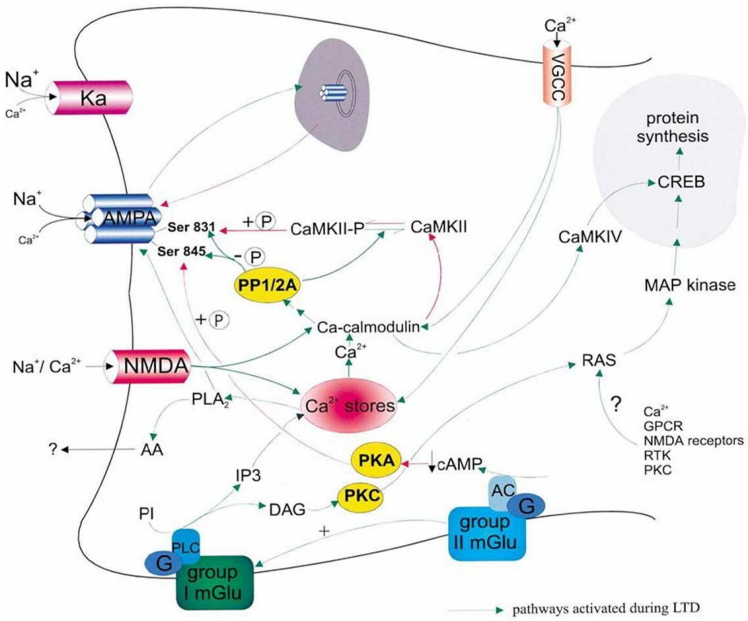

Figure 8) Schematic illustration of the postsynaptic mechanisms involved in LTD. Abbreviations: AC, adenylate cyclase; AA, arachidonic acid; CaMKII, calcium–calmodulin dependent protein kinase II; CREB, cAMP response element binding protein; DAG, diacylglycerol; IP3, inositol triphosphate; KA, kainate receptor; mGlu, metabotropic glutamate receptor; MAP kinase, mitogen-activated protein kinase; PI, phosphatidyl inositol; PLC, phospholipase C, PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PP1/2A, protein phosphatase 1/2A; and TKR, tyrosine kinase receptor. (Adapted from (Kemp and Bashir 2001))

2.2.7. Metaplasticity and the switch between LTP and LTD

The term ‘metaplasticity’ refers to the changes in the fundamental properties of plasticity. The threshold for induction of either of LTP or LTD is changed. In many regions of the brain, the activity-dependent changes in synaptic strength depend on the frequency and timing of presynaptic stimulation and postsynaptic activity, as well as the history of activity at those synapses. The Bienenstock, Cooper and Munro (BCM) theory suggested that there is a sliding threshold for synaptic modification (Fig. 9).

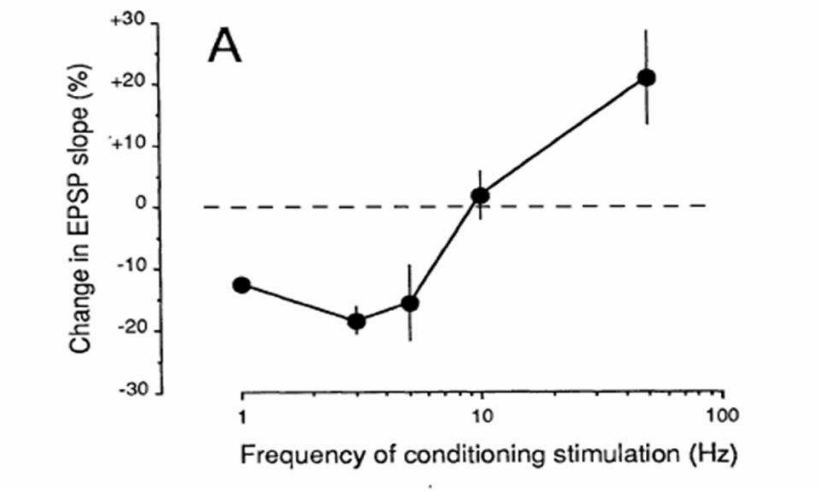

Figure 9) Frequency-dependent synaptic plasticity. Response to 900 pulses delivered at various frequencies (Adapted from (Dudek and Bear 1992))

A typical experiment starts by measuring the strength of a group of synapses. This is done by firing a single action potential in some of the axons that enter this region. These axons make synapses with pyramidal cells and generate a graded excitatory postsynaptic potential (EPSP). The strength of the synapses is defined by the magnitude of the EPSP amplitude and slope. LTP or LTD is then induced by stimulating the axons to fire at high frequency (typically 100 Hz) or low frequency (typically 1 Hz), a stimulus referred to as tetanus. Then depending on the frequency, LTP or LTD can be induced. Another way is to pair postsynaptic action potential (AP) with presynaptic EPSP so that potentiation is induced if a postsynaptic spike repetitively follows a presynaptic spike by a few milliseconds, whereas depression is induced if the temporal order of the spike pairing is reversed (Markram et al. 1997; Froemke and Dan 2002)

The mechanism determining whether LTP or LTD will be induced is imbedded in the common intacellular cascade. Ca^{2+} concentration is crucial in both cases. Many studies (Lisman 1989; Sjostrom et al. 2001; Jedlicka 2002; Sjostrom and Nelson 2002) have suggested that LTP would be induced with high concentration of Ca^{2+} which bind to CaMKII leading to autophosphorylation and subsequent phosphorylation of AMPA receptors. In contrast, LTD would be induced with low concentration of Ca^{2+} that favors activation of PP2B leading to LTD as described above (Fig. 8).

2.3. Inhibition in the amygdala

Fear is a basic evolutionally conserved emotion that triggers a set of defensive mechanisms for adapting to threatening events that is essential for survival. However, fear should not continue when the threatened stimulus is vanished. Therefore, it is crucial to establish a system to control this learning system to bring the system back to its basal level. Indeed, it was shown that most of pathological anxiety disorders are associated with impairment in the inhibitory system. Decreased levels of GABA have consistently been found in patients with depression, panic, and generalized anxiety disorders (Goddard et al. 2001).

In vivo data demonstrated a powerful control through GABAergic inhibition over the activity of projecting principal cells (Lang and Pare 1997; Pape et al. 1998) which renders the role to the GABAergic interneurons in the control of excitation in this region. Indeed, GABAergic interneurons are thought to play a crucial role in information processing in the amygdala (Lang and Pare 1997; Mahanty and Sah 1998) and to participate to the regulation of epileptiform activity (Washburn and Moises 1992; Washburn and Moises 1992) as well as fear and anxiety (Pesold and Treit 1995; Sanders et al. 1995). Converging fast excitatory postsynaptic responses from cortical and thalamic inputs were also found in interneurons of the LA (Szinyei et al. 2000). The cortical excitatory inputs onto interneurons in the lateral and basolateral nucleus of the amygdala were reported to be mediated by AMPA receptors, which show Ca^{2+} permeability that promote a particular form of LTP, whereas NMDAR-mediated signals were reported to be very small or negligible in these types of neurons (Mahanty and Sah 1998). On the contrary, experiments on LA interneurons using pressure application of NMDA showed that the respective receptors are functional in interneurons, although the mediating synaptic inputs were not identified (Danober et al. 2000)

2.3.1. Interneurons in the amygdala

The LA contains two main cell classes, in the LA, pyramidal neurons and nonpyramidal interneurons. The projection neurons in the LA are large pyramidal-like neurons with spiny dendrites that utilize glutamate as an excitatory neurotransmitter (McDonald 1982; Millhouse and DeOlmos 1983; Fuller et al. 1987). The nonpyramidal neurons in the LA

are spine-sparse interneurons that utilize GABA as an inhibitory neurotransmitter (Millhouse and DeOlmos 1983; Carlsen 1988). These subtypes of neurons are mostly round and smaller sized representing about 25% of the all population of LA neurons (McDonald and Augustine 1993). Classically, neurons were distinguished based on intrinsic membrane properties and firing patterns (Washburn and Moises 1992; Rainnie et al. 1993). Excitatory neurons have broad action potentials and show spike frequency adaptation. Inhibitory interneurons show high-frequency firing of action potentials with a distinct afterhyperpolarization after each spike, and no spike frequency adaptation (Fig. 10).

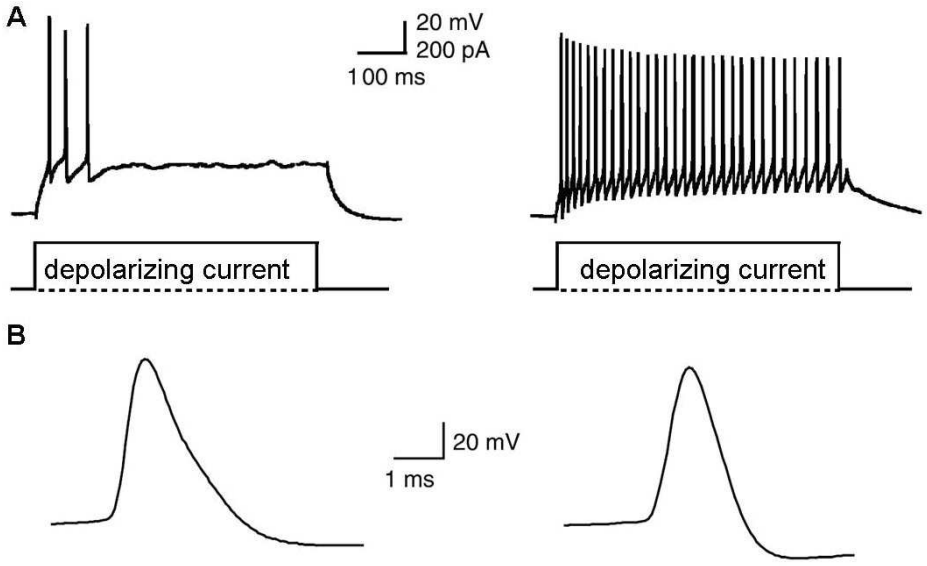

Figure 10) Pyramidal-like neurons and interneurons can be distinguished on electrophysiological grounds. Traces show recordings from typical pyramidal-like neuron and interneuron in the basolateral complex. Traces on the *left* are from a typical pyramidal-like neuron, and those on the *right* are from an interneuron. *A*: injection of a 400-ms depolarizing current injection in pyramidal neurons evokes action potentials that show spike frequency adaptation, while similar current injections into interneurons evoke a high-frequency train of action potentials that do not adapt. *B*: action potentials in interneurons have a shorter duration than in pyramidal cells (Adapted from (Sah et al. 2003))

Although the BLA is a subcortical structure, the anatomy and physiology of its two major cell types, the pyramidal and nonpyramidal neurons, are very similar to their counterparts in the hippocampus and neocortex (McDonald 1992; Washburn and Moises 1992; Rainnie et al. 1993). Similar to cerebral cortex, subpopulations of interneurons in the LA, can be distinguished on the basis of their content of calcium-binding proteins and neuropeptides. Calcium binding proteins are parvalbumin [PV], calbindin [CB], and

calretinin [CR]) and neuropeptides are somatostatin [SOM], neuropeptide Y [NPY], vasoactive intestinal polypeptide [VIP], and cholecystokinin [CCK] (McDonald 1985; Kemppainen and Pitkanen 2000; McDonald and Mascagni 2001; McDonald and Mascagni 2002; Mascagni and McDonald 2003). Double-labeling studies suggest that there are at least four distinct subpopulations of interneurons in both the cortex and BLA in the rat (Kubota et al. 1994; Kubota and Kawaguchi 1997; Kemppainen and Pitkanen 2000; McDonald and Betette 2001; McDonald and Mascagni 2001).

2.3.2. Ionotropic $GABA_A$ receptors

GABA (γ-aminobutyric acid) is synthesized in inhibitory neurons from glutamate by the enzyme glutamic acid decarboxylase (GAD), and is transported into vesicles by a vesicular neurotransmitter transporter (VGAT). Upon activation of interneurons, they start to spike and consequentially release GABA neurotransmitter in the synaptic cleft. The effects of GABA then can be mediated by the activation of either ionotropic or metabotropic receptors, which can be localized either pre- or postsynaptically. GABA signals are terminated by reuptake of the neurotransmitter into nerve terminals and/or into surrounding glial cells by a class of plasma-membrane GABA transporters. Thereafter, GABA is metabolized by a transamination reaction that is catalysed by GABA transaminase (GABA-T). The metabolism of GABA occurs in both neurons and glial cells (for review see (Owens and Kriegstein 2002) (Fig. 11).

The ionotropic receptors are $GABA_A$ and $GABA_C$ receptors. They are closely related pentameric receptors that conduct chloride ions. Whereas $GABA_A$ receptors are composed of combinations of several subunit types, $GABA_C$ receptors are composed of only single or multiple ρ-subunits. Based on the presence of eight subunit families consisting of 21 subunits (α1-6, β1-4, γ1–4, δ, ε, π, θ, ρ1-3), the ionotropic $GABA_A$ receptors display an extraordinary structural heterogeneity. It is thought that most functional $GABA_A$ receptors *in vivo* are formed upon co-assembly of α-, β-, and γ-subunits (Macdonald and Olsen 1994).

Although GABA is best known for its hyperpolarizing action and its inhibitory effect on the neuron output, a depolarizing excitatory action has been also reported (Barker et al. 1975; Gallagher et al. 1978; Gulledge and Stuart 2003). In addition, it was shown that

GABA has different action (excitatory) in immature CNS neurons than the normal inhibitory action in mature CNS neurons (Obata et al. 1978). This has been later clarified by the different developmental expression of Cl^- transporters (Plotkin et al. 1997).

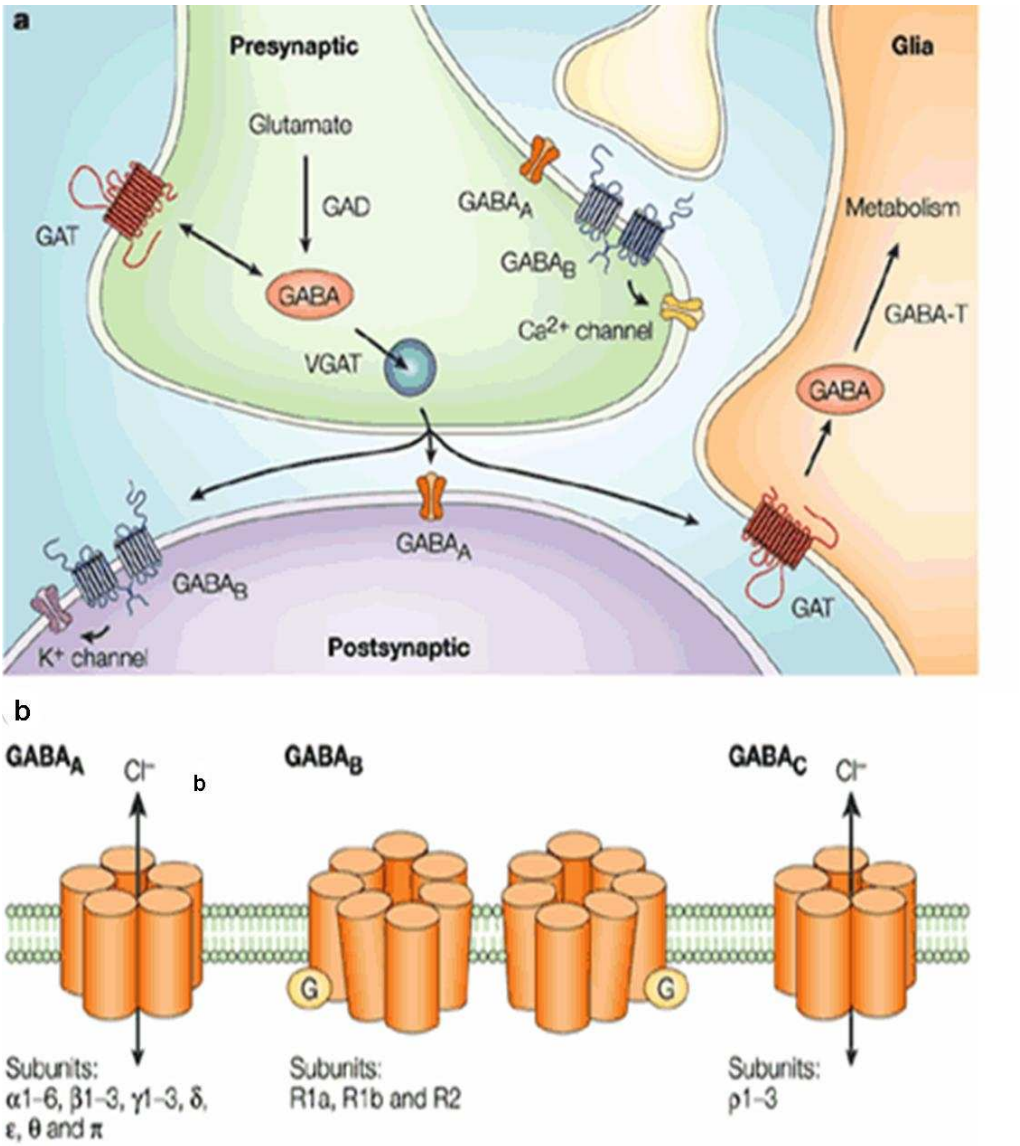

Figure 11) Components of the GABA signalling pathway**.** a) Schematic diagram of the synthesis and transport of GABA at synapses. GABA is synthesized from glutamate by decarboxylase enzyme in interneurons terminals. The released GABA activates ionotropic and metabotropic GABA receptor. Glial cells remove GABA from synaptic cleft by GABA transporter. b) GABA receptors differ in subunit composition and assembly. (Adapted from (Owens and Kriegstein 2002))

2.3.3. Metabotropic $GABA_B$ receptors

Pharmacological discrimination of $GABA_B$ receptors from $GABA_A$ receptors was first demonstrated by Bowery and colleagues in 1980, as receptors that are insensitive to the $GABA_A$ receptor antagonist bicuculline (Bowery et al. 1980). The development of drugs

similar to GABA; but can cross the blood-brain barrier introduced Baclofen. Baclofen shows specificity to $GABA_B$ receptors and was used for treatment of spasticity and skeletal muscle rigidity. $GABA_B$ receptors are abundant in the brain, where they are localized in many neuronal cell types including principle neurons and interneurons. Within the mammalian brain, the highest density of $GABA_B$ receptors is in the thalamic nuclei, the molecular layer of the cerebellum, the cerebral cortex, the interpreduncular nucleus, and the dorsal horn of the spinal cord (Bowery et al. 1987; Chu et al. 1990).
Intracellular in vitro recordings obtained from the basolateral amygdala in rat brain slice preparations show that $GABA_B$ receptors are abundantly present and induce a slow inhibitory component (Rainnie et al. 1991; Asprodini et al. 1992; Karlsson et al. 1992; Washburn and Moises 1992). A recent immunohistochemical study showed that there are high levels of expression of $GABA_B$ receptors in the limbic system (McDonald et al. 2004), which indicates a role in regulating emotional behavior.

2.3.4. Molecular structure of $GABA_B$ receptor

The $GABA_B$ receptor is composed of two subunits, $GABA_{B(1)}$ and $GABA_{B(2);}$ both show similarity to the family 3 heptahelix receptors. These proteins possess two domains, a seven alpha helix transmembrane core and an extracellular domain containing the agonist binding site (Kaupmann et al. 1997; Galvez et al. 2000). This binding domain is likely to fold like bacterial periplasmic binding proteins that are constituted of two lobes that close upon ligand binding (Kaupmann et al. 1998). The initial cloning studies from the rat brain revealed two isoforms of $GABA_{B(1)}$ subunit: $GABA_{B(1a)}$ and $GABA_{B(1b)}$ (Kaupmann et al. 1997). These two isoforms are the most abundant $GABA_B$ receptor isoforms in the CNS. They show a dissimilarity in the extracellular domain. $GABA_{B(1a)}$ has 147 amino acids which are replaced by only 18 amino acids in $GABA_{B(1b)}$ (Isomoto et al. 1998; Peters et al. 1998; Martin et al. 2001). This dissimilarity results from the presence of an alternative transcription initiation site within the $GABA_{B(1a)}$ intron. $GABA_{B(1a)}$ and $GABA_{B(1b)}$ primarily differ by the presence of a pair of sushi repeats in the $GABA_{B(1a)}$ specifc domain (Bettler et al. 1998; Hawrot et al. 1998). These sushi repeats, also known as short consensuses repeats were originally identified in complement proteins as a module that is involved in protein-protein interactions. That is why it is speculated that $GABA_{B(1a)}$ is

targeted to or retained at specific subcellular location by means of interaction of its sushi repeats with proteins in the extracellular matrix or on the surface of the neighboring cells (Bettler et al. 2004). In the rat brain, $GABA_{B(1a)}$ is the prevalent isoform at birth, whereas the $GABA_{B(1b)}$ is more abundant in adult brain tissue.

The absolute functional requirement for $GABA_{B(1)}$ and $GABA_{B(2)}$ heterodimerization was reported in many studies with $GABA_{B(1)}$ knockout mice which are devoid of $GABA_B$ receptor activity (Prosser et al. 2001; Schuler et al. 2001). Also in the transfected cell expression systems, it was shown that only the heterodimer is a fully operative receptor (Ng et al. 1999; Margeta-Mitrovic et al. 2000). It appears that heterodimerization of the two $GABA_B$ receptor proteins occurs predominantly through association of the alpha helical portions of the two C termini, and that this association is essential for trafficking of the receptor (Pagano et al. 2001) (Fig.12). It further appears that the large N-terminal extracellular domain, in particular the $GABA_{B(1)}$ subunit, is the site for ligand binding whereas the $GABA_{B(2)}$ subunit is crucial for effectors coupling (Galvez et al. 2000; Galvez et al. 2000; Galvez et al. 2001).

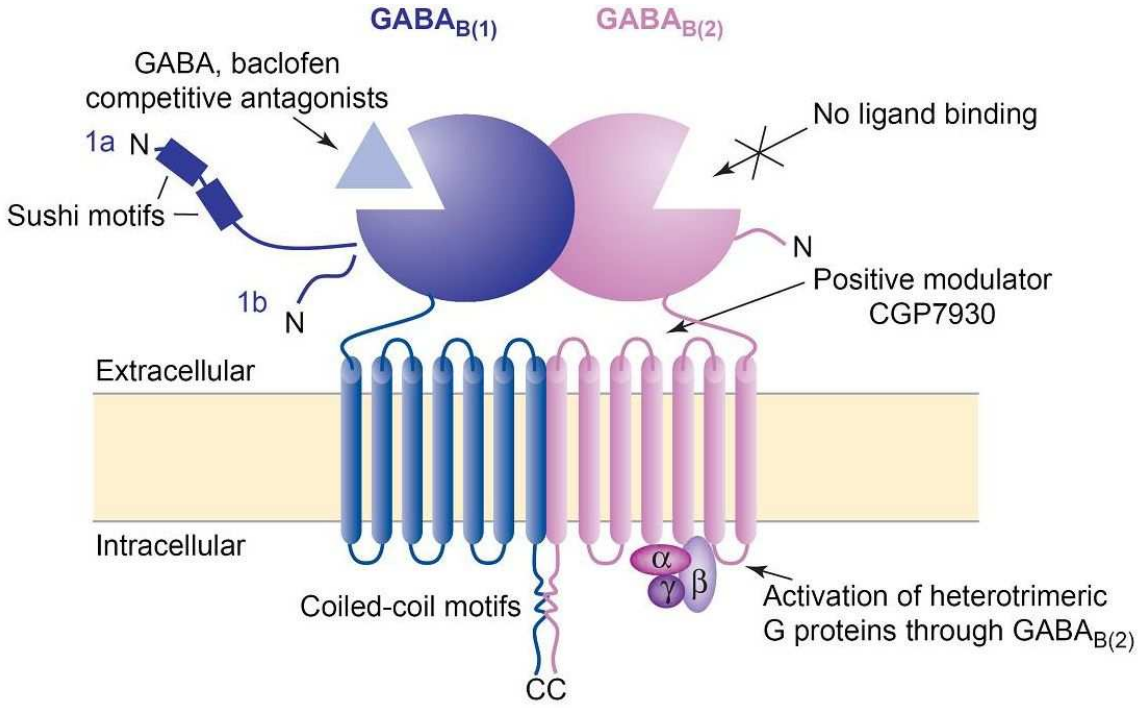

Figure 12) The $GABA_B$ receptor heterodimer. The two different subunits of $GABA_B$ receptor, the two isoforms $GABA_{B(1a)}$ and $GABA_{B(1b)}$ differ in the N-termini with the sushi repeats $GABA_{B(1)}$ subunit contains ligand binding site whereas $GABA_{B(2)}$ subunit coupled to Gi/o protein. (Adapted from (Cryan and Kaupmann 2005))

2.3.5. $GABA_B$ receptor-mediated signaling

In many tissues, $GABA_B$ receptors are negatively coupled to adenylate cyclase activity (Simonds 1999). In some cases, they also enhance the cAMP formation caused by G_S-coupled receptors (Bowery 1993; Bowery et al. 2002; Calver et al. 2002). Both GABA and baclofen have been shown to inhibit forskolin-stimulated cAMP level (Wojcik and Neff 1984). The inhibition of adenylate cyclase shown to be sensitive to pertussis toxin, indicating that $GABA_B$ receptors inhibit cAMP formation through G proteins of the G_i/G_o family (Kaupmann et al. 1997).

Presynaptic $GABA_B$ receptors inhibit the release of GABA, or other neurotransmitters, as well as neuropeptides through inhibition of Ca^{2+} influx by decreasing Ca^{2+} channel conductance (Scholz and Miller 1991; Mintz and Bean 1993). This inhibition is mediated by the interaction of the βγ subunits of the G-protein complex and the Ca^{2+} channel (Filippov et al. 2000). On the basis of electrophysiological and pharmacological criteria, mammalian neuronal Ca^{2+} channels have been classified as L, N, P/Q and T types. Individual channel types differ in their subunit composition. The rapid time course of $GABA_B$ receptor-mediated inhibition of N and P/Q type Ca^{2+} channels indicates a membrane-delimited pathway through the G protein βγ subunits (Mintz and Bean 1993). Such presynaptic inhibition at GABAergic terminals was shown to be involved in the induction of long-term potentiation (Bowery et al. 2002). Interestingly, this presynaptic inhibition of neurotransmitter release is not only effective in the GABAergic synapses (autoreceptor) but also in the glutamatergic synapses (herteroreceptor) (Fig.13).

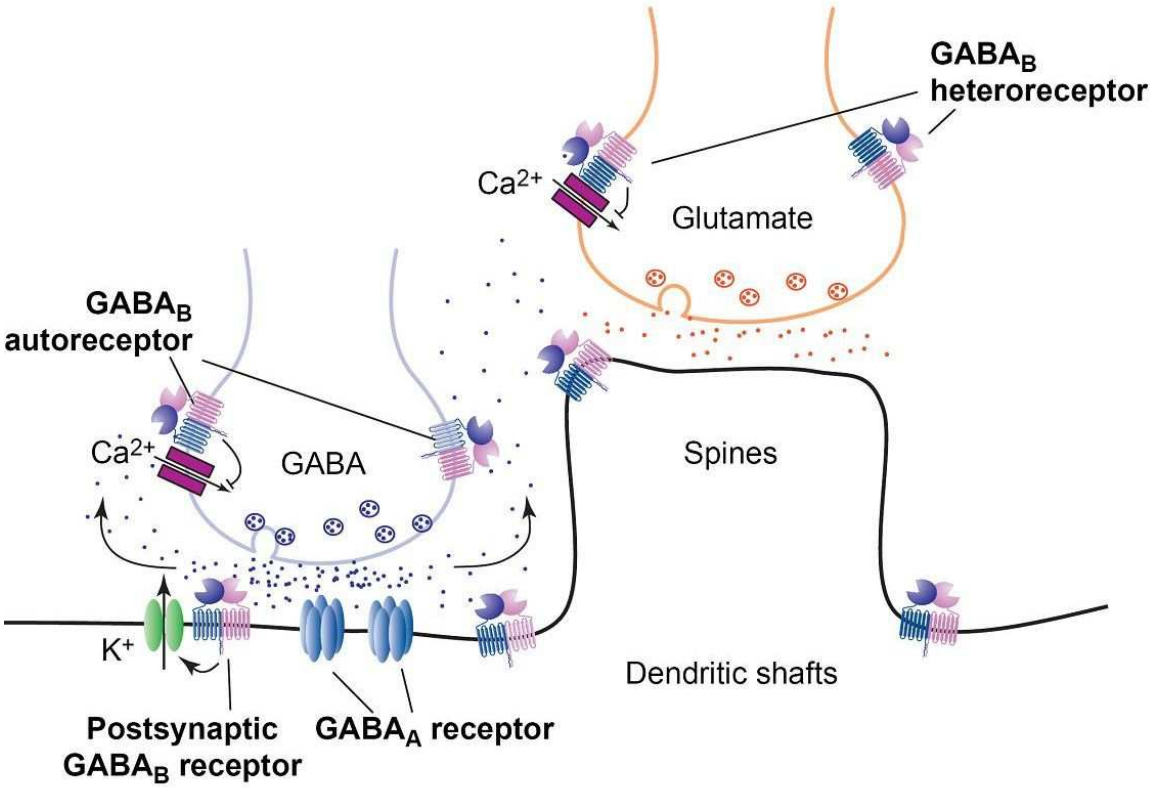

Figure 13) The $GABA_B$ receptor heterodimer and its localization in the brain. In the hippocampus, $GABA_B$ receptors are located presynaptically, postsynaptically and on extrasynaptic membranes. Extrasynaptic receptors are likely to be activated by 'spill-over' of GABA from adjacent synapses. (Adapted from (Cryan and Kaupmann 2005))

Postsynaptically, $GABA_B$ receptor agonists have been shown to hyperpolarize neurons by activating an outward K^+ current. The activation of K^+ channels is sensitive to pertussis toxin and blocked by Ba^{2+} and Cs^{2+}. This indicates an involvement of G-protein in activation of inwardly rectifying K^+ channels of the Kir3.0 family (formerly GIRK) (Luscher et al. 1997; Slesinger et al. 1997). Recent studies with Kir3.2 (subtype of GIRK channel) knockout mice provide strong evidence that native $GABA_B$ receptors couple to K^+ channels assembled with Kir3.2. In Kir3.2 knockout mice, the outward K^+ current evoked by baclofen is completely absent, whereas presynaptic $GABA_B$ receptor responses are unaltered (Luscher et al. 1997) Similarly, in weaver mutant mice, which carry a point mutation in the pore-forming region of the Kir3.2 channel, the amplitude of the $GABA_B$ receptor-activated K^+ current is significantly attenuated (Slesinger et al. 1997).

The activation of postsynaptic $GABA_B$ receptors requires a larger stimulus of the presynaptic terminals than necessary for activation of $GABA_A$ receptors, indicating that the $GABA_B$ response might be relevant under conditions of strong neuronal activity (Otis and Mody 1992). Activation of $GABA_A$ and $GABA_B$ receptors on the postsynaptic membrane generates a biphasic inhibitory postsynaptic potential (IPSP). The fast component of the IPSP ($IPSP_A$) is mediated by $GABA_A$ receptors activation which shunts the transmembrane voltage to the equilibrium potential of chloride, thereby normally leading to a hyperpolarization of the neuron (Owens and Kriegstein 2002). The $GABA_B$ receptor-mediated IPSP ($IPSP_B$) is slow in onset with a prolonged duration (Dutar and Nicoll 1988; Dutar and Nicoll 1988)

2.3.6. $GABA_B$R-mediated inhibition in the LA

$GABA_B$ receptor inhibitory inputs into the amygdala were initially investigated in epilepsy and kindling studies (i.e., over activation of certain brain area). It was shown that Baclofen suppressed the severity and duration of established kindled seizures and increased the intensity of postictal refractoriness. This suggests that Baclofen may be a useful antiepileptic agent (Wurpel et al. 1990). Another study showed that both bicuculline and phaclofen increased the spontaneous rate of firing of amygdaloid neurons (Mello et al. 1992). In the same year, Karlsson showed that synaptically-released GABA activates $GABA_B$ receptors and thereby exerts a depressant effect on kindling development (Karlsson et al. 1992). Moreover, it was shown that late inhibitory postsynaptic potential and the late hyperpolarizing response to GABA arise from a $GABA_B$-mediated increase in potassium (Washburn and Moises 1992). Later on it was shown that paired-pulse depression of the NMDAR-mediated synaptic potentials in the amygdala is mediated by mechanism other than activation of a postsynaptic $GABA_B$ receptor and activation of K^+ conductance (Huang and Gean 1994), suggesting presynaptic inhibition by $GABA_B$ receptors.

Interestingly, it was shown that the activation of $GABA_A$ and $GABA_B$ receptors in the LA differentially regulate glutamatergic synaptic transmission in the auditory thalamo-amygdala pathway (Li et al. 1996). Also Yamada and colleagues showed that the $GABA_B$

receptor agonist baclofen markedly inhibited both EPSCs and IPSCs in a concentration-dependent manner, and that the baclofen-induced inhibition was selectively abolished by the $GABA_B$ receptor antagonist CGP55845A. The paired-pulse ratio of EPSC and IPSC amplitude was increased by baclofen. Moreover, the effect of baclofen was mimicked by lowering the external Ca^{2+} concentration but not by glutamate and $GABA_A$-receptor antagonists. In addition, the frequency but not the mean amplitude of miniature EPSCs and IPSCs was decreased by baclofen. Thus, activation of $GABA_B$ receptors by baclofen reduces the strength of excitatory and inhibitory transmission in the BLA by a presynaptic mechanism (Yamada et al. 1999).

Moreover, in the same study they showed that repetitive conditioning stimulation applied to GABAergic synaptic inputs exerted an inhibitory action on glutamatergic excitatory transmission, and the stimulation-induced inhibition was abolished by CGP55845A. Furthermore, the paired-pulse ratio of EPSCs was increased during the stimulation-induced inhibition. The results in this study provided an evidence that synaptic activation of $GABA_B$ heteroreceptors elicits presynaptic inhibition of glutamatergic excitatory transmission in the BLA (Yamada et al. 1999)

The BLA contains substantial amounts of $GABA_{B(1)}$ and $GABA_{B(2)}$ mRNA (Kaupmann et al. 1997; Bischoff et al. 1999; Durkin et al. 1999; Clark et al. 2000) and exhibits significant $GABA_B$ receptor binding (Bowery et al. 1987; Bischoff et al. 1999). This is consistent with electrophysiological studies which have shown that $GABA_B$ receptors presynaptically modulate glutamate and GABA release from axons in the BLA (Yamada et al. 1999; Szinyei et al. 2000) and postsynaptically mediate a slow, prolonged hyperpolarization of BLA neurons via activation of potassium channels (Rainnie et al. 1991; Washburn and Moises 1992; Sugita et al. 1993).

More recently, investigations with antibodies directed against the $GABA_{B(1)}$ subunit were used to study the neuronal localization of $GABA_B$ receptors in the rat BLA. $GABA_BR$ immunoreactivity was mainly found in all cell types of the BLA with different intensity. Dual-labeling immunofluorescence analysis indicated that each of the four main subpopulations of interneurons exhibited $GABA_BR$ immunoreactivity. Virtually 100% of large CCK+ neurons in the basolateral and lateral nuclei were $GABA_BR$ +. In the basolateral nucleus 72% of somatostatin (SOM), 73% of parvalbumin (PV) and 25% of

VIP positive interneurons were $GABA_BR$ +. In the LA 50% of somatostatin, 30% of parvalbumin and 27% of VIP positive interneurons were $GABA_BR$ +. Electron microscopic (EM) analysis showed the staining of dendritic shafts and spines, most of which probably belonged to spiny pyramidal cells. Very few axon terminals were $GABA_BR$ +. Thus, the distal dendrites of pyramidal cells, and varying percentages of each of the four main subpopulations of interneurons in the BLA, express $GABA_B$ receptors (McDonald et al. 2004). Thus, these receptors may play an important role in the physiology and pathophysiology of the BLA.

2.3.7. GABAergic modulation of synaptic plasticity in the LA

A previous study has shown that blockade of GABAergic inhibition in the region of the LA in rats elicits physiological changes associated with a defence reaction, which suggests that endogenous GABA acts tonically at $GABA_A$ receptors in the BLA to inhibit anxiety response (Sanders and Shekhar 1995). Moreover, Pare showed that interneuron intercalated cells located between the basal lateral amygdala and central nucleus are gating the flow of the information between the two nuclei (Pare et al. 2003; Pare et al. 2004). These cell populations were also suggested to play an important role in extinction of fear memory (Royer and Pare 2002). Thus, inhibition is crucial in the LA circuit and firmly controlling the induction of synaptic plasticity within the LA and consequently the formation of fear memory.

The relevance of GABAergic modulation in the LA was highlighted by Shumyatsky et al. (2002), showing gastrin-releasing peptide (GRP) and its receptor GRPR. GRP is specifically expressed in the LA and in regions sending synaptic projection to the LA, whereas GRP receptors are expressed by a subset of GABAergic interneurons in the LA. Application of GRP in vitro excites interneurons and increase GABA release onto pyramidal cells (Fig. 14). GRPR Knockout mice result in disinhibition of principal neurons and facilitates the induction of LTP in cortical inputs, which is accompanied by persistent great fear memory. Thus, this tight control of glutamatergic synapses in the neural circuitry of fear conditioning regulates the formation of fear memory in the amygdala. Indeed, other studies demonstrate fear conditioning–induced reductions in the amygdala expression level of 65-kD isoform of glutamate decarboxylase (GAD65), an

enzyme important for GABA synthesis, and concomitant reduction in GABA release (Pape and Stork 2003). Furthermore, a recent study (Chhatwal et al. 2005) showed that gephyrin protein needed to stabilize $GABA_A$ receptors at synapses was down regulated after fear conditioning, indicating a decrease in $GABA_A$ inhibition during formation of fear memory.

Another system modulating GABAergic inputs in the amygdala is conducted by dopamine. The LA receives massive dopaminergic projections from the ventral tegmental area (VTA) (Nestler 2001). Application of dopamine in *in vitro* slices was shown to suppress feed-forward inhibition of principal cells or to facilitate inhibition of interneurons via activation of D2 receptors (Bissiere et al. 2003). This inhibition of D2 receptors prevents enhancement of LTP by dopamine and blocks acquisition of fear memory (Greba et al. 2001). Thus, induction of LTP at the amygdala synapses may implicate co- activation of dopaminergic fibers projecting to the LA (Fig.14).

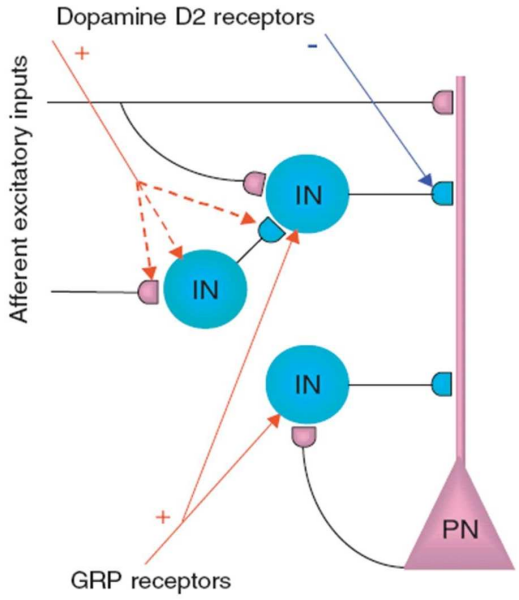

Figure. 14) Regulation of inhibition in the LA. Activation of dopamine D2 receptors leads to the suppression of inhibitory inputs to principal neurons (PN) via two mechanisms. First, it suppresses feed-forward inhibition by decreasing GABA release at synapses formed by local interneurons (IN) on principal cells. Second, it promotes inhibition of interneurons either via an increase of excitatory input to disinhibitory interneurons or by an increase in excitability of disinhibitory interneurons, or strengthening of interneuron-interneuron synapses. Gastrin-releasing peptide (GRP) is expressed by neurons projecting to the amygdala and principal cells within the amygdala. Activation of GRP receptors may excite interneurons mediating feed-forward and feedback inhibition and leading to stronger inhibition of principal cells. For the sake of simplicity, interneurons mediating disinhibition, feed-forward, and feedback inhibition are shown as distinct entities, although their functions may overlap.(Adapted from (Dityatev and Bolshakov 2005))

2.3.8. The loss of inhibition and anxiety

Although fear is crucial for survival, excessive or inappropriate fear can become an illness. Anxiety is a mental state that is elicited in anticipation of threat or potential threat. Excessive anxiety has been treated primarily with drugs that have calming properties, including alcohol, barbiturates, opiates, beta-blockers and benzodiazepines (Nemeroff 2003). Benzodiazepines are the most specific and effective, and are therefore widely used to treat both normal and pathological anxiety. Benzodiazepines increase the potency of GABA by modulating the function of $GABA_A$ receptors (Martin 1987). Therefore, it has been proposed that excessive excitatory neurotransmission is an important physiological hallmark of anxiety (McNaughton 1997). An increased brain activity in response to anxiety-provoking stimuli in the amygdala, parahippocampal gyrus and frontal cortex has been reported (Davidson et al. 1999). Studies on mice with genetically engineered $GABA_A$ receptors, which specifically lack the benzodiazepine-binding site, showed that $GABA_A$ receptors, which contain α2 subunit, are primarily responsible for the anxiolytic effects of these drugs. These $GABA_A$ receptors are located in the hippocampus, cortex and amygdala, (Low et al. 2000). Furthermore, it has been shown that $GABA_B$ receptors are down regulated as a result of amygdala kindling, which could contribute to the enhancement of excitatory transmission in kindled animals (Asprodini et al. 1992; Karlsson et al. 1992). Together, all these studies indicate that the amygdala might be a site of increased excitatory neurotransmission in anxiety disorders.

3. The aim of the study

We focused on $GABA_BR$-mediated modulation of synaptic plasticity in the LA. To clarify this kind of modulation, we need first to understand the mode of action and the physiological function of the $GABA_B$ receptor itself. Using knockout mice, we investigated whether $GABA_{B(1)}$ can participate in functional $GABA_B$ receptors in the absence of $GABA_{B(2)}$ subuint. Then we invistigated the localization of the two isomers $GABA_{B(1a)}$ and $GABA_{B(1b)}$ in the cortical and thalamic synapses. The subcellular localization of these two subunit isomers was never investigated in the LA. Via electrophysiological tools we tested whether they are differently distributed in the pre- versus postsynapstic loci. To investigate the effect of $GABA_BR$-mediated inhibition on the induction of LTP we established a subthreshold induction protocol. With this induction protocol, we explored the properties of synaptic plasticity at thalamic and cortical afferents. Then we used this induction protocol to elaborate the role of inhibition by $GABA_B$ receptor on the induction of homosynaptic LTP at thalamic and cortical afferents. Finally, in collaboration with behavioral specialist, we invistigated the relevance of $GABA_BR$- mediated modulation of synaptic plasticity on the mice behavior.

4. Materials and methods

4.1. Mouse brain Slice Preparation

Standard procedures were used to prepare 350 μm thick coronal slices from three to four week old male C57BL/6J mice following a protocol approved by the Veterinary Department of the Canton of Basel-Stadt. Briefly, the brain was dissected in ice-cold artificial cerebrospinal fluid (ACSF), mounted on an agar block and sliced with a vibratome at 4°C. Slices were maintained for 45 min. at 35°C in an interface chamber containing ACSF equilibrated with 95% O_2/5% CO_2 and containing (in mM): 124 NaCl, 2.7 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 0.4 NaH_2PO_4, 18 glucose, 2.25 ascorbate, and then for at least 45 min. at room temperature before being transferred to a superfusing recording chamber. In some preparation 2mM kynurenic acid was added to prevent glutamate toxicity.

4.2. Electrophysiology

In this study whole-cell patch-clamp recordings were obtained from projection neurons in the dorsolateral portion of the LA (Fig. 15) at 30°C–32°C in a superfusing chamber. Neurons were visually identified with infrared video microscopy using an upright microscope equipped with a x40 objective (Olympus). Patch electrodes (3-5 MΩ) were pulled from borosilicate glass tubing and were filled with a solution for each individual experiment as follows:

Monosynaptic EPSCs-IPSCs (in mM): 140 CsCl, or 140 Cs-gluconate, 10 HEPES, 10 phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP, 20 KCl (pH adjusted to 7.25 with CsOH, 295 mOsm). For current-clamp experiments CsCl was replaced by equiosmolar K-gluconate in the patch electrode. Isolated monosynaptic IPSC were recorded in the presence of the AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM) and the N-methyl-d-aspartate (NMDA) receptor antagonist 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; 10 μM). In current-clamp recordings, membrane potential was kept manually at −70 mV. Monosynaptic EPSPs exhibiting constant 10%–90% rise times and latencies were elicited by stimulation of afferent fibers with a bipolar twisted platinum/10% iridium wire (25 μm diameter).

Bipolar stimulating electrodes were placed on afferent fibres from the internal capsule (containing thalamic afferents) or from the external capsule (containing cortical afferents) (Fig.15).

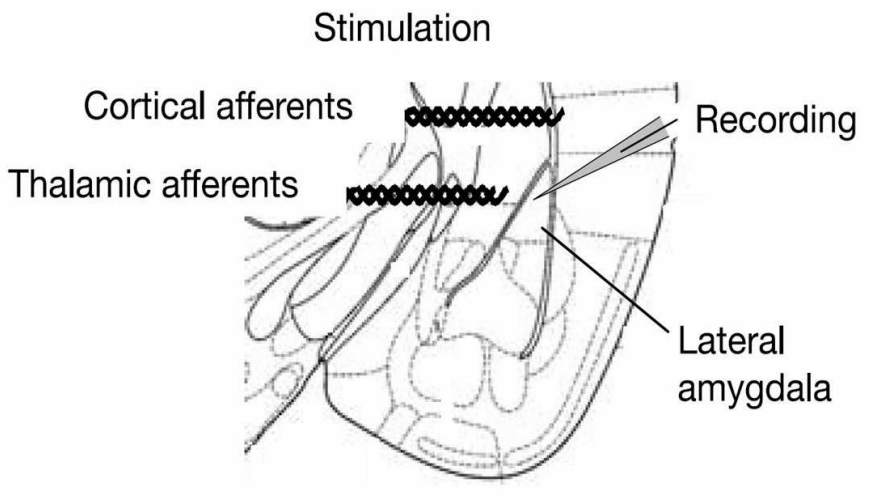

Figure.15) Placement of stimulating and recording electrodes (see text above).

Disynaptic EPSC-IPSC recording: (in mM): 155 K-gluconate, 10 HEPES, 10 phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP , 0.5 KCl (pH adjusted to 7.25 with KOH, 280-285 mOsm). LTP experiments were performed in the presence of NMDA receptor antagonist 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP; 10 μM).

LTP induction protocol

To mimic the physiological activity of converging thalamic and cortical afferents during fear conditioning (Quirk et al. 1997; Rosenkranz and Grace 2002), both afferents were stimulated simultaneously or separately for 1.5 s at an average frequency of 30 Hz using two different stimulation protocols containing Poisson-distributed stimuli (Fig. 16).

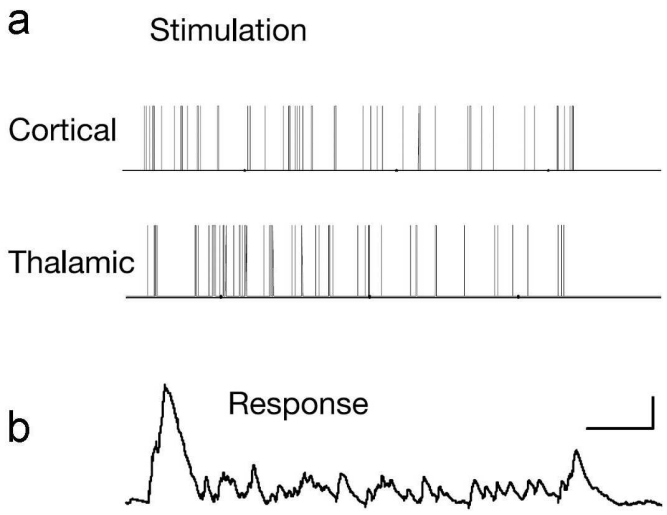

Figure 16) Random stimulation protocol. A) Poisson-train stimulation used for LTP induction. Each train consisted of 45 stimuli at an average frequency of 30 Hz. B) The trace shows a typical postsynaptic response. Scale bars, 10 mV and 250 ms

4.3. Data analysis

Data were acquired and analyzed with: pClamp9.0 (Axon Instruments, Union City, CA, USA), Mini Analysis Program (Synaptosoft, Decatur, GA, USA), and the LTP Program (W. Anderson, University of Bristol, UK)1. Poisson-trains were generated using custom software obtained from N.Buchs (University of Bern, Switzerland). Data were recorded with an Axopatch200B, filtered at 2 kHz and digitized at 10 kHz. Series resistance was monitored throughout the experiments by applying a hyperpolarizing pulse, and if it changed more than 15%, the data were not included in the analysis.

LTP was quantified for statistical comparisons by normalizing and averaging EPSP slopes during the last 10 min of experiments relative to 5–10 min of baseline. Depicted traces show averaged EPSPs or EPSCs for 2 min of baseline and 2 min of LTP (20–25 min after pairing). All values are expressed as means s.e.m. Statistical comparisons were done with paired or unpaired Student's t-test as appropriate (two-tailed $P < 0.05$ was considered significant).

4.4. Behavioral experiments

Adult male Balb/c mice were purchased from RCC, Novartis, Basel. The animals were individually housed in plastic cages with ad-lib access to food and water with a 12/12-h light/dark cycle. All studies took place during the light portion of the cycle. Mice were handled gently for 2-3 min/day during 5 days, to minimize nonspecific stress. Animal care, and behavioral tests were conducted in accordance with the Veterinary Authority of Basel-Stadt, Switzerland

Fear conditioning and testing took place in two different context (Context A and B). Context A consisted of a square transparent Plexiglas box (27 cm side, 80 cm high) with a shock grid floor made of stainless steel rods. The whole system was placed inside a sound-attenuating and temperature-regulated cubicle. A speaker was positioned on the top of the cubicle. The shock grid was connected to a current generator and scrambler to provide a 1 s footshock. The conditioning box and the floor were cleaned with 70 % ethanol before and after each session. Testing was performed in context B, which consists of a circular transparent Plexiglas box (27 cm diameter, 80 cm high) placed inside a sound-attenuating and temperature-regulated cubicle. A speaker was positioned on the top of the cubicle. This context was washed with 1 % acetic acid before and after each session. To maximize discrimination between the two contexts, light intensity was reduced during fear conditioning. The behavior of each mouse was monitored and videotaped during all the phases of the experiment. To score freezing behavior we used an automatic infrared beam detection system placed on the bottom of the experimental chambers (Coulbourn Instruments, Allentown, PA) for which the threshold was set to detect 2 s epochs of freezing behavior.

On the conditioning day, all mice were submitted to a discriminative fear conditioning protocol in which a 30 s tone CS+ (7.5 kHz, 80 dB) was systematically paired to a 1 s footshock US (0.6 or 0.9 mA, 7 CS+/US pairings; inter-trial interval: 20-180 s). The onset of the US coincided with the offset of the CS+. A second unconditioned 30 s tone CS- (3 kHz, 80 dB) was presented after each CS+/US association but was never reinforced (7 CS- presentations, inter-trial interval: 20-180 s). Conditioned mice were tested 24 hr later in context B with 4 presentations of the CS- and the CS+. Mice were allowed to explore the environment freely for 2 minutes before testing. During CS

presentation, a mouse was considered to freeze when it adopted a motionless posture, refraining from all but respiratory movements (Blanchard & Blanchard, 1969). Freezing was scored using a time-sampling procedure. mouse was determined to be freezing or not freezing by an experimenter who was blind to the experimental history of each mouse.
In completion of the fear conditioning experiments, mice were submitted to a series of increasing strength 1-s foot-shocks in context A. (0.1 to 0.8 mA range, 0.1 mA steps). Nociception threshold was calculated by scoring the first noticeable flitching behavior and stress-induced vocalization of animals.

4.5. Drugs

KT2321, Rp-cAMPs and BAPTA-AM were initially dissolved in DMSO and then diluted to its final concentration in ACSF. DMSO concentration in ACSF was 0.1%. All lipophilic PKA inhibitors compound were pre-incubateed with the brain slice one hour before recording. BAPTA-AM was applied together with probenecid (1 mM) and 2-hydroxypropyl-β-cyclodextran (0.7 mM) to prevent extrusion and to stabilize the chelator. BAPTA-AM, Rp-cAMPs, U73122, CPP, CNQX, (S)-(–)-sulpiride, (–)-quinpirole, dihydrexidine, genistein, PP1, SKF38393, and SCH23390 were from Tocris-Cookson (Bristol, UK), TTX from Latoxan (Valence, France). All other drugs were from Fluka/Sigma (Buchs, Switzerland).Baclofen, GS3732, CGP54484, and CGP were kind gift from Dr. Klemens , Novartis

5.1. Presynaptic induction of heterosynaptic associative plasticity in the mammalian brain

Humeau Y, Shaban H, Bissiere S, Lüthi A.

[1]Friedrich Miescher Institute, Maulbeerstrasse 66, CH-4058 Basel, Switzerland
[2]Department of Pharmacology/Neurobiology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

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This work was done in collaboration with Yann Humeau and Stephanie Bissiere. We investigated the associativity and interaction between thalamic and cortical afferents. I had the opportunity to participate with my data, by investigating the effect of increasing the time intervals between the two afferent stimulations. I could show that the change in EPSP slope at cortical afferent synapses was highest when the delay between the two trains was zero and declined with increasing this delay (Fig.21a). Moreover, the highest change was observed with ~30 Hz (Fig. 21b). I also did some investigations to check if the glutamate uptake blocker TBOA would allow LTP induction by stimulation of cortical afferents alone (Fig. 19d). Finally, I did experiments that showed NMDAR-mediated current potentiation after applying LTP_{HA} induction protocol but in the presence of AMPAR antagonist (Fig. 19c).

Summary

The induction of associative synaptic plasticity in the mammalian central nervous system classically depends on coincident presynaptic and postsynaptic activity. According to this principle, associative homosynaptic long-term potentiation (LTP) of excitatory synaptic transmission can be induced only if synaptic release occurs during postsynaptic depolarization. In contrast, heterosynaptic plasticity in mammals is considered to rely on activity-independent, non-associative processes. Here we describe a novel mechanism underlying the induction of associative LTP in the lateral amygdala (LA). Simultaneous activation of converging cortical and thalamic afferents specifically induced associative, *N*-methyl-D-aspartate (NMDA)-

receptor-dependent LTP at cortical, but not at thalamic, inputs. Surprisingly, the induction of associative LTP at cortical inputs was completely independent of postsynaptic activity, including depolarization, postsynaptic NMDA receptor activation or an increase in postsynaptic Ca^{2+} concentration, and did not require network activity. LTP expression was mediated by a persistent increase in the presynaptic probability of release at cortical afferents. Our study shows the presynaptic induction and expression of heterosynaptic and associative synaptic plasticity on simultaneous activity of converging afferents. Our data indicate that input specificity of associative LTP can be determined exclusively by presynaptic properties.

Bipolar stimulating electrodes were placed on afferent fibres from the internal capsule (containing thalamic afferents) (LeDoux 2000; Bauer et al. 2002) or from the external capsule (containing cortical afferents) (Huang and Kandel 1998) in coronal slices prepared from 3–4-week-old male C57BL/6J mice (Fig. 17a). Whole-cell current-clamp recordings were obtained from projection neurons in the dorsolateral portion of the LA (Fig. 17a). Low-frequency baseline stimulation in the presence of the $GABA_A$ receptor antagonist picrotoxin (100 µM) elicited monosynaptic excitatory postsynaptic potentials (EPSPs) of similar amplitudes and slopes at both afferent inputs (thalamic, 5.6 ±0.4 mV, 1.07 ±0.11 mV ms^{-1}; cortical, 5.7 ±0.4 mV, 1.04 ±0.11 mV ms^{-1}; n = 13). To mimic the physiological activity of converging thalamic and cortical afferents during fear conditioning (Quirk et al. 1997; Rosenkranz and Grace 2002), both afferents were stimulated simultaneously for 1.5 s at an average frequency of 30 Hz using two different stimulation protocols containing Poisson-distributed stimuli ('Poisson-train'; Fig. 17b; see Methods). Simultaneous Poisson-train stimulation resulted in the induction of LTP at cortical (151 ±10% of baseline, n = 13, $P < 0.01$), but not at thalamic, afferent synapses (98 ±5%, n = 13, $P > 0.05$; Fig. 17c). Inverting the two stimulation patterns to assess stimulation protocol-specific effects did not affect the input-specific induction of LTP at cortical input synapses (cortical, 152 ±16% of baseline, n = 6, $P < 0.05$; thalamic, 106 ±12%, n = 6, $P > 0.05$). The induction of LTP was associative, in that stimulation of both

the thalamic and cortical afferents was required. Stimulation of either pathway on its own did not induce LTP at cortical afferents (cortical, 106 ±15% of baseline, $n = 6$, $P > 0.05$; thalamic, 101 ±8%, $n = 5$, $P > 0.05$; Fig. 17d), or at thalamic afferents (cortical, 100 ±8% of baseline, $n = 7$, $P > 0.05$; thalamic, 105 ±16%, $n = 5$, $P > 0.05$; see supplementary information), indicating that the stimulation protocols applied were below threshold for the induction of homosynaptic (Huang and Kandel 1998; Weisskopf et al. 1999) and heterosynaptic LTP at cortical afferents.

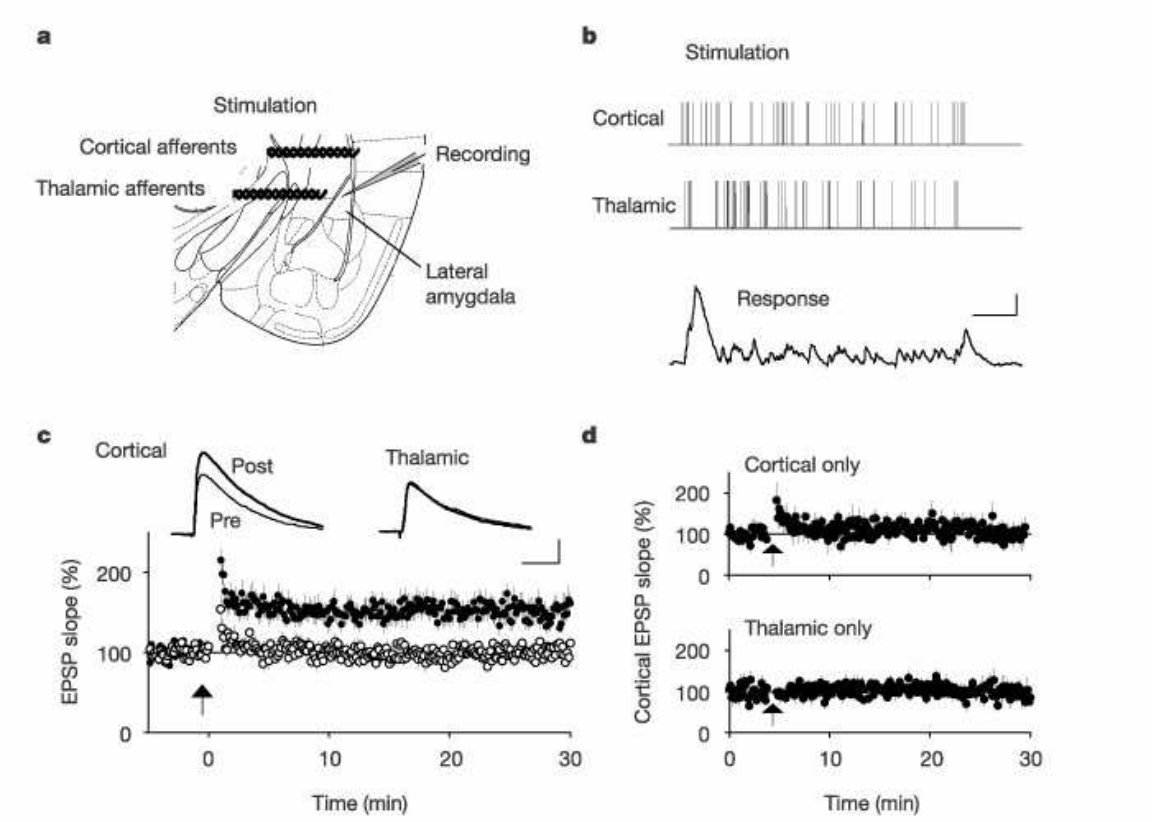

Figure 17) Induction of LTP_{HA} at cortical, but not at thalamic, afferent synapses by simultaneous Poisson-train stimulation of thalamic and cortical afferents. **a**, Placement of stimulating and recording electrodes. **b**, Poisson-train stimulation used for LTP induction. Each train consisted of 45 stimuli at an average frequency of 30 Hz. The trace shows a typical postsynaptic response. Scale bars, 10 mV and 250 ms. **c**, Time course of synaptic changes after simultaneous Poisson-train stimulation (arrow) of cortical (filled circles) and thalamic (open circles) afferents. Scale bars, 2 mV and 50 ms. **d**, Time course of synaptic changes occurring at cortical afferent synapses upon Poisson-train stimulation (arrow) of either cortical or thalamic afferents alone.

Associative LTP in the hippocampus (Bliss and Collingridge 1993) and the amygdala (Huang and Kandel 1998; Bauer et al. 2002) depends largely on the activation of NMDA

receptors and an increase in the postsynaptic Ca2+ concentration. Accordingly, heterosynaptic, associative LTP (LTP_{HA}) at cortical afferents could not be induced in the presence of the competitive NMDA receptor antagonist 3-(((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP) at 20 µM (control, 151 ±10% of baseline, n = 13; CPP, 88 ±8% of baseline, n = 9, P > 0.05; Fig. 18a). To assess whether NMDA receptor activation in conjunction with Poisson-train stimulation of cortical afferents was sufficient for the induction of LTP_{HA}, we applied NMDA locally in the vicinity of the projection neuron from which we were recording by using a pressure application system. Whereas puff-application of NMDA in the absence of cortical afferent activity did not result in the induction of LTPHA (98 ±4% of baseline, n = 5, P > 0.05; Fig. 18b), combining the application of NMDA with Poisson-train stimulation of cortical afferents resulted in a potentiation of cortical afferent synapses (157 ±12% of baseline, n = 4, P < 0.05; Fig. 18b). In contrast, pairing NMDA application with Poisson-train stimulation of thalamic afferents did not induce LTP at thalamic afferents (99 ±10% of baseline, n = 3, P > 0.05; Fig. 18b).

To determine whether an increase in postsynaptic Ca^{2+} concentration was required for LTP_{HA} induction we dialysed the postsynaptic neuron with the Ca^{2+} chelator BAPTA (10–50 mM). Surprisingly, postsynaptic dialysis with BAPTA did not prevent the induction of LTP_{HA} (152 ±17% of baseline, n = 14, P < 0.05; Fig. 18c). Given that activation of NMDA receptors is required for the induction of LTP_{HA}, this finding suggests that they are not located on the postsynaptic neuron or, alternatively, that they can signal in a Ca^{2+}-independent way. To test these possibilities we dialysed the postsynaptic cell with the NMDA receptor open-channel blocker MK-801, and stimulated cortical and thalamic afferents while holding the postsynaptic cell at +30 mV (Berretta and Jones 1996). This procedure completely blocked postsynaptic NMDA receptors (Fig. 18d). However, even the complete blockade of postsynaptic NMDA receptors did not interfere with the induction of LTP_{HA} (134 ±9% of baseline, n = 4, P < 0.05; Fig. 18d). To test whether Ca^{2+} signalling was required, we next incubated the slices with BAPTA-acetoxymethyl ester (BAPTA-AM; 50 µM), a membrane-permeant form of the Ca^{2+} chelator BAPTA. BAPTA-AM completely abolished LTP_{HA} induction (86 ±8% of baseline, n = 7, P > 0.05; Fig. 18e). In conclusion, the induction of LTP_{HA} was

independent of postsynaptic activity including depolarization, postsynaptic NMDA receptor activation and increase in intracellular Ca^{2+} concentration, but still required NMDA receptor activation and Ca^{2+} signalling.

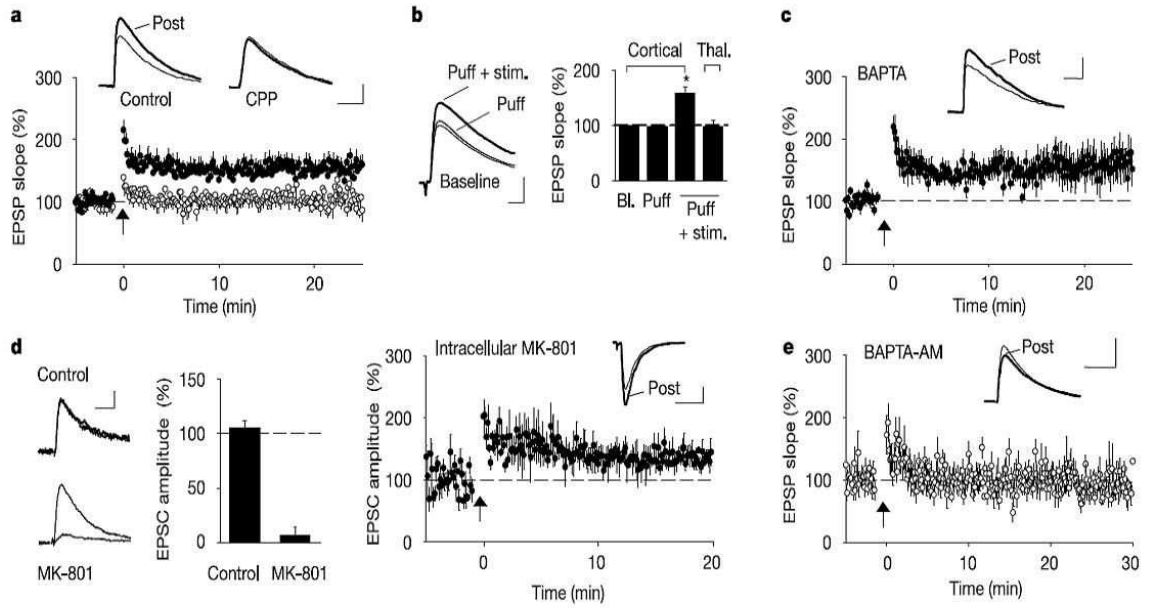

Figure 18) Induction of LTP_{HA} does not depend on postsynaptic activity, but is dependent on NMDA receptor activation and Ca^{2+}. **a**, Induction of LTP_{HA} is blocked in NMDA receptor antagonist CPP (control, same data as in Fig. 1c). Filled circles, control; open circles, CPP. Scale bars, 2 mV and 50 ms. **b**, Left, averaged EPSPs from 2 min of baseline, after pressure application of NMDA (Puff), and 25 min after pairing of NMDA application with cortical afferent stimulation (Puff + stim.). Scale bars, 2 mV and 25 ms. Right, changes in EPSP slope induced by pressure application of NMDA alone, and in conjunction with Poisson-train stimulation of cortical or thalamic afferents. **c**, Induction of LTP_{HA} is independent of increase in postsynaptic Ca^{2+}. Scale bars, 2 mV and 50 ms. **d**, Left, intracellular dialysis with MK-801 blocks NMDA-receptor-mediated EPSCs recorded at +30 mV in the presence of NBQX. Traces show averaged NMDA EPSCs for the first and last five stimulations. Scale bars, 20 pA and 100 ms. Middle, MK-801-induced blockade of NMDA-receptor-mediated EPSCs at cortical and thalamic afferents (pooled data). Right, LTP_{HA} is not affected by blockade of postsynaptic NMDA receptors. Scale bars, 50 pA and 20 ms. **e**, Induction of LTP_{HA} is blocked in the presence of BAPTA-AM. Scale bars, 2 mV and 50 ms.

We considered the possibility that LTP_{HA} induction might represent a network phenomenon involving NMDA receptors located on other neurons within the LA. We therefore sought to decrease network excitability strongly in the LA, a brain structure that is tightly controlled by GABA-mediated inhibition (Lang and Pare 1997; Szinyei et al. 2000), by application of the $GABA_A$ receptor agonist muscimol (5 µM) during LTP induction. Indeed, heterosynaptic forms of plasticity mediated by network activity have been shown to be strongly reduced by activation of $GABA_A$ receptors (Abraham and

Wickens 1991; Scanziani et al. 1996). Muscimol clamped the membrane potential at or near the chloride equilibrium potential (Fig. 19a), thereby preventing action potential initiation in all neurons expressing $GABA_A$ receptors, including projection neurons and local inhibitory interneurons(Farb et al. 1995; Lang and Pare 1998). However, LTP_{HA} induction was not affected by the presence of muscimol (146 ±14% of baseline, $n = 6$, $P < 0.05$; Fig. 19b), but we could not exclude the possibility that some unknown factor released by neurons not expressing $GABA_A$ receptors would be required for the induction of LTP_{HA}. To investigate this further, we strongly suppressed network activity by application of the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(f)quinoxaline; 20 µM). However, even when AMPA-receptor-mediated synaptic transmission was completely blocked, LTP_{HA} could still be induced as monitored by NMDA-receptor-mediated excitatory postsynaptic currents (EPSCs) recorded at +30 mV (122 ±8% of baseline, $n = 7$, $P < 0.05$; Fig. 19c). Thus, most parsimoniously, our data are consistent with the possibility that glutamate released by thalamic afferents might directly activate NMDA receptors located on presynaptic terminals of cortical afferents, a hypothesis supported by electron-microscopic studies indicating the presence of the NMDA receptor subunit NR1 on presynaptic terminals in the LA (Farb et al. 1995; Farb and Ledoux 1999).

If activation of NMDA receptors on presynaptic terminals of cortical afferents underlies the induction of LTP_{HA}, this raises the question why Poisson-train stimulation of cortical afferents alone does not induce LTP. One possible explanation is that glutamate released at cortical afferents would be rapidly cleared by glutamate uptake. Indeed, we found that a single Poisson-train stimulation of cortical afferents was able to induce LTP in the presence of a low concentration of the glutamate uptake blocker TBOA (D,L-*threo*-β-benzyloxyaspartate; 20 µM; 133 ±9% of baseline, $n = 5$, $P < 0.05$; Fig. 19d). To assess whether the facilitation of LTP induction at cortical afferents in the presence of TBOA was due to the activation of presynaptic NMDA receptors by increased ambient glutamate levels, we checked whether TBOA affected the presynaptic properties of cortical afferents (see below) or spontaneous excitatory network activity. However, TBOA did not significantly affect paired-pulse facilitation (PPF) at cortical afferents

(control, 1.35 ±0.15; TBOA, 1.44 ±0.22; $n = 3$, $P > 0.05$) or the frequency of spontaneous EPSPs (control, 3.0 ±0.7 Hz; TBOA, 3.2 ±1.1 Hz; $n = 5$, $P > 0.05$).

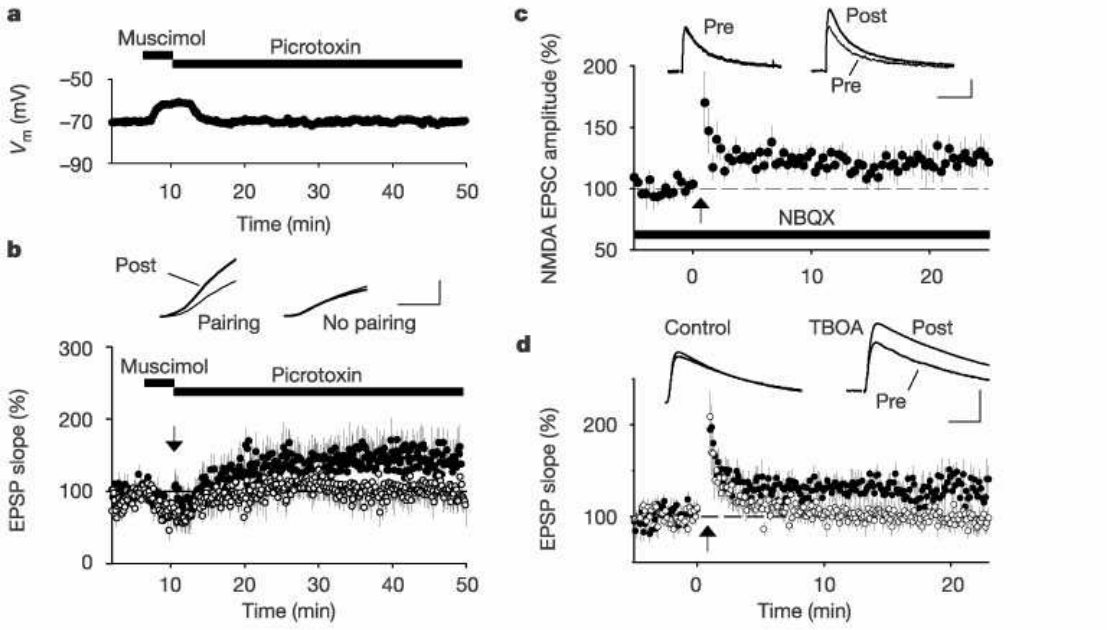

Figure 19) Network activity is not required for the induction of LTP_{HA}. **a**, Average time course of the membrane potential (V_m) during muscimol application. **b**, Induction of LTP_{HA} in the presence of muscimol. Time course of cortical EPSP slope after Poisson-train stimulation and in the absence of Poisson-train stimulation. Picrotoxin was used to terminate shunting induced by tonic $GABA_A$ receptor activation. Filled circles, pairing; open circles, no pairing. Scale bars, 1.3 mV and 2 ms. **c**, Induction of LTP_{HA} in the presence of NBQX. The plot shows a time course of NMDA-receptor-mediated EPSCs at cortical afferents recorded at +30 mV. Scale bars, 20 pA and 100 ms. **d**, Induction of homosynaptic LTP at cortical afferents in the presence of the glutamate uptake blocker TBOA. Open circles, control; filled circles, TBOA. Scale bars, 3 mV and 25 ms.

Given the induction mechanism of LTP_{HA}, we reasoned that thalamic afferent stimulation should affect presynaptic function of cortical afferents in an NMDA-receptor-dependent manner. We therefore compared PPF (Hess et al. 1987) in response to double stimulation of cortical afferents (inter-stimulus interval 50 ms) before and after tetanic stimulation (45 stimuli at 30 Hz) of thalamic afferents. Tetanic stimulation of thalamic afferents resulted in a transient decrease in PPF at cortical afferents (82 ±6% of baseline, $n = 7$, $P < 0.05$; Fig. 20a–c). In contrast, tetanic stimulation of cortical afferents did not affect PPF at thalamic afferents (104 ±4% of baseline, $n = 5$, $P > 0.05$; Fig. 20c). Furthermore, the decrease in cortical PPF after thalamic afferent stimulation was completely abolished by the NMDA receptor antagonist CPP (20 µM; 107 ±5% of baseline, $n = 6$, $P > 0.05$; Fig.

20a, c). These results indicate that repeated stimulation of thalamic afferents transiently increases the probability of release (P_r) at cortical afferents in an NMDA-receptor-dependent manner and that the expression of LTP_{HA} might involve a more persistent increase in P_r at cortical synapses. Indeed, LTP_{HA} was associated with a persistent decrease in PPF (81 ±5% of baseline, $n = 6$, $P < 0.05$; Fig. 20d). Moreover, if LTP_{HA} expression were mediated by an increase in P_r, we reasoned that it should be occluded at high initial P_r. Therefore; we increased P_r by increasing the extracellular Ca^{2+} concentration from 2 to 8 mM. This induced an increase in EPSP amplitude to 175 ±19% ($n = 8$, $P < 0.05$) and occluded the further induction of LTP_{HA} after adjusting EPSP amplitude to control values (111 ±11% of baseline, $n = 5$, $P > 0.05$). Finally, using analysis of fluctuations of the postsynaptic response amplitude, we determined the quantal parameters modified upon induction of LTP_{HA} (Humeau et al. 2002; Tsvetkov et al. 2002). The plot of 1/(c.v.) (Bliss and Collingridge 1993) (where c.v. is the coefficient of variation) against mean response amplitude shows that LTP_{HA} induction fits best with an increase in P_r (Fig. 20e). Finally, because 1/(c.v.) (Bliss and Collingridge 1993) is independent of the quantal amplitude (q), we directly assessed possible changes in q by monitoring the amplitude of asynchronously released quanta in the presence of Sr^{2+} under control conditions and after the induction of LTP_{HA}. The amplitude of stimulation-induced miniature EPSCs at cortical afferents was not significantly affected by the induction of LTP_{HA} (control, -6.7 ±0.2 pA; LTP, -7.4 ±1.1 pA; $n = 4$, $P > 0.05$). Because homosynaptic LTP at cortical afferent synapses has previously been shown to be mediated by a presynaptic increase in P_r (Huang and Kandel 1998; Tsvetkov et al. 2002), our results indicate that heterosynaptic LTP_{HA} could share the same expression mechanism. Indeed, we found that the prior induction of homosynaptic LTP by pairing afferent stimulation at 2 Hz with postsynaptic depolarization (Huang and Kandel 1998; Tsvetkov et al. 2002), occluded the subsequent induction of LTP_{HA} using Poisson-train stimulation (Fig.21d,e). However, in contrast to the induction of homosynaptic LTP, which depends on postsynaptic NMDA receptor activation and can be blocked by postsynaptic BAPTA (30 mM; 103 ±10% of baseline, $n = 5$) (Huang and Kandel 1998; Tsvetkov et al. 2002), LTP_{HA} is induced and expressed presynaptically.

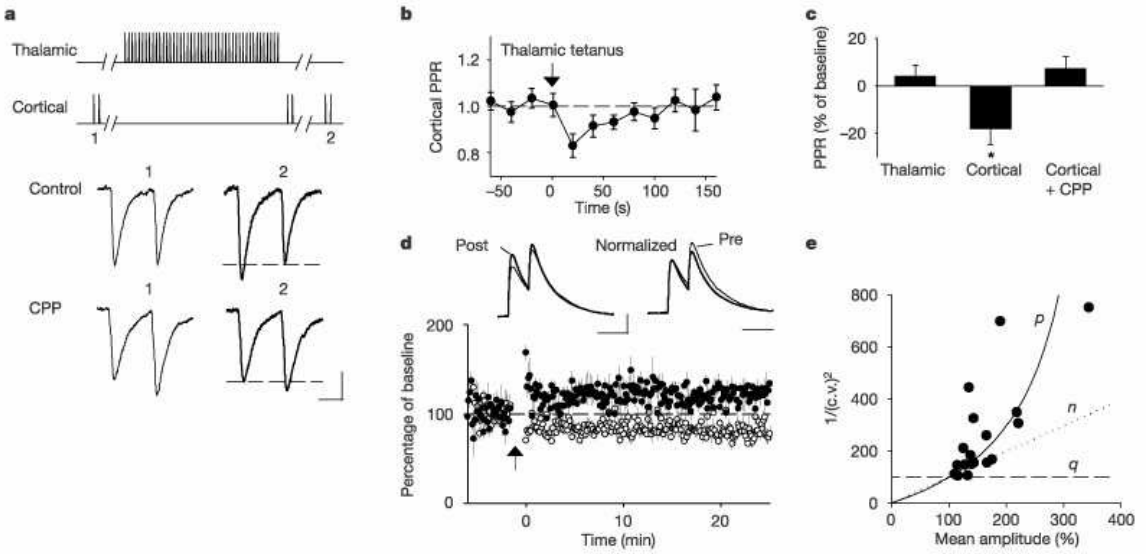

Figure 20) Heterosynaptic, NMDA-receptor-dependent increase of release probability at cortical afferents after stimulation of thalamic afferents. **a**, Top, stimulation protocols. Bottom, EPSCs recorded in response to paired-pulse stimulation (50-ms inter-stimulus interval) of cortical afferents before (point 1) and 10 s after (point 2) tetanic stimulation of thalamic afferents in control conditions or in the presence of CPP. Dashed lines represent the initial amplitude (point 1) of the first response before thalamic stimulation. Scale bars, 40 pA and 20 ms. **b**, Average time course of PPF changes at cortical afferent synapses after tetanic stimulation of thalamic afferents. **c**, PPF changes at thalamic and at cortical afferents after tetanic stimulation of the other input. **d**, Average time course of cortical EPSP slope (filled circles) and PPF (open circles) after induction of LTP_{HA}. Scale bars, 2.5 mV and 75 ms. **e**, Variance analysis of EPSP amplitude fluctuations illustrating that LTP_{HA} expression fits best with an increase in the probability of release (*p*).

The physiological relevance of LTP_{HA} for fear learning is supported by two observations. First, thalamic and cortical afferents to the lateral amygdala are simultaneously active during fear conditioning and can interact (LeDoux 2000; Doyere et al. 2003). Second, electrophysiological experiments *in vivo* show that postsynaptic hyperpolarization does not completely abolish LTP induced by pairing of sensory stimulation with foot-shocks (Rosenkranz and Grace 2002)suggesting that LTP induction independent of postsynaptic activity does occur *in vivo*. The physiological stimulation patterns used in this study suggest that LTP_{HA} might be induced on subthreshold activity elicited by simultaneous sensory input by means of thalamic and cortical afferents. LTP_{HA} might therefore serve as a priming mechanism to increase the impact of selective cortical afferents on the subsequent induction of homosynaptic hebbian plasticity at neighbouring synapses, which requires stronger afferent activity and/or the induction of postsynaptic action

potentials (Markram et al. 1997; Huang and Kandel 1998; Weisskopf et al. 1999; Blair et al. 2001; Bissiere et al. 2003)

Our study indicates that the input specificity of associative LTP can be entirely determined by presynaptic properties. Heterosynaptic associative modifications of synaptic efficacy add a level of complexity to the classical hebbian forms of synaptic plasticity, and open a new perspective for understanding integrative processes between converging afferent pathways in the mammalian central nervous system.

Methods

Coronal slices from 3–4-week-old male C57BL/6J mice were prepared as described (Bissiere et al. 2003). Slices were maintained for 45 min at 35 °C in an interface chamber containing artificial cerebrospinal fluid equilibrated with 95% O2/5% CO2 and containing (in mM): 124 NaCl, 2.7 KCl, 2 CaCl2, 1.3 MgCl2, 26 NaHCO3, 0.4 NaH2PO4, 10 glucose, 4 ascorbate; and then kept for at least 45 min at 21–25 °C before being transferred to a superfusing recording chamber. Whole-cell recordings were performed at 30–32 °C. Neurons were identified visually with infrared videomicroscopy. Patch electrodes (3–5 MΩ) were normally filled with a solution containing (in mM): 120 potassium gluconate, 20 KCl, 10 HEPES, 10 phosphocreatine, 4 Mg-ATP, 0.3 Na-GTP, pH 7.25, 295 mOsM. All experiments were performed in the presence of picrotoxin (100 µM) unless indicated otherwise. Monosynaptic EPSPs exhibiting constant 10–90% rise times and latencies were elicited by stimulation of afferent fibres with a bipolar twisted platinum/10% iridium wire (25 µm diameter). LTP was quantified for statistical comparisons by normalizing and averaging EPSP slopes during the last 10 min of experiments relative to 5–10 min of baseline. Depicted traces show averaged EPSPs or EPSCs for 2 min of baseline and 2 min of LTP (20–25 min after pairing). All values are expressed as means ±s.e.m. Statistical comparisons were done with paired or unpaired Student's t-test as appropriate (two-tailed $P < 0.05$ was considered significant). For details on experimental conditions and analysis see Supplementary Information.

Supplementary Methods

Data were acquired and analyzed with: pClamp9.0 (Axon Instruments, Union City, CA, USA), Mini Analysis Program (Synaptosoft, Decatur, GA, USA), and the LTP Program (W. Anderson, University of Bristol, UK)1. Poisson-trains were generated using custom software obtained from N.Buchs (University of Bern, Switzerland). Data were recorded with an Axopatch200B, filtered at 2 kHz and digitized at 10 kHz. Series resistance was monitored throughout the experiments by applying a hyperpolarizing pulse, and if it changed more than 15%, the data were not included in the analysis. Amplitudes of postsynaptic responses can be described as the binomial distribution of three parameters2 (n: number or active release sites; Pr: average release probability; q: amplitude of miniature postsynaptic responses). Accordingly, mean EPSP amplitude EPSPmean = n*Pr*q and the variance of the fluctuations in EPSP amplitude is Var = n*Pr*[1 - Pr]*q2. EPSPmean and Var were determined from EPSPs recorded during expression of LTPHA. The factor 1/CV2 = EPSPmean2/Var was calculated. 1/CV2 = n*Pr/(1 – Pr). This expression is independent of q, evolves linearly when n is changed and is a curve when Pr is changed. Computed reference lines are drawn in Fig. 20e. Mean amplitude (Amean) and CV2 values were obtained by analysis of 10 min epochs (80 events) before, and 20 min after induction of LTP_{HA}.

LTP was quantified for statistical comparisons by normalizing and averaging EPSP slopes during the last 10 min of experiments relative to 5–10 min of baseline. Depicted traces show averaged EPSPs or EPSCs for 2 min of baseline and 2 min of LTP (20–25 min after pairing). All values are expressed as means s.e.m. Statistical comparisons were done with paired or unpaired Student's t-test as appropriate (two-tailed $P < 0.05$ was considered significant).

Experimental conditions: For experiments using pressure application of NMDA (Poisson stimulation for 1.5 s; average frequency: 30 Hz; each pulse was 20 psi; duration 6 ms), a patch pipette (containing 1 mM NMDA and 20 µM glycine in extracellular perfusion medium) was placed near the postsynaptic cell. Experiments using pressure application of

NMDA were performed in the presence of 10 mM postsynaptic BAPTA to prevent depolarization-induced plasticity. When applied externally, BAPTA-AM was initially dissolved in DMSO and then diluted to its final concentration in ACSF. DMSO concentration in ACSF was 0.1%. BAPTA-AM was applied together with probenecid (1 mM) and 2-hydroxypropyl-β-cyclodextran (0.7 mM) to prevent extrusion and to stabilize the chelator. Control experiments showed that DMSO, probenecid, and cyclodextran did not affect LTP induction at the above concentrations. Application of BAPTA-AM initially reduced synaptic release by 32 ± 6% (n = 7). However, after synaptic responses had stabilized and stimulation intensity had been adjusted to evoke an EPSP of similar amplitude than under control conditions, BAPTA-AM did not significantly affect baseline synaptic transmission during the time course of an LTP experiment (11 ± 15% decrease during the experiment; n = 3; $P > 0.05$).

Induction of associative LTP in the lateral amygdala (LA): The induction of associative synaptic plasticity in the mammalian central nervous system classically depends on coincident presynaptic and postsynaptic activity (Gustafsson 1990; Bliss and Collingridge 1993). According to this principle, associative homosynaptic long-term potentiation (LTP) of excitatory synaptic transmission can be induced only if synaptic release occurs during postsynaptic depolarization(Gustafsson 1990; Bliss and Collingridge 1993). In contrast, heterosynaptic plasticity in mammals is considered to rely on activity-independent, non-associative processes (Bailey et al. 2000; Nishiyama et al. 2000).

The changes in EPSP slope at cortical afferent synapse was highest when the delay between the two train is zero and decline with increasing this delay (Fig. 21a)

The highest change was also observed with ~30 HZ (Fig.21 b), and by using 30 HZ tetani and changing the number of stimuli within the tetani; the highest changes observed was with around 45 stimuli (Fig.21c). Induction of LTP_{HA} was occluded by prior induction of homosynaptic LTP induced by pairing postsynaptic depolarization with afferent stimulation at 2 Hz (Fig.21d, e).

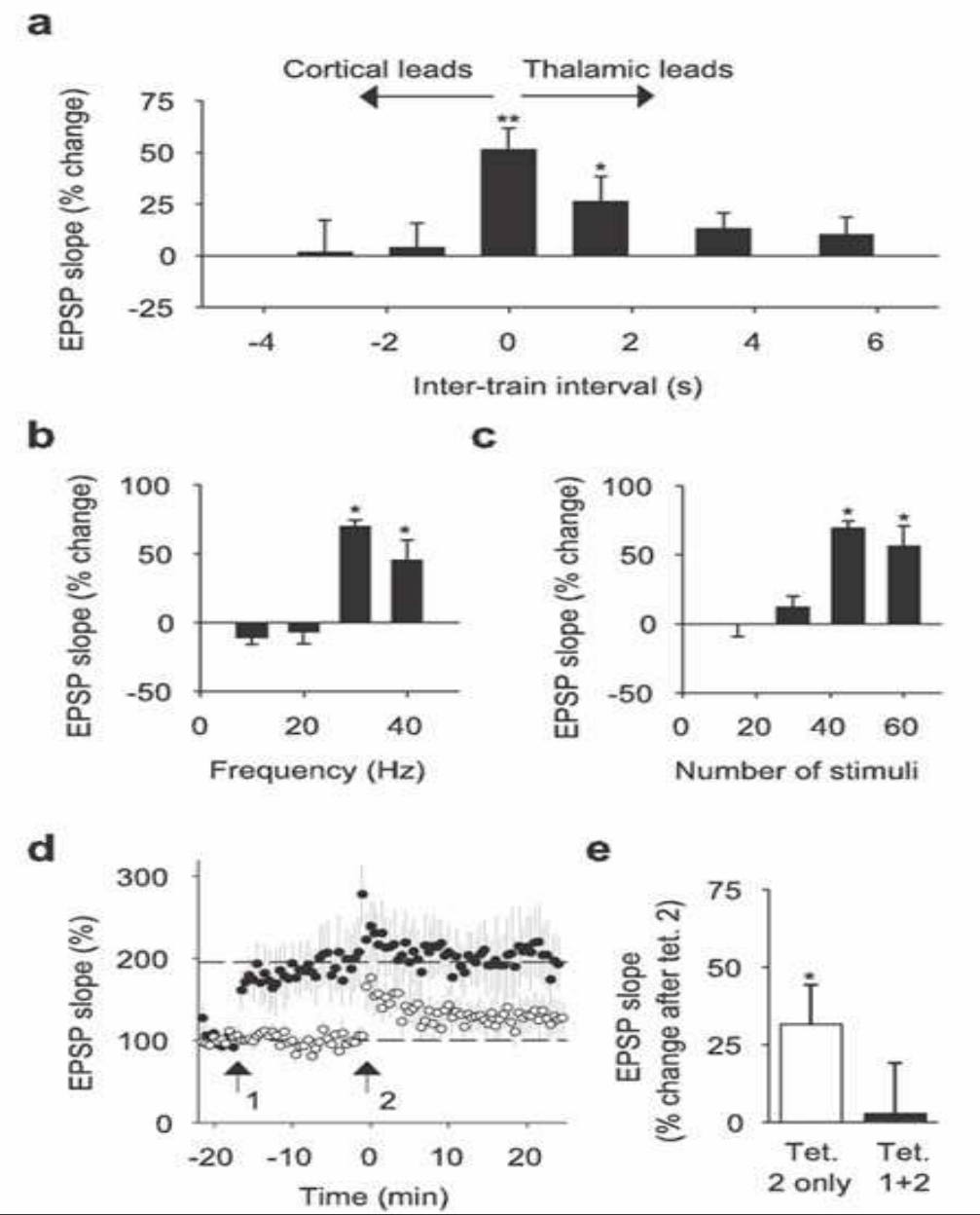

Figure 21) (a) Summary graph illustrating changes in EPSP slope at cortical afferent synapses induced by combined Poisson-train stimulation of cortical and thalamic afferents with various delays between the start of the cortical and thalamic train (-3.5 s: $n = 10$; -1.5 s: $n = 5$; 0 s: $n = 13$; 1.5 s: $n = 5$; 3.5 s: $n = 7$; 5.5 s: $n = 12$). (**b**) Changes in EPSP slope at cortical afferent synapses induced by combined tetanic stimulation of cortical and thalamic afferents at various frequencies (10 Hz: $n = 5$; 20 Hz: $n = 8$; 30 Hz: $n = 7$; 40 Hz: $n = 5$). (**c**) Changes in EPSP slope at cortical afferent synapses induced by combined stimulation of cortical and thalamic afferents with tetani containing increasing numbers of stimuli at 30 Hz (15 stim.: $n = 3$; 30 stim.: $n = 4$; 45 stim.: $n = 4$; 60 stim: $n = 3$). (**d, e**) Induction of LTP_{HA} (tet. 2) is occluded by prior induction of homosynaptic LTP induced by pairing postsynaptic depolarization with afferent stimulation at 2 Hz (tet. 1; filled symbols; $n = 5$). Controls (open symbols) illustrate the induction of LTP_{HA} using combined Poisson-train stimulation of cortical and thalamic afferents without prior induction of homosynaptic LTP ($n = 9$). $P^* < 0.05$, $P^{**} < 0.01$.

5.2. $GABA_{B(1a)}$ heteroreceptors modulate associative properties of presynaptic LTP and learning

Hamdy Shaban[1],Yann Humeau[1,2], Cyril Herry[1], Stephane Ciocchi[1], Gilles Sansig[3], Samuel Barbieri[4], Herman van der Putten[3], Bernhard Bettler[4], and Andreas Lüthi[1]

[1]Friedrich Miescher Institute, CH-4058 Basel, Switzerland, [2]UPR2356, CNRS, F-67084 Strasbourg, France, [3]Novartis Institutes for Biomedical Research, Novartis Pharma AG, CH-4002 Basel, Switzerland, [4]Pharmazentrum, Department of Clinical-Biological Sciences, University of Basel, CH-4056 Basel, Switzerland

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This work was done in collaboration with Yann Humeau who contributed some electrophysiological experiments. Cyril Herry and Stephane Ciocchi did the behavioral investigations. Genotyping was done by Gilles Sansig.

5.2.1. Summary

Classical fear conditioning in rodents, a simple form of associative learning, is thought to induce N-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation (LTP) at thalamic and cortical sensory afferents converging on projection neurons in the lateral amygdala (LA). LTP at cortical afferents can be induced presynaptically by associative co-activation of thalamic and cortical afferents in vitro. We show here that the associativity of cortical afferent LTP is regulated by presynaptic $GABA_B$ heteroreceptors. Using whole-cell recordings from LA projection neurons in acute mouse brain slices we found that pharmacological $GABA_B$ receptor blockade facilitated the induction of homosynaptic, non-associative cortical afferent LTP independent of simultaneous thalamic afferent activity and NMDA receptor activation. Moreover, by taking a genetic approach in mice, we demonstrate that presynaptic inhibition at cortico-amygdala afferents is specifically mediated by $GABA_{B(1a)}$ receptors. Strikingly, this facilitation of non-associative LTP induction was accompanied, at the behavioral level, with a generalization of conditioned fear to non-conditioned stimuli. Thus, our findings indicate that the

specificity of information processing in the LA is controlled by presynaptic inhibition mediated by specific $GABA_B$ receptors.

5.2.2. Introduction

Induction of associative NMDA receptor-dependent LTP in the LA is thought to underlie the acquisition of classical fear conditioning in rodents (LeDoux 2000; Maren and Quirk 2004; Rumpel et al. 2005). Projection neurons in the LA receive converging thalamic and cortical sensory afferents that are simultaneously active during sensory experience (LeDoux 2000). While LTP at thalamic afferents is induced and expressed postsynaptically (Weisskopf et al. 1999; Bissiere et al. 2003; Humeau et al. 2005; Rumpel et al. 2005), we have recently shown that associative co-activation of thalamic and cortical afferents induces presynaptic LTP at cortical afferents involving the heterosynaptic activation of presynaptic NMDA receptors (Humeau et al. 2003). Thus, by means of presynaptic NMDA receptors, cortical afferents are able to detect and integrate coincident activity of neighboring excitatory inputs. Given that postsynaptic integration of excitatory transmission in the LA is tightly controlled by local GABAergic circuits (Sugita et al. 1993; Li et al. 1996; Lang and Pare 1997; Bissiere et al. 2003) this raises the question whether presynaptic integration is also subject to inhibitory control.

Presynaptic GABAergic inhibition of excitatory synaptic transmission can be mediated by ionotropic $GABA_A$ receptors (Kullmann et al. 2005) and metabotropic $GABA_B$ receptors (Thompson et al. 1993). In the LA, $GABA_A$ receptor-mediated inhibition plays a major role in postsynaptic integration (Sugita et al. 1993; Li et al. 1996; Lang and Pare 1997). Accordingly, induction of postsynaptic LTP at thalamic afferents is facilitated by $GABA_A$ receptor blockade (Bissiere et al. 2003). Presynaptic LTP at cortical afferents, however, is insensitive to $GABA_A$ receptor-mediated inhibition (Humeau et al. 2003), suggesting that presynaptic $GABA_A$ receptors do not play a major role at cortico-amygdala afferents. Indeed, there is accumulating evidence suggesting a role for $GABA_B$ receptors in regulating amygdala-dependent fear and anxiety behavior (Cryan and Kaupmann 2005). However, whereas the role of $GABA_B$ heteroreceptors for presynaptic inhibition and integration at glutamatergic synapses is well documented in other brain

areas (Thompson et al. 1993; Dittman and Regehr 1997; Vogt and Nicoll 1999), nothing is known about their role in the LA.

Functional $GABA_B$ receptors are generally thought to be heterodimers containing $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits (Schuler et al. 2001; Bettler et al. 2004; Gassmann et al. 2004). The $GABA_{B(1)}$ subunit exists in two differentially expressed isoforms, $GABA_{B(1a)}$ and $GABA_{B(1b)}$ differing by the presence of two N-terminal "sushi" domains in the $GABA_{B(1a)}$ isoform (for review see: (Bettler et al. 2004)). In the LA, $GABA_B$ receptors are expressed at moderate to high levels (Bischoff et al. 1999; Fritschy et al. 1999; McDonald et al. 2004), and can be activated by afferent stimulation *in vitro* and *in vivo* (Sugita et al. 1993; Li et al. 1996; Lang and Pare 1997). These studies, however, focused on the activation of postsynaptic $GABA_B$ receptors that modulate synaptic transmission by activating postsynaptic inwardly rectifying Kir3-type K^+ channels giving rise to a slow inhibitory postsynaptic potential (Luscher et al. 1997). To study the role of $GABA_B$ heteroreceptors in presynaptic integration at cortical afferents to the LA we have used a combined pharmacological and genetic approach in mice. We find that presynaptic $GABA_B$ heteroreceptors, predominantly comprised of $GABA_{B(1a)}$ subunits, critically determine associative properties of presynaptic cortical LTP. In the absence of functional presynaptic $GABA_B$ heteroreceptors, an NMDA receptor-independent, non-associative form of presynaptic LTP is unmasked. Strikingly, the loss of associativity of cortico-amygdala LTP is accompanied by a generalization of conditioned fear at the behavioral level. This indicates that the specificity of information processing in the LA is controlled by presynaptic inhibition mediated by specific $GABA_B$ receptors.

5.2.3. Methods

Electrophysiology. Standard procedures were used to prepare 350 µm thick coronal slices from three to four week old male C57BL/6J or BALB/c mice following a protocol approved by the Veterinary Department of the Canton of Basel-Stadt (Humeau et al. 2003). Briefly, the brain was dissected in ice-cold artificial cerebrospinal fluid (ACSF), mounted on an agar block and sliced with a Dosaka vibratome (Kyoto, Japan) at 4ºC. Slices were maintained for 45 min at 35ºC in an interface chamber containing ACSF

equilibrated with 95% O_2/5% CO_2 and containing (in mM): 124 NaCl, 2.7 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 0.4 NaH_2PO_4, 18 glucose, 4 ascorbate, and then for at least 45 min. at room temperature before being transferred to a superfusing recording chamber. Whole-cell recordings from LA projection neurons were performed at 30-32ºC in a superfusing chamber. Neurons were visually identified with infrared videomicroscopy using an upright microscope equipped with a x40 objective (Olympus). Patch electrodes (3-5 MΩ) were pulled from borosilicate glass tubing and normally filled with a solution containing (in mM): 120 K-gluconate, 20 KCl, 10 HEPES, 10 phosphocreatine, 4 Mg-ATP, and 0.3 Na-GTP (pH adjusted to 7.25 with KOH or CsOH, respectively, 295 mOsm). For voltage-clamp experiments K-gluconate was replaced by equimolar Cs-gluconate. All experiments were performed in the presence of picrotoxin (100 μM) unless indicated otherwise. In current-clamp recordings membrane potential was kept manually at –70 mV (not corrected for junction potentials). Data were recorded with an Axopatch200B, filtered at 2 kHz and digitized at 10 kHz. In all experiments, series resistance was monitored throughout the experiment by applying a hyperpolarizing current or voltage pulse, and if it changed by more than 15%, the data were not included in the analysis. Data were acquired and analyzed with ClampEx9.0 and ClampFit9.0 (Axon Instruments, CA). Monosynaptic EPSPs or EPSCs exhibiting constant 10-90% rise times and latencies were elicited by stimulation of afferent fibers with a bipolar twisted platinum/10% iridium wire (25 μm diameter). LTP was induced by delivering Poisson-distributed 45 stimulations at an average frequency of 30 Hz (Humeau et al. 2003). LTP was quantified for statistical comparisons by normalizing and averaging EPSP slopes during the last 5 min of experiments relative to 5 min of baseline. Depicted traces show averaged EPSPs for 2 min of baseline and 2 min of LTP (25-30 min after pairing). All values are expressed as means ± s.e.m. Statistical comparisons were done with paired or unpaired Student's *t*-test as appropriate (two-tailed $P < 0.05$ was considered significant).

Behavior. Adult male wild-type and mutant BALB/c mice were individually housed in plastic cages with ad-lib access to food and water with a 12/12-h light/dark cycle. All studies took place during the light portion of the cycle. Mice were handled gently for 2-3 min/day during 5 days, to minimize nonspecific stress. Fear conditioning and testing took place in two different contexts (Context A and B). The conditioning and testing boxes

and the floor were cleaned with 70% ethanol, or 1% acetic acid before and after each session, respectively. To score freezing behavior an automatic infrared beam detection system placed on the bottom of the experimental chambers (Coulbourn Instruments, Allentown, PA) was used. The animals were considered freezing if no movement was detected for 2 s. Mice were submitted to a discriminative fear conditioning protocol in which a 30 s tone conditioned stimulus (CS^+)(7.5 kHz, 80 dB) was systematically paired to a 1 s foot-shock (unconditioned stimulus; US) (0.9 mA, 7 CS^+/US pairings; inter-trial interval: 20-180 s). The onset of the US coincided with the offset of the CS^+. A second non-conditioned 30 s tone (CS^-; 3 kHz, 80 dB) was presented after each CS^+/US association but was never reinforced (7 CS^- presentations, inter-trial interval: 20-180 s). Conditioned mice were tested 24 h later in context B with 4 presentations of the CS^- and the CS^+. To determine pain sensitivity mice were submitted to a series of increasing strength 1 s foot-shocks in context A (0.1 to 0.8 mA range, 0.1 mA steps). Nociception threshold was calculated by scoring the first noticeable flinching behavior and stress-induced vocalization.

Reagents. BAPTA, BAPTA-AM, CPP, and NBQX were from Tocris-Cookson (Bristol, UK). CGP55845A and GS3732 were a gift from Novartis Pharma AG (Basel, Switzerland). All other drugs were from Fluka/Sigma (Buchs, Switzerland).

5.2.4. Results

$GABA_B$ receptor blockade facilitates non-associative homosynaptic LTP. Whole-cell current clamp recordings from projection neurons showing spike frequency adaptation upon depolarizing current injection were obtained in the dorsal subdivision of the LA (Weisskopf et al. 1999; Bissiere et al. 2003). Stimulation of afferent fibers from the internal capsule, containing thalamic afferents (Weisskopf et al. 1999), or from the external capsule, containing cortical afferents (Huang and Kandel 1998; Tsvetkov et al. 2002) elicited monosynaptic excitatory postsynaptic potentials (EPSPs) of similar amplitudes and slopes at both inputs. Simultaneous stimulation of cortical and thalamic afferents with a single Poisson-train (45 stimuli at an average frequency of 30 Hz) resulted in the pathway-specific induction of LTP at cortical afferent synapses (cortical:

157 ± 14% of baseline, $n = 11$, $P < 0.05$; thalamic: 101 ± 10% of baseline, $n = 11$, $P > 0.05$)(Fig. 22*B*)(Humeau et al. 2003). Under these conditions, cortical LTP was associative since Poisson-train stimulation of cortical afferents alone (ie in the absence of concomitant thalamic afferent stimulation) did not result in long-lasting changes in synaptic efficacy (105 ± 11% of baseline, $n = 10$, $P > 0.05$)(Fig. 22*C*)(Humeau et al. 2003). The requirement for associative interactions between cortical and thalamic afferent activity during LTP induction was obviated by the application of the specific $GABA_B$ receptor antagonist CGP55485A. In the presence of CGP55845A (10 μM) homosynaptic LTP could be induced in a non-associative manner by a single train delivered to cortical afferents (153 ± 15% of baseline, $n = 6$, $P < 0.05$)(Fig. 22*C*). Like heterosynaptic, associative LTP induced by co-stimulation of both inputs, homosynaptic LTP at cortical afferents was associated with a decrease in paired-pulse facilitation (PPF; 73 ± 6% of baseline, $n = 10$, $P < 0.05$)(Fig. 22*D*) suggesting a presynaptic expression mechanism (Humeau et al. 2003). To directly test whether homosynaptic, non-associative LTP was mediated by the same expression mechanism we performed occlusion experiments. Prior induction of homosynaptic LTP in the presence of a $GABA_B$ receptor antagonist completely occluded the subsequent induction of heterosynaptic, associative LTP ($n = 8$)(Fig. 22*E*). In the reverse experiment, homosynaptic LTP was partially occluded by prior induction of associative LTP ($n = 5$)(Fig. 22*F*) indicating that associative LTP induction requiring NMDA receptor-dependent interactions between thalamic and cortical afferents (Humeau et al. 2003) was more specific and only induced at a subset of the activated inputs. Thus, these experiments show that $GABA_B$ receptor-mediated inhibition serves as a constraint for the induction of homosynaptic, non-associative LTP at cortical afferents, thereby making concomitant thalamic input a necessary requirement for LTP induction.

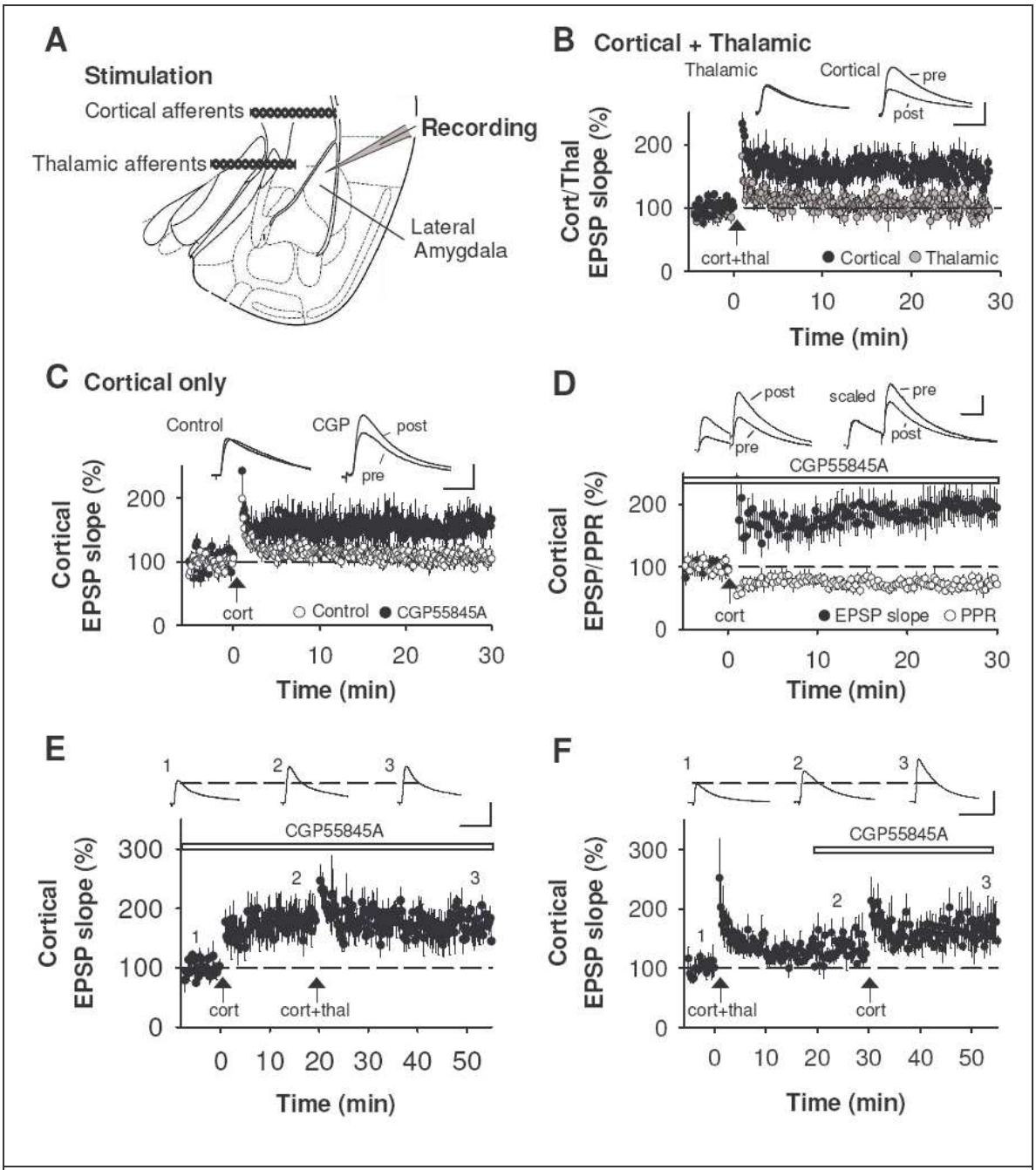

Figure.22) $GABA_B$ receptor blockade enables the induction of homosynaptic, non-associative LTP at cortical afferents to the lateral amygdala. (*A*) Placement of stimulation and recording electrodes. (*B*) Time course of synaptic changes after simultaneous tetanic stimulation of cortical and thalamic afferent fibers. Heterosynaptic, associative LTP is induced at cortical, but not at thalamic afferents ($n = 11$). (*C*) A single tetanus delivered to cortical afferents only does not induce LTP ($n = 10$). In the presence of the $GABA_B$ receptor antagonist CGP55845A (10 μM) homosynaptic, non-associative LTP is induced by the same protocol ($n = 6$). (*D*) Induction of homosynaptic LTP (filled circles) is associated with a decreased paired-pulse ratio (PPR; open circles; $n = 10$). (*E*) Prior induction of homosynaptic LTP occludes subsequent induction of heterosynaptic LTP ($n = 8$). (*F*) The converse experiment reveals a partial occlusion of LTP by prior induction of heterosynaptic LTP ($n = 5$). Scale bars: 4 mV/20 ms.

Mechanisms of presynaptic LTP induction. Since the induction of heterosynaptic, associative LTP depends on the activation of presynaptic NMDA receptors by glutamate released from thalamic afferents, and since efficient glutamate uptake prevents NMDA receptor activation by tetanic stimulation of cortical afferents alone (Humeau et al. 2003), one might predict that homosynaptic LTP should be NMDA receptor-independent. Indeed, application of the competitive NMDA receptor antagonist CPP (20 μM) did not prevent induction of homosynaptic LTP (159 ± 18% of baseline, $n = 7$, $P < 0.05$)(Fig. 23*A*). LTP could even be induced in the presence of the non-specific glutamate receptor antagonist kynurenate (3 mM) (191 ± 27% of baseline, $n = 4$, $P < 0.05$)(Fig. 23*B*). This potentiation was not caused by a rebound after washing out kynurenate, since synaptic transmission returned to baseline levels if no tetanic stimulation was applied (105 ± 17% of baseline, $n = 3$, $P > 0.05$)(Fig. 23*B*). To test if postsynaptic Ca^{2+} signaling was required for the induction of homosynaptic LTP, recorded neurons were loaded with the Ca^{2+} chelator BAPTA (30 mM). This BAPTA concentration completely abolishes postsynaptic LTP induction at thalamic inputs (Humeau et al. 2005). Homosynaptic LTP at cortical inputs, however, was resistant to postsynaptic BAPTA perfusion (157 ± 19% of baseline, $n = 6$, $P < 0.05$)(Fig. 23*C*) indicating a presynaptic induction mechanism. Consistent with this model, we found that, unlike thalamic LTP (Bissiere et al. 2003), homosynaptic cortical LTP could be induced in the absence of the $GABA_A$ receptor antagonist picrotoxin (148 ± 17% of baseline, $n = 6$, $P < 0.05$). To determine if homosynaptic cortical LTP was Ca^{2+}-dependent, we bath applied BAPTA-AM (50 μM), a membrane-permeant form of BAPTA. Stimulation strength was set to compensate for the BAPTA-AM induced reduction in baseline EPSP amplitude. Under these conditions, BAPTA-AM completely abolished LTP induction (96 ± 10% of baseline, $n = 6$, $P < 0.05$)(Fig. 23*D*) indicating a requirement for presynaptic Ca^{2+} signaling during homosynaptic LTP induction.

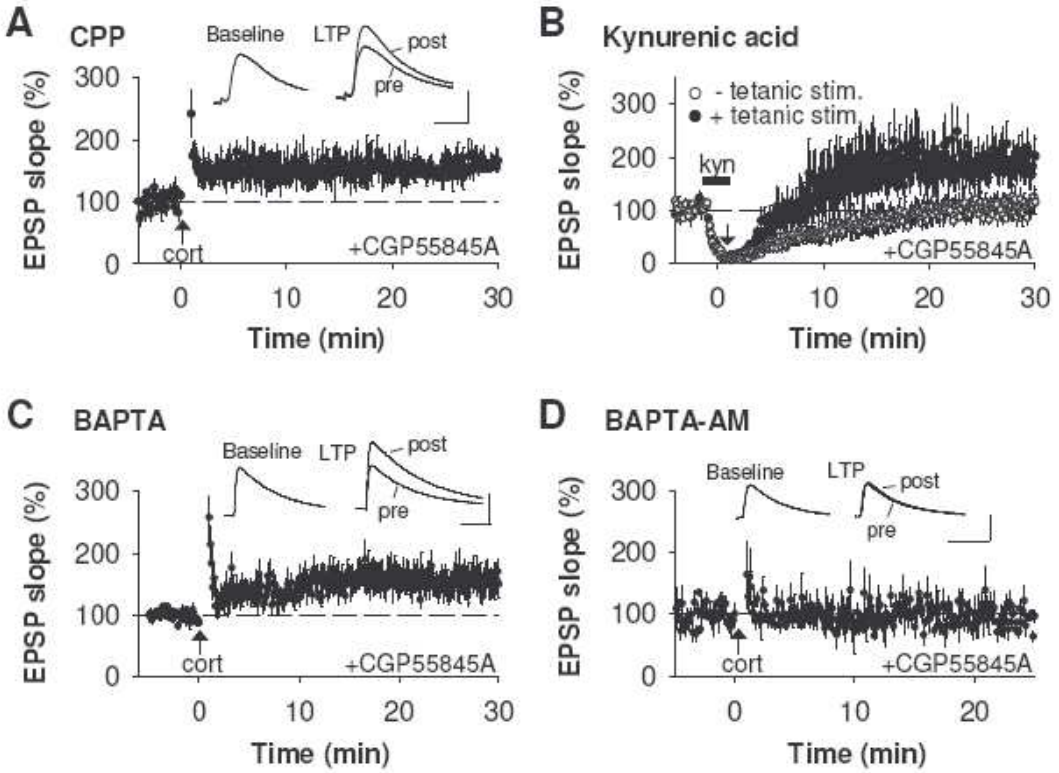

Figure 23) Induction of homosynaptic LTP at cortical afferents is independent of NMDA receptor activation and postsynaptic Ca^{2+}. (*A*) Homosynaptic cortico-amygdala LTP is independent of NMDA receptor activation (n = 7). (*B*) Complete blockade of glutamatergic transmission by kynurenate (3 mM) does not interfere with homosynaptic LTP induction (n = 4; filled symbols). In the absence of tetanic stimulation synaptic responses recover to baseline levels after wash-out of kynurenate (n = 3; open symbols). (*C*) LTP induction is not affected by postsynaptic perfusion with BAPTA (30 mM; n = 6). (*D*) Application of the membrane-permeant BAPTA-AM (50 μM) completely blocks LTP induction (n = 6). Scale bars: 3 mV/20 ms.

Since NMDA receptors could not be the source of presynaptic Ca^{2+} entry, and since L-type voltage-dependent Ca^{2+} channels (L-VDCCs) have been previously shown to play a role during induction of LTP at thalamic (Weisskopf et al., 1999; Bauer et al., 2002; Shinnick-Gallagher et al., 2003; Humeau et al., 2005) and cortical afferents (Tsvetkov et al., 2002; Humeau et al., 2005), we bath applied verapamil (50 μM), a L-VDCC antagonist. Verapamil prevented the induction of homosynaptic cortical LTP (98± 11.6% of baseline, n = 6, $p > 0.05$; Fig. 24A), indicating a role for presynaptic L-VDCCs during LTP induction and/or expression.

Previous reports have demonstrated postsynaptic induction of homosynaptic cortical LTP by much stronger induction paradigms, such as repeated high-frequency tetanic

stimulations (Huang and Kandel 1998; Huang et al. 2000), or pairing protocols (Tsvetkov et al. 2002; Humeau et al. 2003; Tsvetkov et al. 2004; Humeau et al. 2005). Postsynaptically induced LTP, however, occludes further induction of presynaptic LTP by simultaneous thalamic and cortical afferent stimulation (Humeau et al. 2003), and requires activation of protein kinase A (PKA) (Huang and Kandel 1998; Huang et al. 2000). To test if presynaptically induced homosynaptic cortical LTP converged on the same signal transduction pathways we applied the adenylate cyclase (AC) activator forskolin (50 μM). Forskolin application increased excitatory synaptic transmission at cortical afferents (197 ± 16.8% of pre-drug baseline, $n = 6$, $p < 0.05$; Fig. 24B). Consistent with a forskolin-induced increase in the presynaptic release probability (Kaneko and Takahashi 2004), the increase in EPSP amplitude was associated with a decrease in PPF (71 ± 6.8% of pre-drug baseline, $n = 6$, $p < 0.05$; Fig. 24*B*). Forskolin-induced potentiation of synaptic transmission completely occluded induction of homosynaptic cortical LTP (101 ± 6.5% of baseline, $n = 6$, $p < 0.05$; Fig. 24*B*), suggesting a rise in presynaptic cAMP during LTP induction. To directly test this idea, we applied the non-hydrolysable cAMP analogue Rp-cAMPS (100 μM). In slices pre-treated for 45 min. with Rp-cAMPS cortical LTP could not be induced (104 ± 8.6% of baseline, $n = 7$, $p > 0.05$; Fig. 24C). This effect could not be attributed to an action of Rp-cAMPS in the postsynaptic cell since intracellular perfusion with the membrane-impermeable analogue Rp-8-OH-cAMPS (5 mM) did not interfere with LTP induction (143 ± 7.1% of baseline, $n = 6$, $p < 0.05$; Fig. 24*C*). Finally, to assess whether the Rp-cAMPS effect was due to blockade of PKA we tested if the PKA inhibitor KT5720 (2 μM) also blocked LTP. In the presence of KT5720 homosynaptic cortical LTP was completely abolished (99.5 ± 9.4% of baseline, $n = 8$, $p > 0.05$; Fig. 24*D*). These results suggest that presynaptically induced homosynaptic cortical LTP involves the activation of presynaptic AC and PKA eventually resulting in an increase probability of release.

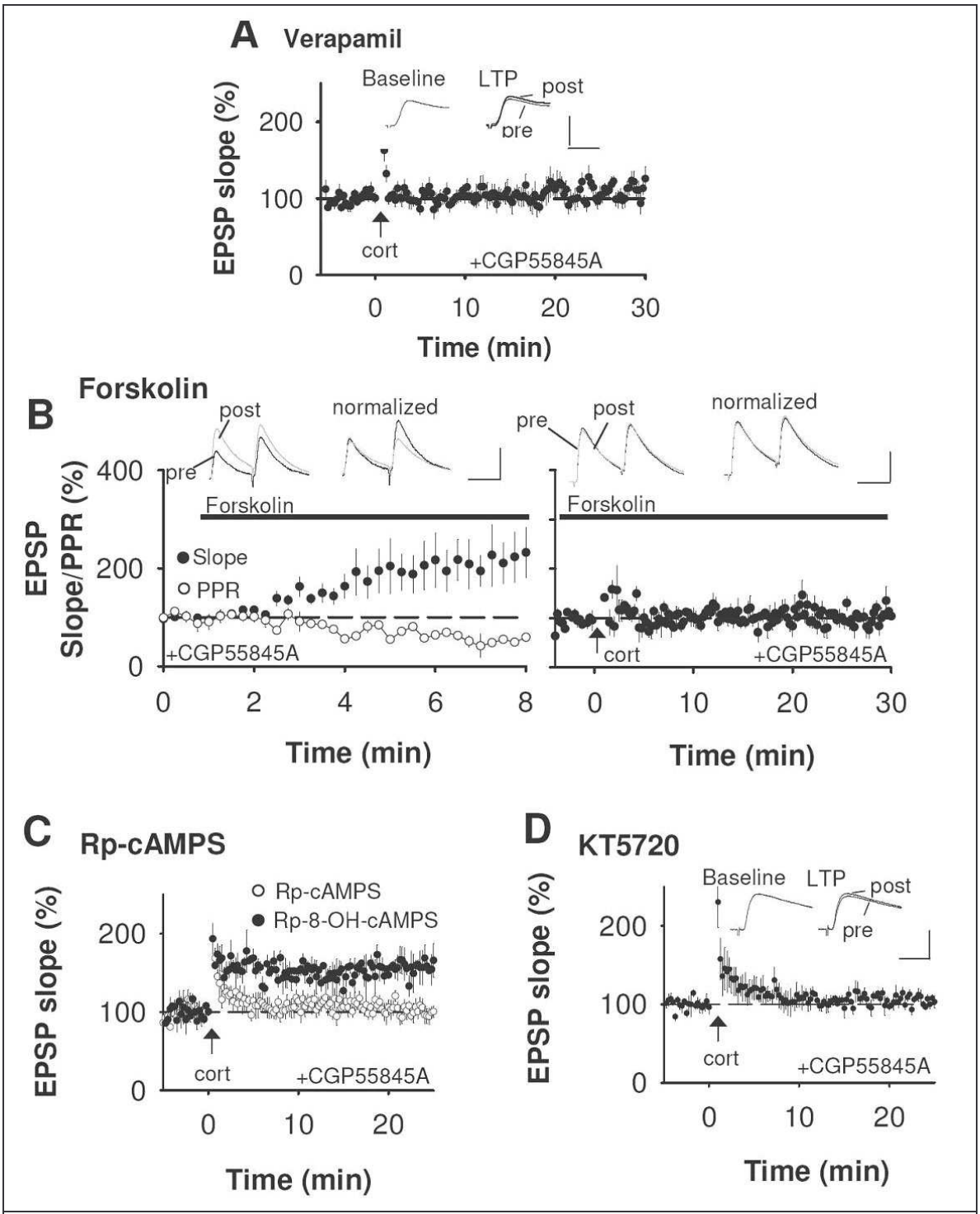

Figure 24) Induction of homosynaptic LTP at cortical afferents activates the cAMP/PKA signal cascade pathway. (A) Cortical EPSP is not potentiated in the presence of L-type Ca^{2+} blocker (Verapamile) 100 µM, n=6. (B) Forskolin-induced potentiation of Cortical EPSP is associated with a decrease in PPF (n = 7). Forskolin-induced potentiation occlude homosynaptic LTP (C) Blockade of presynaptic PKA block induction of presynaptic LTP at cortical afferents (n = 4; open symbols) whereas blockade of postsynaptic PKA doesnot occlude induction of LTP (n = 5; filled symbols). (D) The PKA catalytic subunit blocker (KT5720) abolish LTP induction (30 mM; n = 6).

$GABA_{B(1a)}$ receptors mediate presynaptic inhibition at cortical afferents. Although we could not find any evidence for a postsynaptic contribution to the induction of homosynaptic LTP in the presence of a $GABA_B$ receptor antagonist, this does not conclusively demonstrate a role for presynaptic as opposed to postsynaptic $GABA_B$ receptors. Since it is not possible to pharmacologically discriminate between pre- and postsynaptic $GABA_B$ receptors, we took a genetic approach using mice deficient for the $GABA_{B(1)}$ subunit isoforms $GABA_{B(1a)}$ and $GABA_{B(1b)}$ (Jarrell et al. 1987). Recent experiments carried out in the CA1 area of the hippocampus revealed that presynaptic heteroreceptors on glutamatergic terminals are exclusively comprised of $GABA_{B(1a)}$ and $GABA_{B(2)}$ subunits, whereas postsynaptic $GABA_B$ receptors appear to be predominantly comprised of $GABA_{B(1b,2)}$ heterodimers (McDonald et al. 2004; Vigot 2005). To test the contribution of $GABA_{B(1a)}$ vs. $GABA_{B(1b)}$ containing receptors to presynaptic inhibition at cortico-amygdala afferents, we assessed the effect of the $GABA_B$ receptor agonist baclofen (50 μM) on EPSCs elicited by cortical afferent stimulation. Whereas baclofen-induced inhibition of excitatory synaptic transmission was only slightly reduced in $GABA_{B(1b)}^{-/-}$ mice (wild-type: 86 ± 2% inhibition, n = 20; $GABA_{B(1b)}^{-/-}$: 75 ± 3% inhibition, n = 16, $P < 0.01$)(Fig. 25*A,B*), $GABA_{B(1a)}^{-/-}$ mice exhibited a strongly decreased presynaptic inhibition (27 ± 4% inhibition, n = 18; $P < 0.001$)(Fig. 25*A,B*). Consistent with earlier findings in the CA1 area of the hippocampus (Schuler et al. 2001) baclofen had no effect on synaptic transmission in $GABA_{B(1)}^{-/-}$ animals (-1 ± 11% inhibition, n = 5; $P < 0.001$)(Fig. 25*B*). In all genotypes presynaptic inhibition induced by activation of adenosine receptors was not affected (Fig. 25*A*). This demonstrates that $GABA_{B(1a)}^{-/-}$ mice largely lack functional $GABA_B$ receptors on cortical afferent terminals.

In contrast to the predominant role of $GABA_{B(1a)}$ subunits in presynaptic inhibition of excitatory synaptic transmission, postsynaptic inhibition mediated by the induction of an outward current (at -50 mV) activation of GIRK-type K^+ channels was equally reduced in $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ animals (wild-type: 61 ± 8 pA, n = 21; $GABA_{B(1)}^{-/-}$: 2 ± 6 pA, n = 5, $P < 0.01$; $GABA_{B(1a)}^{-/-}$: 37 ± 5 pA, n = 20, $P < 0.05$; $GABA_{B(1b)}^{-/-}$: 34 ± 6

pA, n = 16, $P < 0.05$)(Fig. 26*C*). Again, adenosine-induced postsynaptic activation of GIRK currents was not affected in all genotypes (data not shown).

We next analyzed inhibitory synaptic transmission elicited by local stimulation within the LA in the presence of ionotropic glutamate receptor antagonists. While activation of $GABA_B$ autoreceptors on interneurons was completely abolished in $GABA_{B(1)}^{-/-}$ mice (wild-type: 83 ± 7% inhibition, n = 5; $GABA_{B(1)}^{-/-}$: 0 ± 1% inhibition, n = 6, $P < 0.001$), presynaptic inhibition on interneurons was not different from wild-type animals in $GABA_{B(1a)}^{-/-}$ and in $GABA_{B(1b)}^{-/-}$ mice ($GABA_{B(1a)}^{-/-}$: 79 ± 6% inhibition, n = 5; $GABA_{B(1b)}^{-/-}$: 86 ± 3% inhibition, n = 8). This indicates that $GABA_{B(1a)}$- and $GABA_{B(1b)}$-mediated presynaptic inhibition on GABAergic terminals is either redundant, or that $GABA_{B(1a)}$ subunits are able to compensate for the loss $GABA_{B(1b)}$ subunits and vice versa. Taken together, these results show that $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice can be used as a tool to discriminate between pre- and postsynaptic $GABA_B$ receptor-mediated inhibition at cortical afferents to the LA.

To address the question if the facilitation of homosynaptic LTP induction in the presence of a $GABA_B$ receptor antagonist was due to the blockade of presynaptic heteroreceptors on cortical afferents we induced LTP in $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice in the absence of CGP55485A. Since $GABA_{B(1a)}^{-/-}$ and $GABA_{B(1b)}^{-/-}$ mice are only viable in the BALB/c background, we first verified the pharmacological findings in wild-type BALB/c animals. Like in C57BL/6J mice CGP55845A (10 μM) facilitated the induction of homosynaptic LTP in BALB/c animals (wild-type: 105 ± 4% of baseline, n = 18, $P > 0.05$; wild-type + CGP55845A: 150 ± 15% of baseline, n = 10, $P < 0.05$). Consistent with a predominant role of $GABA_{B(1a)}$ –containing receptors in presynaptic inhibition at cortical afferents, we found that homosynaptic LTP induction was facilitated in $GABA_{B(1)}^{-/-}$ and in $GABA_{B(1a)}^{-/-}$, but not in $GABA_{B(1b)}^{-/-}$ mice ($GABA_{B(1)}^{-/-}$: 153 ± 17% of baseline, n = 5, $P < 0.05$; $GABA_{B(1a)}^{-/-}$: 130 ± 8% of baseline, n = 18, $P < 0.01$; $GABA_{B(1b)}^{-/-}$: 90 ± 11% of baseline, n = 13, $P > 0.05$)(Fig. 25*D,E*). LTP induction in $GABA_{B(1a)}^{-/-}$ animals could not be significantly enhanced by application of CGP55485A (139 ± 15% of baseline, n = 4, $P > 0.05$), demonstrating that blockade of $GABA_{B(1a)}$-

containing receptors largely underlies pharmacological facilitation of homosynaptic LTP. Finally, in line with the results using pharmacological $GABA_B$ receptor blockade, LTP was consistently associated with a decrease in PPF ($GABA_{B(1)}^{-/-}$: -25 ± 6% PPF change, $n = 5$, $P > 0.05$; $GABA_{B(1a)}^{-/-}$: -13 ± 3%, $n = 18$, $P < 0.01$; $GABA_{B(1b)}^{-/-}$: 2 ± 6%, $n = 13$, $P > 0.05$). In conclusion, using mice deficient for the $GABA_{B(1)}$ isoforms $GABA_{B(1a)}$ and $GABA_{B(1b)}$ we were able to dissociate pre- and postsynaptic $GABA_B$ receptor function at cortical afferents to the LA. Our experiments show that presynaptic $GABA_{B(1a)}$-containing heteroreceptors are critical in determining the associative properties of cortical afferent LTP.

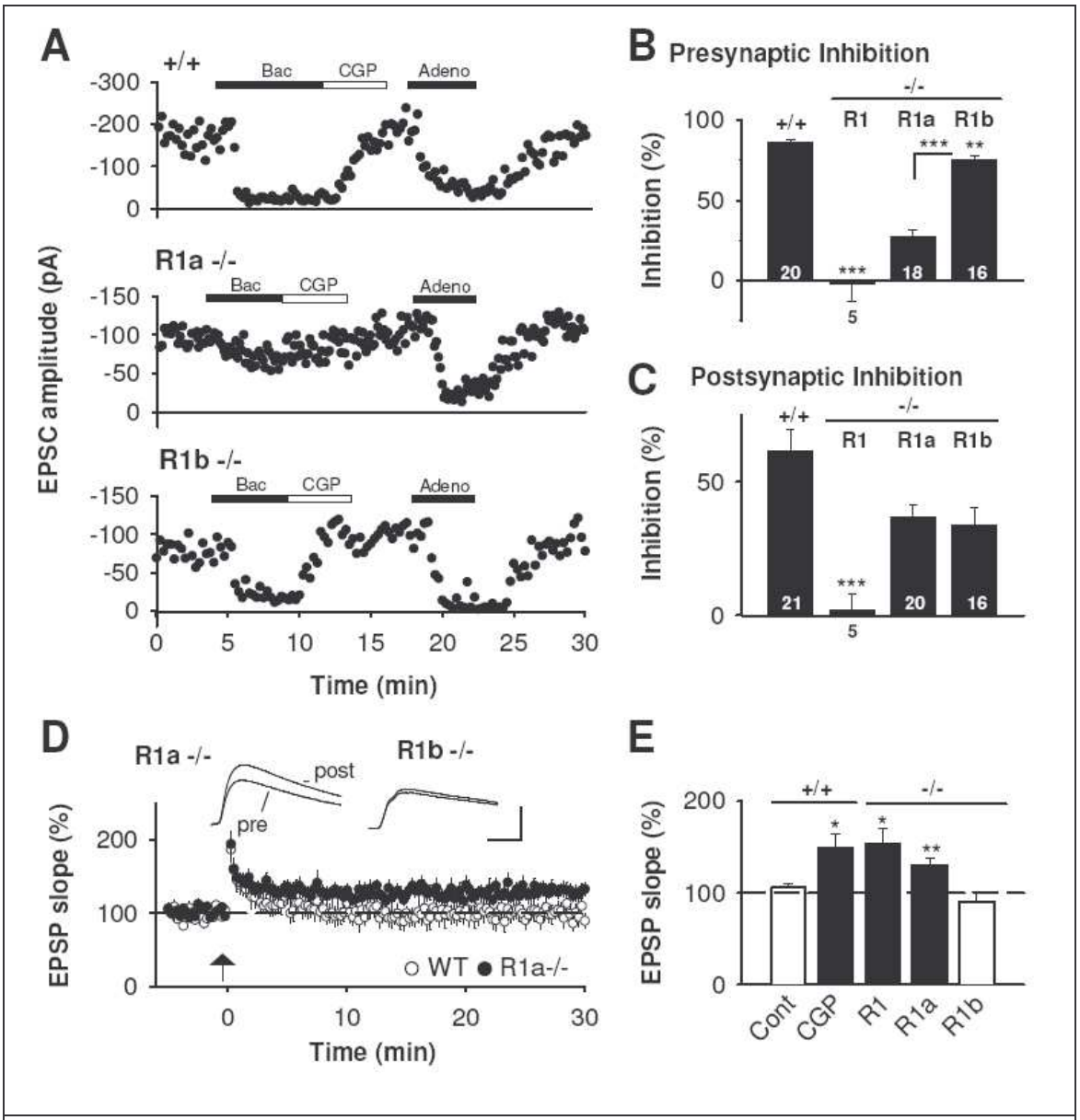

Figure 25) $GABA_{B(1a)}$ receptors mediate presynaptic inhibition at cortical afferents. (*A*) Baclofen-induced presynaptic inhibition at cortical afferents is strongly reduced in $GABA_{B(1a)}^{-/-}$ mice (middle) as compared to wild-type (top) or $GABA_{B(1b)}^{-/-}$ animals (bottom). The effect of adenosine (100 μM) is similar in all genotypes. (*B*) Top: Summary graph illustrating the predominant contribution of $GABA_{B(1a)}$-containing receptors to baclofen-induced presynaptic inhibition. Presynaptic inhibition is completely absent in $GABA_{B(1)}^{-/-}$ mice. (*C*) Baclofen-induced postsynaptic K^+ currents are completely absent in $GABA_{B(1)}^{-/-}$ mice (n = 5), and equally reduced in $GABA_{B(1a)}^{-/-}$ (n = 20) or $GABA_{B(1b)}^{-/-}$ animals (n = 16). (*D*) Induction of homosynaptic LTP in the absence of CGP55845A is facilitated in $GABA_{B(1a)}^{-/-}$ (n = 18), but not in $GABA_{B(1b)}^{-/-}$ animals (n = 13). (C) Summary graph illustrating the facilitation of homosynaptic LTP induction in wild-type BALB/c animals in the presence of CGP55845A (n = 10). In the absence of CGP55845A, LTP induction is facilitated in $GABA_{B(1)}^{-/-}$ (n = 5) and $GABA_{B(1a)}^{-/-}$ (n = 18), but not in $GABA_{B(1b)}^{-/-}$deficient mice (n = 13). Scale bars: 3 mV/10 ms.

Generalization of conditioned fear in $GABA_{B(1a)}$–deficient mice. To assess the behavioral impact of altered presynaptic inhibition and facilitation of non-associative LTP induction we subjected $GABA_{B(1a)}^{-/-}$ animals to an auditory fear conditioning paradigm. Given that previous studies have implicated the cortico-amygdala pathway in stimulus discrimination and generalization of conditioned fear ((Jarrell et al. 1987; Armony et al. 1997), but see:(Klausberger et al. 2003)) mice were trained using a differential fear conditioning paradigm where only one of two tones , the CS^+ (7.5 kHz, 30 s, 80 dB), but not the CS^- (3 kHz, 30 s, 80 dB), was paired to the unconditioned stimulus (US; 0.9 mA, 1.5 s). Training consisted of seven CS^+-US pairings with 7 interleaved CS^- presentations. When tested 24 hrs later, wild-type animals exhibited clear discrimination between the CS^+ and the CS^- ($n = 6$; $P < 0.05$)(Fig. 26*A*). In contrast, $GABA_{B(1a)}^{-/-}$ mice showed indistinguishable freezing behavior in response to the CS^+ and the CS^- ($n = 6$; $P > 0.05$)(Fig. 26*A*). It is unlikely that the increased freezing in response to the CS^- was caused by a general increase in stress or anxiety levels in $GABA_{B(1a)}^{-/-}$ mice because the animals did not freeze more than wild-type mice when they were exposed for the first time to a novel context ($n = 5$; $P > 0.05$)(Fig. 26*A*). Moreover, $GABA_{B(1a)}^{-/-}$ mice exhibited no difference in the threshold for the US to induce rapid movements or vocalization ($n = 5$; $P > 0.05$)(Fig. 26*B*). Thus, in parallel to the loss of accociativity of cortical afferent LTP, $GABA_{B(1a)}^{-/-}$ mice exhibit a striking deficit in associative stimulus discrimination at the behavioral level.

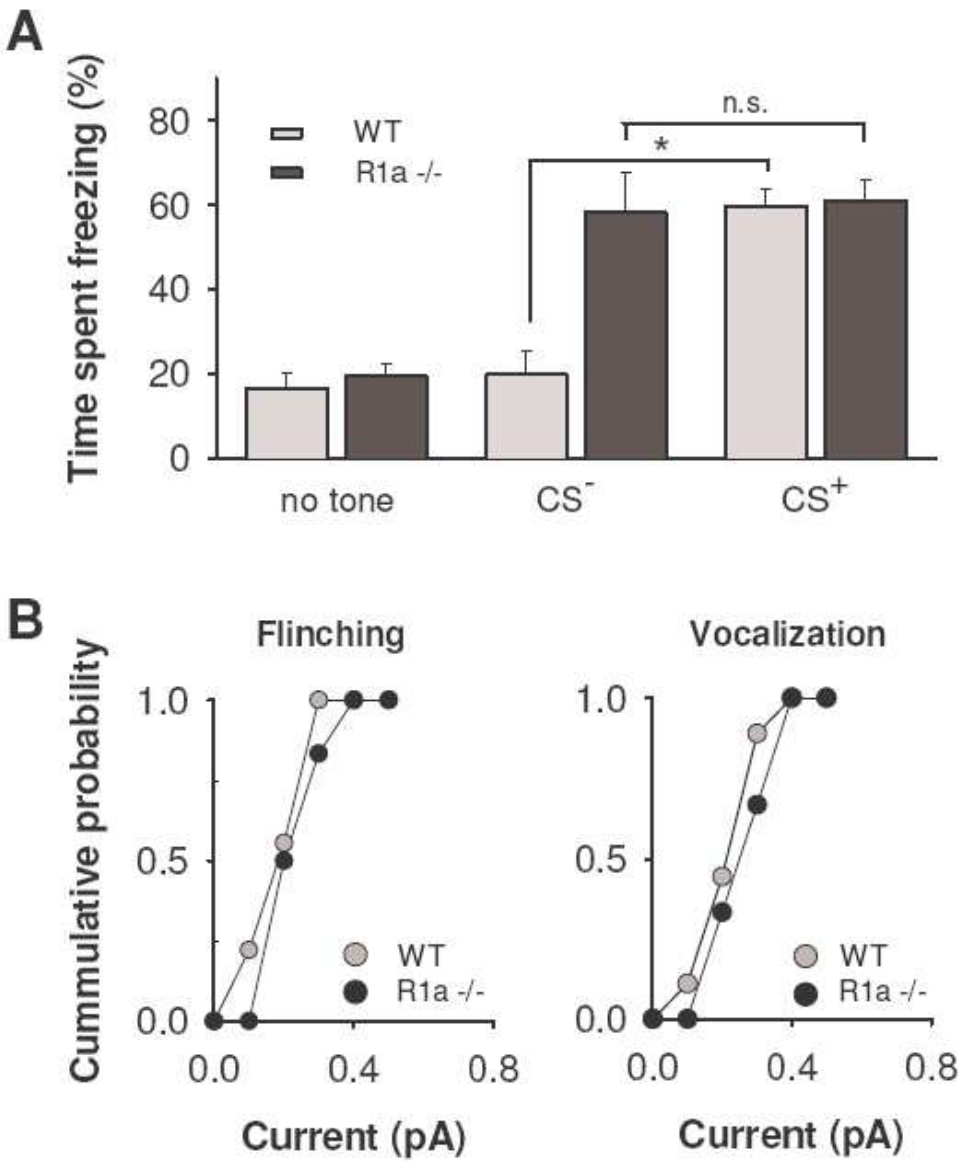

Figure 26) Generalization of conditioned fear in $GABA_{B(1a)}^{-/-}$ mice. (*A*) $GABA_{B(1a)}^{-/-}$ mice exhibit normal freezing levels to CS^+ presentations (24 h after conditioning), but a complete absence of stimulus discrimination as evidenced by equally high freezing levels during CS^- presentations (n = 6 for each genotype). (*B*) $GABA_{B(1a)}^{-/-}$ mice exhibit no difference in the threshold of foot-shock-induced movements or vocalization (n = 4 for each genotype).

5.2.4. Discussion

Our present results show that associativity of presynaptic LTP at cortico-amygdala afferents not only depends on presynaptic NMDA receptor activation, but also requires presynaptic $GABA_B$ heteroreceptor-mediated inhibition to prevent induction of homosynaptic, non-associative plasticity. In the absence of presynaptic $GABA_B$ receptor function, an NMDA receptor-independent, non-associative form of presynaptic LTP is unmasked. This may suggest that the level of $GABA_B$ receptor-mediated presynaptic inhibition, and hence associativity of presynaptic cortical LTP, may be determined by the activity of local inhibition. Thereby, changes in inhibitory transmission associated with distinct patterns of network activity (Nicoll and Malenka 1995) will result in a shift of the relative threshold for the induction of homo- vs. heterosynaptic LTP. Thus, cortical afferent boutons are endowed with presynaptic ionotropic and metabotropic receptor complements allowing the detection and integration of concomitant excitatory and inhibitory activity in a heterosynaptic manner. Similar properties have been reported for other types of synapses in the brain exhibiting presynaptic long-term plasticity, such as hippocampal mossy fiber synapses (Linden and Ahn 1999; Schmitz et al. 2003), or parallel fiber-Purkinje cell synapses in the cerebellum (Dittman and Regehr 1997), suggesting that heterosynaptic modulation of plasticity thresholds, a form of metaplasticity, may be a general feature of presynaptic plasticity underlying many forms of integrative computation.

Interestingly, presynaptic LTP at all these synapses requires activation of cAMP/PKA-dependent signaling (Nicoll and Malenka 1995; Huang and Kandel 1998; Linden and Ahn 1999). It remains to be shown, however, at which level induction of heterosynaptic and homosynaptic forms of presynaptic LTP converge at cortico-amygdala synapses. Potentially, presynaptic $GABA_B$ receptor-mediated inhibition could influence LTP induction threshold at multiple levels, such as direct inhibition of Ca^{2+} channels, activation of K^+ channels, or by multiple downstream effects triggered by the lowering of presynaptic cAMP levels (Bettler et al. 2004).

Using mice deficient for the $GABA_{B(1a)}$ and $GABA_{B(1b)}$ isoforms, we found that presynaptic heteroreceptors on cortico-amygdala boutons predominantly contain the $GABA_{B(1a)}$ isoform. A segregation of $GABA_{B(1a)}$ and $GABA_{B(1b)}$ to pre- and postsynaptic compartments has been previously suggested based on layer-specific localization of 1a and 1b receptors in the cerebellum (Billinton et al. 1999), a hypothesis that has been directly confirmed by recent electrophysiological analysis of pre- and postsynaptic $GABA_B$ receptor-mediated inhibition in the CA1 area of the hippocampus of $GABA_{B(1a)}$ and $GABA_{B(1b)}^{-/-}$ mice (Vigot 2005). It is not clear, why presynaptic heteroreceptors should be specifically of the $GABA_{B(1a)}$ subtype, since 1a and 1b isoforms do not exhibit any difference in their intracellular domains coupling to downstream signaling, but rather differ in terms of alternative N-termini with the presence of a pair of sushi repeats in the $GABA_{B(1a)}$-specific domain. Sushi repeats are thought to be involved in extracellular protein-protein interactions (Bettler et al. 2004), suggesting that $GABA_{B(1a)}$ receptors might be specifically retained in the presynaptic compartment by means of interactions with these sushi repeats.

The differential contribution of $GABA_{B(1a)}$- and $GABA_{B(1b)}$-containing receptors to pre- and postsynaptic inhibition, and the finding that $GABA_B$ autoreceptor-mediated inhibition is not affected in $GABA_{B(1a)}^{-/-}$ or $GABA_{B(1b)}^{-/-}$ mice, allowed us to specifically address the role of presynaptic heteroreceptors *in vivo*. Consistent with previous reports demonstrating a role for cortical pathways in stimulus discrimination during Pavlovian conditioning (Jarrell et al. 1987; Armony et al. 1997), but see: (Klausberger et al. 2003), we found that $GABA_{B(1a)}$-deficient mice exhibit a striking generalization of conditioned fear to non-conditioned stimuli. While it has been shown that conditioned changes in tone-evoked single-unit activity in the LA discriminate between conditioned and non-conditioned stimuli (Collins and Pare 2000), our data do not exclude that $GABA_{B(1a)}$-containing receptors play an important role for stimulus discrimination in a brain area upstream from the amygdala such as the auditory cortex or the auditory thalamus. Nevertheless, the modulation of associative properties of cortico-amygdala LTP by presynaptic $GABA_B$ heteroreceptors offers an attractive mechanism by which concomitant homosynaptic thalamic and heterosynaptic cortical afferent plasticity could

contribute to determine stimulus-discrimination during Pavlovian conditioning paradigms. Moreover, by modulation of inhibitory network activity, activation of distinct neuromodulatory input, associated with specific behavioral states, may modulate the degree of fear generalization appropriate for specific behavioral demands under certain conditions.

5.3. Postsynaptic $GABA_{B(1b)}$ receptors modulate the induction of homosynaptic LTP at thalamic afferents

Hamdy Shaban[1],Yann Humeau[1,2], Cyril Herry[1], Stephane Ciocchi[1], Gilles Sansig[3], Samuel Barbieri[4], Herman van der Putten[3], Bernhard Bettler[4], and Andreas Lüthi[1]

[1]Friedrich Miescher Institute, CH-4058 Basel, Switzerland, [2]UPR2356, CNRS, F-67084 Strasbourg, France, [3]Novartis Institutes for Biomedical Research, Novartis Pharma AG, CH-4002 Basel, Switzerland, [4]Pharmazentrum, Department of Clinical-Biological Sciences, University of Basel, CH-4056 Basel, Switzerland

Manuscript in preparation

In this work, I did most of the electrophysiological investigations. Some experiments are contributed by Yann Humeau. Behavior analysis was performed by Cyril Herry and Stephane Ciocchi.

5.3.1. Summary

Synaptic plasticity in the lateral amygdala (LA) is believed to underlie the formation and storage of fear memory. The activity in the LA excitatory cells is under firm control of inhibitory inputs. $GABA_B$ receptors are abundant in LA and were shown to modulate synaptic plasticity in the hippocampus. Here we show that the blockade of $GABA_B$ receptors facilitates the induction of long term potentiation (LTP) at thalamic afferents to LA. This LTP was NMDAR-dependent and needs postsynaptic Ca^{2+} influx. To discriminate between pre- and postsynaptic $GABA_B$ receptor-mediated effect, we used mice deficient in either one of the two isoforms $GABA_{B(1a)}$ and $GABA_{B(1b)}$. These two subunit isoforms are believed to be localized differentially at the subcellular level. Genetic knockout of the $GABA_{B(1a)}$ isoform revealed no LTP facilitation at the thalamic afferents, whereas $GABA_{B(1b)}$ knockout mice exhibited LTP facilitation specifically at thalamic afferents. Again this homosynaptic LTP at thalamic afferents in $GABA_{B(1B)}$ -/- mice required postsynaptic Ca^{2+} influx. Moreover, disynaptic IPSCs recorded from $GABA_{B(1a)}$ knockout mice revealed LTP facilitation only at the cortical afferents whereas $GABA_{B(1b)}$ knockout mice revealed

LTP facilitation only at the thalamic afferents. Behavior analysis of $GABA_{B(1b)}$ -/- mice shows a clear deficit in fear memory formation. Thus, $GABA_B$ receptors modulate synaptic plasticity at both inhibitory and excitatory LA synapses and thereby play important role in fear memory formation in the LA.

5.3.2. Introduction

Classical Pavlovian conditioning (Pavlov; 1927) is widely used as a model system for understanding how the brain associates and stores information about fear experiences. In fear conditioning paradigms a subject (mouse) is exposed to the unconditioned stimulus (US), a noxious stimulus, such as footshock, in concurrence with conditioned stimulus (CS), a neutral stimulus, such as tone or light. After training the tone (on next occurrence) acquires aversive properties and will trigger fear responses. In rodents, such responses include freezing behavior observed by complete arrest of the movements. The neural basis of fear conditioning points to the amygdala as a key player in processing and storage of emotional memory (Davis 1994; Fanselow and Kim 1994; LeDoux 1994; Lang et al. 2000; LeDoux 2000). Sensory information about stimuli that are harmful enter the amygdala by way of the LA (LeDoux et al. 1990). The LA, in turn, projects to other amygdala areas (Pitkanen et al. 1997), which control defence responses (Dalgleish 2004; Dityatev and Bolshakov 2005)

Activity-dependent Hebbian plasticity at cortical and/or thalamic afferents to LA projection neurons is generally thought to underlie Pavlovian fear conditioning (LeDoux 2000; Maren 2001; Tsvetkov et al. 2002). The most intensively studied and best-characterized sensory pathway is a direct projection from the medial geniculate nucleus of the thalamus to the dorsal portion of the LA of the amygdala (LeDoux and Farb 1991). It was shown that fear conditioning induces LTP at the glutamatergic synapses in the LA of the amygdala (Clugnet and LeDoux 1990; McKernan and Shinnick-Gallagher 1997).

In vivo data demonstrated a powerful control through GABAergic inhibition over the activity of projecting cells in the LA (Lang and Pare 1997; Pape et al. 1998). Furthermore, the ability to induce LTP in excitatory cells in vitro depends on the strength of the local inhibitory network (Marsicano et al. 2002; Shumyatsky et al. 2002; Bissiere et al. 2003). Indeed, GABAergic interneurons are thought to play a crucial role in

information processing in the amygdala (Lang and Pare 1997; Mahanty and Sah 1998; Bissiere et al. 2003). Converging fast excitatory postsynaptic responses from cortical and thalamic inputs were also found in interneurons of the LA (Mahanty and Sah 1998; Mahanty and Sah 1999; Szinyei et al. 2000). Furthermore, heterosynaptic LTP of inhibitory interneurons were recently observed in the LA (Bauer and LeDoux 2004). GABA is the predominant inhibitory neurotransmitter in the mammalian nervous system, which upon release from interneuron activates two types of receptors. $GABA_A$ receptors are ligand-gated ion channels that mediate fast synaptic inhibition (Bowery et al. 1987; Macdonald and Olsen 1994). $GABA_B$ receptors are coupled to G-proteins and exert their inhibitory action by activating postsynaptic inwardly rectifying Kir3-type K^+ channels and by inactivating presynaptic voltage-gated ca^{2+} channels (Wojcik and Neff 1984; Bowery et al. 1987; Slesinger et al. 1997; Marshall et al. 1999).

In hippocampal neurons, pre- and postsynaptic $GABA_B$ receptor-mediated synaptic inhibition has been shown to play a crucial role during the induction of homosynaptic LTP (Olpe and Karlsson 1990; Olpe et al. 1993; Perkel and Nicoll 1993). Immunohistochemical studies showed that $GABA_B$ receptors are strongly present in the LA (Fritschy et al. 1999; McDonald et al. 2004). Moreover, electrophysiological studies showed that $GABA_A$ and $GABA_B$ receptors differentially regulate glutamatergic synaptic transmission in the auditory thalamo-amygdala pathway (Rainnie et al. 1991; Li et al. 1996). Here, we are investigating the role of pre- and postsynaptic $GABA_B$ receptors during the induction of synaptic plasticity at the thalamic afferent to the LA.

5.3.3. Methods

Electrophysiology. Standard procedures were used to prepare 350 μm thick coronal slices from three to four week old male C57BL/6J or BALB/c mice following a protocol approved by the Veterinary Department of the Canton of Basel-Stadt (Humeau et al. 2003). Briefly, the brain was dissected in ice-cold artificial cerebrospinal fluid (ACSF), mounted on an agar block and sliced with a Dosaka vibratome (Kyoto, Japan) at 4ºC. Slices were maintained for 45 min at 35ºC in an interface chamber containing ACSF

equilibrated with 95% O_2/5% CO_2 and containing (in mM): 124 NaCl, 2.7 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 0.4 NaH_2PO_4, 18 glucose, 4 ascorbate, and then for at least 45 min. at room temperature before being transferred to a superfusing recording chamber. Whole-cell recordings from LA projection neurons were performed at 30-32ºC in a superfusing chamber. Neurons were visually identified with infrared videomicroscopy using an upright microscope equipped with a x40 objective (Olympus). Patch electrodes (3-5 MΩ) were pulled from borosilicate glass tubing and normally filled with a solution containing (in mM): 120 K-gluconate, 20 KCl, 10 HEPES, 10 phosphocreatine, 4 Mg-ATP, and 0.3 Na-GTP (pH adjusted to 7.25 with KOH or CsOH, respectively, 295 mOsm). For voltage-clamp experiments K-gluconate was replaced by equimolar Cs-gluconate. All experiments were performed in the presence of picrotoxin (100 μM) unless indicated otherwise. In current-clamp recordings membrane potential was kept manually at –70 mV (not corrected for junction potentials). Data were recorded with an Axopatch200B, filtered at 2 kHz and digitized at 10 kHz. In all experiments, series resistance was monitored throughout the experiment by applying a hyperpolarizing current or voltage pulse, and if it changed by more than 15%, the data were not included in the analysis. Data were acquired and analyzed with ClampEx9.0 and ClampFit9.0 (Axon Instruments, CA). Monosynaptic EPSPs or EPSCs exhibiting constant 10-90% rise times and latencies were elicited by stimulation of afferent fibers with a bipolar twisted platinum/10% iridium wire (25 μm diameter). LTP was induced by delivering Poisson-distributed 45 stimulations at an average frequency of 30 Hz (Humeau et al. 2003). LTP was quantified for statistical comparisons by normalizing and averaging EPSP slopes during the last 5 min of experiments relative to 5 min of baseline. Depicted traces show averaged EPSPs for 2 min of baseline and 2 min of LTP (25-30 min after pairing). All values are expressed as means ± s.e.m. Statistical comparisons were done with paired or unpaired Student's *t*-test as appropriate (two-tailed $P < 0.05$ was considered significant).

Behavior. Adult male wild-type and mutant BALB/c mice were individually housed in plastic cages with ad-lib access to food and water with a 12/12-h light/dark cycle. All studies took place during the light portion of the cycle. Mice were handled gently for 2-3 min/day during 5 days, to minimize nonspecific stress. Fear conditioning and testing took place in two different contexts (Context A and B). The conditioning and testing boxes

and the floor were cleaned with 70% ethanol, or 1% acetic acid before and after each session, respectively. To score freezing behavior an automatic infrared beam detection system placed on the bottom of the experimental chambers (Coulbourn Instruments, Allentown, PA) was used. The animals were considered freezing if no movement was detected for 2 s. Mice were submitted to a discriminative fear conditioning protocol in which a 30 s tone conditioned stimulus (CS^+)(7.5 kHz, 80 dB) was systematically paired to a 1 s foot-shock (unconditioned stimulus; US) (0.9 mA, 7 CS^+/US pairings; inter-trial interval: 20-180 s). The onset of the US coincided with the offset of the CS^+. A second non-conditioned 30 s tone (CS^-; 3 kHz, 80 dB) was presented after each CS^+/US association but was never reinforced (7 CS^- presentations, inter-trial interval: 20-180 s). Conditioned mice were tested 24 h later in context B with 4 presentations of the CS^- and the CS^+. To determine pain sensitivity mice were submitted to a series of increasing strength 1 s foot-shocks in context A (0.1 to 0.8 mA range, 0.1 mA steps). Nociception threshold was calculated by scoring the first noticeable flinching behavior and stress-induced vocalization.

Reagents. BAPTA, BAPTA-AM, CPP, and NBQX were from Tocris-Cookson (Bristol, UK). CGP55845A and GS3732 were a gift from Novartis Pharma AG (Basel, Switzerland). All other drugs were from Fluka/Sigma (Buchs, Switzerland).

5.3.3. Results

Pre- and postsynaptic inhibition by $GABA_B$ receptors in the LA

To detect functional $GABA_B$ receptors in the LA, we recorded EPSCs evoked by stimulating the external capsule (cortical afferents) or the internal capsule (thalamic afferents). Pharmacological activation of $GABA_B$ receptors by applying the specific $GABA_B$ agonist Baclofen (50 µM) reduced EPSCs amplitude in both thalamic (54.2 ± 5.3% inhibition; $n = 5$; $p < 0.001$) and cortical (52.2 ± 4.1% inhibition; $n = 5$; $p < 0.001$). The decrease of EPSCs amplitude is thought to be due to activation of presynaptic $GABA_B$ receptors located at glutamatergic terminals (Kombian et al. 1996; Takahashi et al. 1998). This response to $GABA_B$ receptor was antagonized by application of the specific $GABA_B$ receptor antagonist CGP55845A indicating activation and blockade of

presynaptic $GABA_B$ heteroreceptors (Schuler et al. 2001) (Fig. 27A). Furthermore, synaptic activation of $GABA_B$ receptor by three subsequent pulses at 20 Hz and application of CGP55845A decreased the pair pulse ratio (PPR). This indicates presynaptic mechanism of inhibition (Fig. 27B). Postsynaptic $GABA_B$ receptors are known to activate a Kir3-mediated K^+ conductance in CA1 pyramidal neurons (Luscher et al. 1997; Schuler et al. 2001). That $GABA_BR$-activated K^+ conductance underlies the late IPSP (Luscher et al. 1997). Indeed, activation of $GABA_B$ receptors by baclofen elicited outward current that is antagonized by CGP55845A application. Moreover, this outward current can be elicited with synaptic activation of postsynaptic $GABA_BR$ by endogenous GABA (Fig. 27C, D).

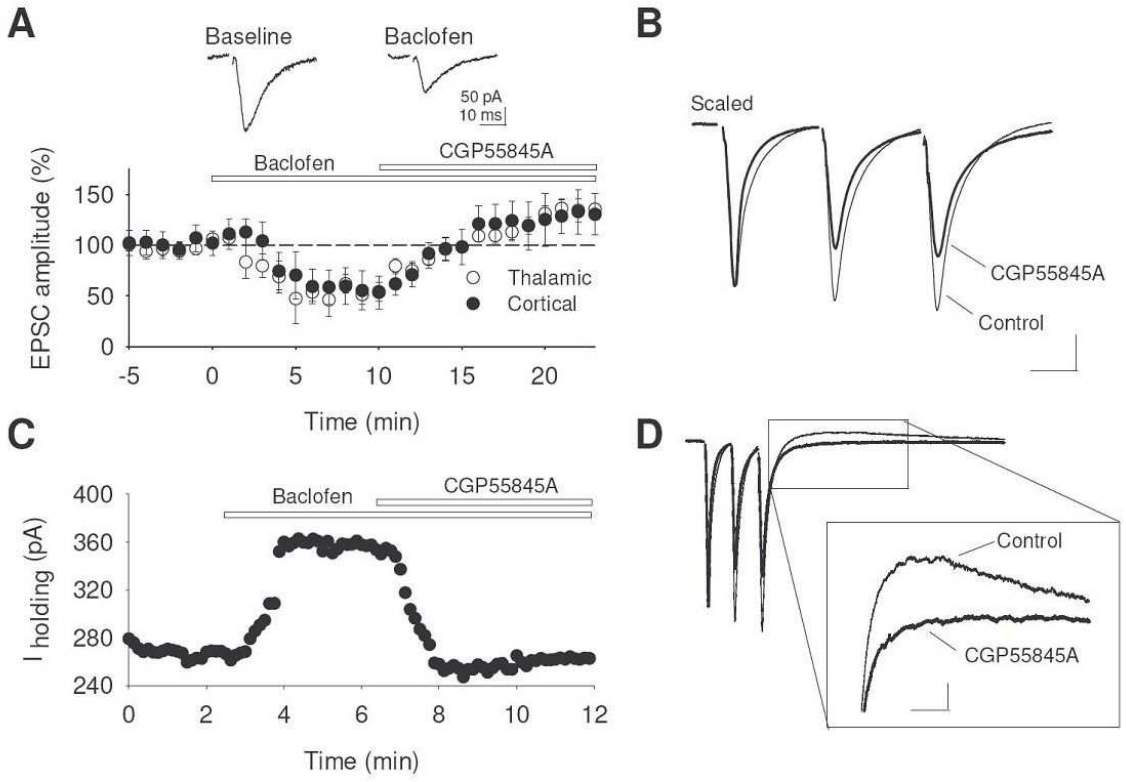

Figure 27) $GABA_B$ receptor-mediated inhibition in the LA A,B: Presynaptic inhibition of excitatory synaptic transmission. A: Application of the $GABA_B$ agonist baclofen (50 µM) decreases EPSC amplitude at cortical and thalamic afferent synapses. **B**: Application of the $GABA_B$ antagonist CGP55458A (10 µM) increases glutamate release as monitored by a decrease in the paired-pulse ratio, scale bar: 100pA, 100 ms. **C, D:** Postsynaptc inhibition. C: Application of baclofen (50 µM) activates an outward current in postsynaptic LA pyramidal cells. **D:** This current can be activated synaptically upon repetitive stimulation. It is completely blocked by CGP55458A (10 µM; inset).

Facilitation of postsynaptic LTP induction upon $GABA_B$ receptor blockade

To investigate the impact of $GABA_BR$ inhibitory inputs, we tested synaptic plasticity at thalamic and cortical afferents. In agreement with a previous report (Bissiere et al. 2003), thalamic afferent EPSP was not potentiated under intact $GABA_A$ receptors but blocked $GABA_BRS$, (EPSP slope:102.8 ± 5.3% of the baseline, $n = 6$ $p < 0.001$). Surprisingly, cortical afferent was significantly potentiated; (EPSP slope: 182.8 ± 25.8% of the baseline, $n = 6$ $p < 0.001$) (Fig. 28A).

Our previous study showed that upon simultaneous subthreshold stimulation of both thalamic and cortical afferents a heterosynaptic LTP was induced only at the cortical afferents (Humeau et al. 2003). Indeed, homosynaptic LTP induction was not possible in both inputs when only $GABA_A$ receptors are blocked by picrotoxin (100 µM) (Fig. 28B). Blockade of $GABA_B$ receptors by CGP55845A facilitated the induction of homosynaptic LTP (induced by 45 stimulations at 30 Hz) at thalamic afferents synapses (Fig. 28B). The EPSP slope was potentiated (EPSP slope: 155 ± 6.1% of the baseline, n=11; $p < 0.001$) compared to control condition (EPSP slope 103 ± 4.3% of the baseline, n=11; $p < 0.001$). This form of LTP at thalamic afferents was associated without any significant change in paired-pulse facilitation suggesting that it was not mediated by presynaptic mechanisms (n = 8; $P < 0.05$) (Fig. 28C). These findings suggest a postsynaptic expression mechanism. To test the mechanism of induction, we applied the NMDAR antagonist CPP (20 µM) or perforated the postsynaptic cell with the Ca^{2+} chelator BAPTA (30 µM) (Fig. 28D, E). CPP blocked LTP at thalamic afferents, (EPSP slope: 101 ± 5.1% of the baseline, n = 7; $P < 0.05$) and postsynaptic perfusion with BAPTA blocked the induction of LTP, (EPSP slope 103 ± 4.6% of the baseline, n = 9; $P < 0.05$). These results would suggest that thalamic homosynaptic LTP required postsynaptic NMDA activation and postsynaptic Ca^{2+} influx

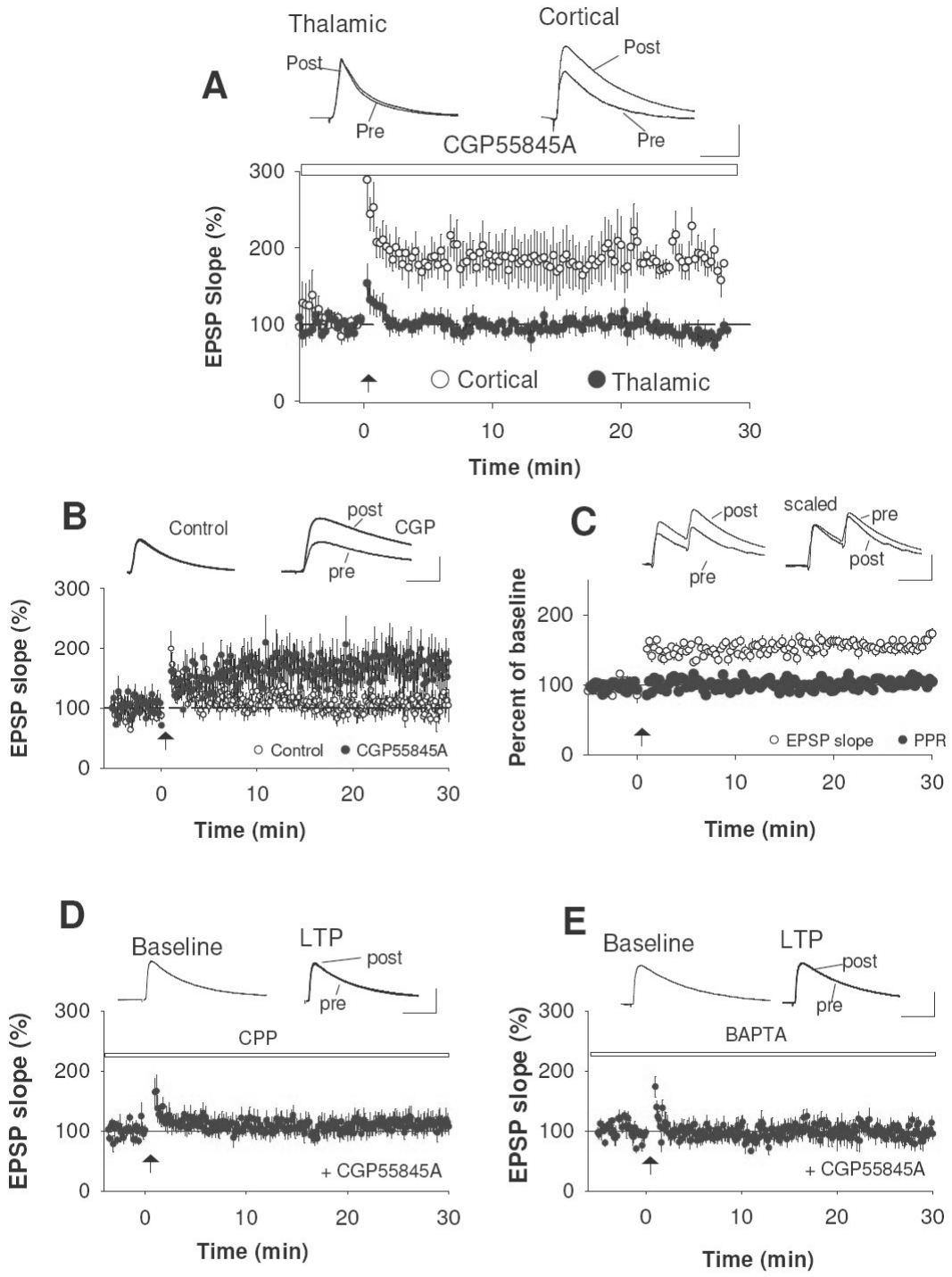

Figure 28) Blockade of $GABA_B$ receptor facilitates the induction of homosynaptic LTP.
A: Blockade of $GABA_B$Rs but not $GABA_A$Rs by leaving out PTX and application of the specific $GABA_B$Rs antagonist CGP55845A facilitates LTP induction only at cortical but not thalamic afferents in wild-type BALB/c mice. **B:** Blockade of $GABA_B$Rs and $GABA_A$Rs by CGP55845A and PTX facilitates the induction of LTP (induced by 45 stimulations at 30 Hz) at thalamic afferents synapses. **C:** Induction of LTP is associated with no change in paired-pulse facilitation at thalamic afferents. **D:** The NMDA receptor antagonist blocks LTP at thalamic afferents. **E:** postsynaptic perfusion with the Ca^{2+} chelator BAPTA blocks the induction of homosynaptic LTP at thalamic scale bar: 5 mV, 50msec.

Presynaptic inhibition in $GABA_{B(1a)}$ -/- mice is impaired

To investigate whether pre- or postsynaptic $GABA_B$Rs mediated-inhibition are suppressing the induction of homosynaptic LTP we used transgenic mice. These mice where genetically modified so that the $GABA_{B(1)}$ subunit or one of its isomer variant $GABA_{B(1a)}$ and $GABA_{B(1b)}$ were knocked out. In previous work we have shown that presynaptic inhibition in $GABA_{B(1a)}$-/- mice are impaired in cortical afferents. To investigate if we could obtain the same result at thalamic afferents, evoked EPSCs were recorded in the LA by stimulating the internal capsule. It was shown that the amplitude of the EPSCs were reduced upon activation of $GABA_B$ heteroreceptors or A1 adenosine receptors that inhibit glutamate release (Schuler et al., 2001). Accordingly, in slices from wild-type mice, both baclofen (100 µM) and adenosine (100 µM) evoked the expected depression of the EPSCs, (baclofen: 87.0 ± 2.5% inhibition, n = 21; $p < 0.01$, adenosine: 86.5 ± 5.8% inhibition, $n = 21$; $p < 0.01$). Surprisingly, at thalamic afferents baclofen induced inhibition was strongly reduced in slices from $GABA_{B(1a)}$ -/- mice compared to adenosine induced inhibition, (baclofen: 41.5 ± 3.6% inhibition, $n = 18$; $p < 0.001$, adenosine: 85.5 ± 2.9% inhibition, $n = 18$; $p < 0.001$)(Fig. 29A).

In $GABA_{B(1)}$ -/- mice, baclofen induced inhibition of glutamate synaptic transmission was completely abolished, (baclofen: 1.7 ± 12.7% inhibition, $n = 6$; $p < 0.01$), adenosine: 71.6 ± 8.6% inhibition, $n = 6$; $p < 0.01$). This demonstrates that $GABA_{B(1a)}$ -/- mice largely lack functional $GABA_B$ heteroreceptors at thalamic terminals, whereas adenosine receptors are still operational and inhibit glutamate release (Fig. 29B). The response to baclofen was only slightly reduced in $GABA_{B(1b)}$ -/- mice, (baclofen: 77.2 ± 2.5% inhibition, $n = 22$; $p < 0.01$, adenosine: 89.3 ± 3.6% inhibition, $n = 22$; $p < 0.01$). The activation of $GABA_B$ autoreceptors on interneurons attenuated IPSCs recorded from LA projection neurons of wild-type mice, IPSCs amplitude; (baclofen: 83.7 ± 7.0% inhibition, $n = 5$; $p < 0.001$). In contrast, baclofen was unable to inhibit IPSCs in $GABA_{B(1)}$ -/- mice, IPSCs; (baclofen: -0.2 ± 1.4% inhibition, $n = 6$; $p < 0.01$). $GABA_{B(1a)}$-/- and $GABA_{B(1b)}$ -/- mice were similar to wild type mice, $GABA_{B(1a)}^{-/-}$ mice IPSCs; (baclofen: 79.5 ± 6.1% inhibition, $n = 18$, $p < 0.001$), $GABA_{B(1b)}^{-/-}$ mice IPSCs; (baclofen: 86.1 ± 2.9% inhibition, $n = 22$, $p < 0.001$). This suggests that LA interneurons express $GABA_B$ autoreceptors which might be heterodimerized from any of both isomers,

$GABA_{B(1a)}$ -/- or $GABA_{B(1b)}$ -/- with $GABA_{B(2)}$ subunit. Presynaptic inhibition of glutamatergic transmission by baclofen (50 µM) is impaired in $GABA_{B(1a)}$-/- mice which indicates that a high proportion of presynaptic $GABA_B$ heteroreceptors at thalamic afferents are mostly heterodimerized from $GABA_{B(1a)}$ isomer with $GABA_{B(2)}$ subunit.

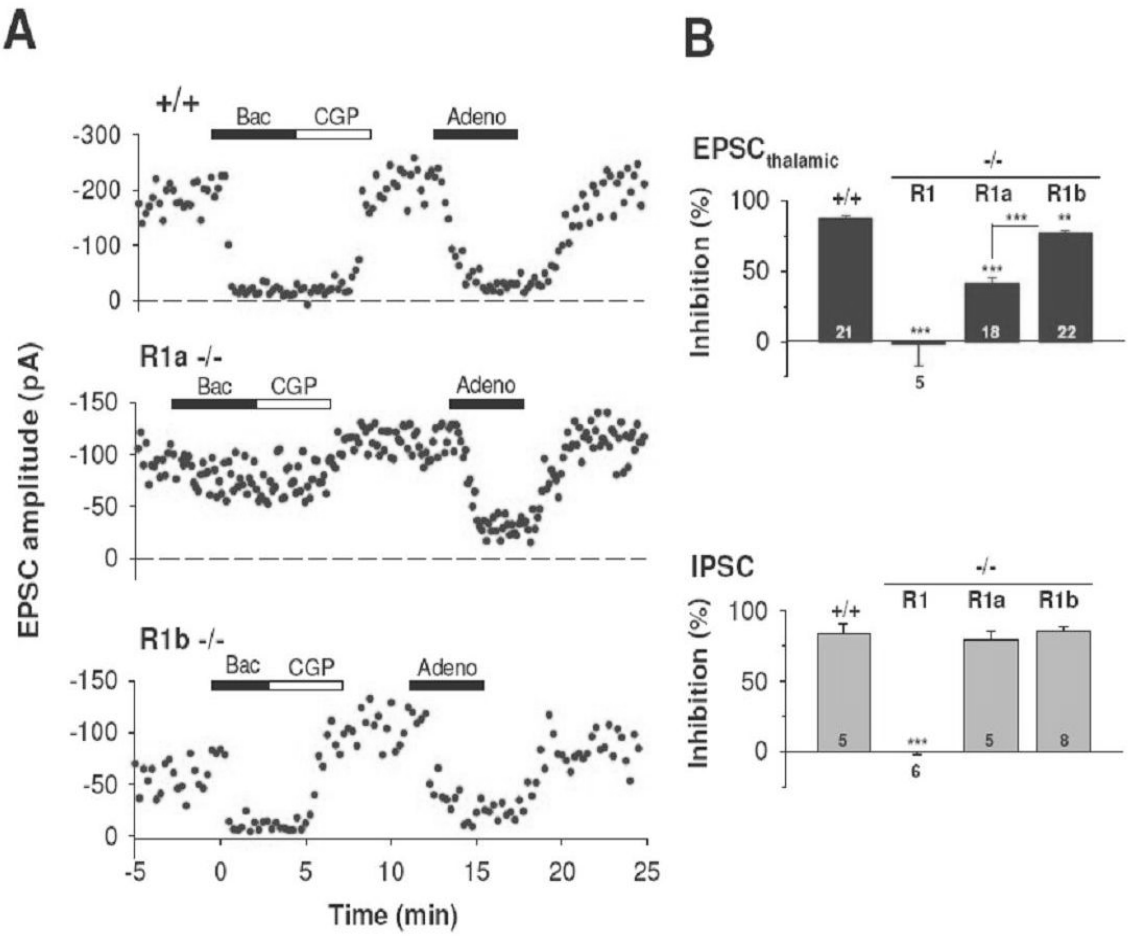

Figure 29) Presynaptic inhibition impairment in $GABA_{B(1a)}$-/- mice at thalamic afferents. **A:** Presynaptic inhibition of glutamatergic transmission by the $GABA_B$ receptor agonist baclofen (50 µM) is impaired in $GABA_{B(1a)}$-/- mice. Graphs show amplitudes of EPSCs evoked by thalamic afferent stimulation. Adenosine-induced inhibition was not different.. **B:** top**,** Both pre- and postsynaptic $GABA_B$-mediated inhibition is completely abolished in global $GABA_{B(1)}$ knock-outs. bottom, Presynaptic inhibition of inhibitory transmission (IPSCs) was not affected ((n) are indicated in the bars)

Input-specific facilitation of LTP induction at glutamatergic synapses in $GABA_{B(1a)}$ -/- and $GABA_{B(1b)}$ -/- mice

To test whether the facilitation of LTP induction at thalamic inputs is due to pre, or postsynaptic $GABA_B$ receptors, LTP was induced in both knockout mice.

If homosynaptic LTP at thalamic afferent is facilitated by blockade of presynaptic $GABA_B$ receptors, then we expect facilitation of LTP induction in $GABA_{B(1a)}$ -/-mice but if it is postsynaptic $GABA_B$ receptors, then we expect facilitation of LTP induction in both genotypes. Surprisingly, thalamic LTP induction was facilitated only in $GABA_{B(1b)}$ -/- (in the absence of CGP55845A)(Fig. 30B). Wild type mice; (EPSP slope: 100.9 ± 4.8% of the baseline, $n = 11$; $p < 0.001$), and with CGP55845A; (EPSP slope: 153.9 ± 20.7% of the baseline, $n = 11$; $p < 0.001$). In $GABA_{B(1b)}$ -/- mice, the EPSP slope was potentiated; (EPSP slope: 148.2 ± 10.5% of the baseline, $n = 19$; $p < 0.001$), whereas in $GABA_{B(1a)}$ -/- mice, the EPSP slope was not significantly changed; (EPSP slope: 105.8 ± 4.3% of the baseline, $n = 21$; $p < 0.001$). In mice with deleted $GABA_{B(1)}$ subunits (including the two isomers), similar potentiation was shown (EPSP slope: 141.0 ± 4.6% of the baseline, $n = 6$; $p < 0.001$)(Fig. 30C). Furthermore, this form of LTP in $GABA_{B(1b)}$ -/- mice is associated with no change in PPR (Fig. 30D). This is Consistent with our previous results that we observed in wild type mice where we observed facilitation of thalamic LTP in the presence of $GABA_B$ antagonist is associated with no change in PPR. Interestingly, this thalamic LTP in $GABA_{B(1b)}$ -/- mice was also dependent on postsynaptic Ca^{2+} influx. While it was abolished by perforating BAPTA (30 mM) in the intracellular solution, (EPSP slope: 108.0 ± 4.2% of the baseline, $n = 6$; $p < 0.001$) (Fig. 30E). This suggests that an increase in the postsynaptic Ca^{2+} concentration is required for the LTP induction mechanism

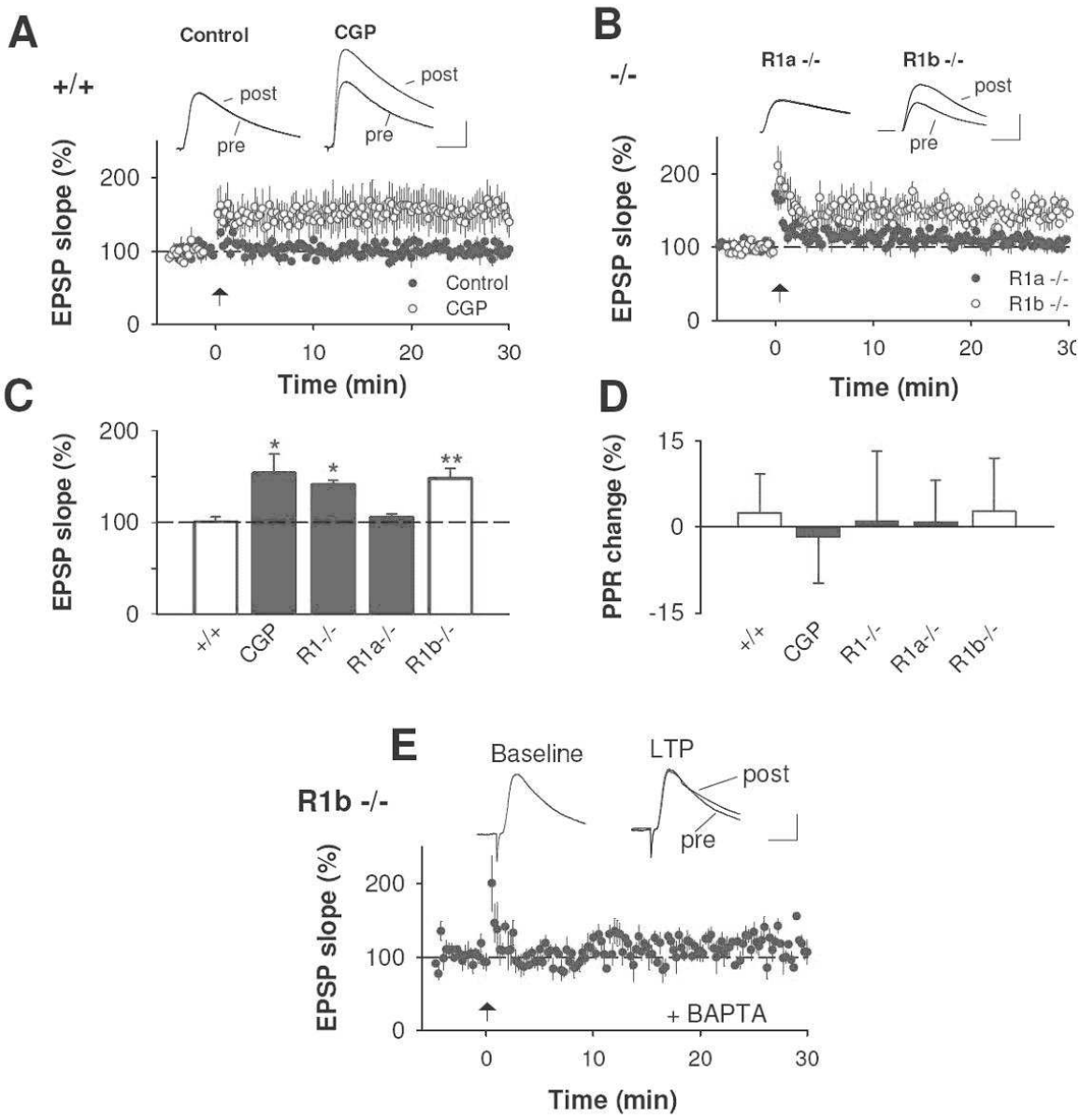

Figure 30) Input-specific facilitation of LTP induction $GABA_{B(1a)}$ -/- and $GABA_B$(1b) -/- mice **A:** Blockade of $GABA_B$ receptors by application of the specific $GABA_B$ receptor antagonist CGP55845A facilitates LTP induction at thalamic afferents in wild-type BALB/c mice. **B:** LTP at thalamic afferents is facilitated in $GABAB_{(1b)}$ -/- but not in $GABA_{B(1a)}$ -/- animals in the absence of CGP55845A. **C:** Summary graph illustrating input-specific facilitation of LTP induction in $GABA_{B(1a)}$-/- and $GABAB_{(1b)}$ -/- animals. **D:** No changes in PPR associated with LTP in all genotypes. **E:** LTP at thalamic afferents in $GABA_{B(1b)}$ -/- mice is abolished by intracellular BAPTA. scale bars: 5 mV, 50 ms.

Input-specific facilitation of LTP induction in disynaptic inhibitory inputs in $GABA_{B(1a)}$ -/- and $GABAB_{(1b)}$ -/- mice

Stimulation of thalamic or cortical afferents produces an IPSP in excitatory cells mediated by $GABA_A$ and $GABA_B$ receptors (Li et al. 1996; Lang and Pare 1998; Stutzmann and LeDoux 1999; Szinyei et al. 2000). LTP can be induced at thalamic excitatory synapses, to test the effect of induction of LTP at thalamic afferent on the feed-forward inhibition conducted by interneurons. To see the change of inhibitory inputs due to induction of LTP onto thalamic synapses, we recorded disynaptic GABAergic IPSC in LA excitatory neurons. By holding the projection neuron on a depolarized membrane potential (-50 mV) and stimulating thalamic and/ or cortical afferents. This elicited a clear EPSC followed by an IPSC (Fig. 31A). In wild type mice, disynaptic IPSCs were not changed from the baseline after applying the same induction protocol, (thalamic IPSC amplitude: 101.5 ± 5.8% of the baseline, $n = 8$, $p < 0.001$, cortical IPSC amplitude: 105.8 ± 8.3% of the baseline, $n = 8$, $p < 0.001$). $GABA_{B(1a)}$ -/- mice showed a clear LTP facilitation only at the cortical disynaptic IPSC (IPSC amplitude:175.4 ± 11.8% of the baseline, $n = 8$ $p < 0.001$), but not in thalamic (IPSC amplitude:102.6 ± 9.3% of the baseline, $n = 8$, $p < 0.001$)(Fig. 31B). In contrast, $GABA_{B(1b)}$ -/- mice showed significant LTP facilitation only in the thalamic disynaptic IPSC; (IPSC amplitude: 195.4 ± 17.3% of the baseline, $n = 9$, $p < 0.001$), but not in thalamic disynaptic IPSC; (IPSC amplitude:105.6 ± 6.8% of the baseline, $n = 9$ $p < 0.001$) (Fig. 31).

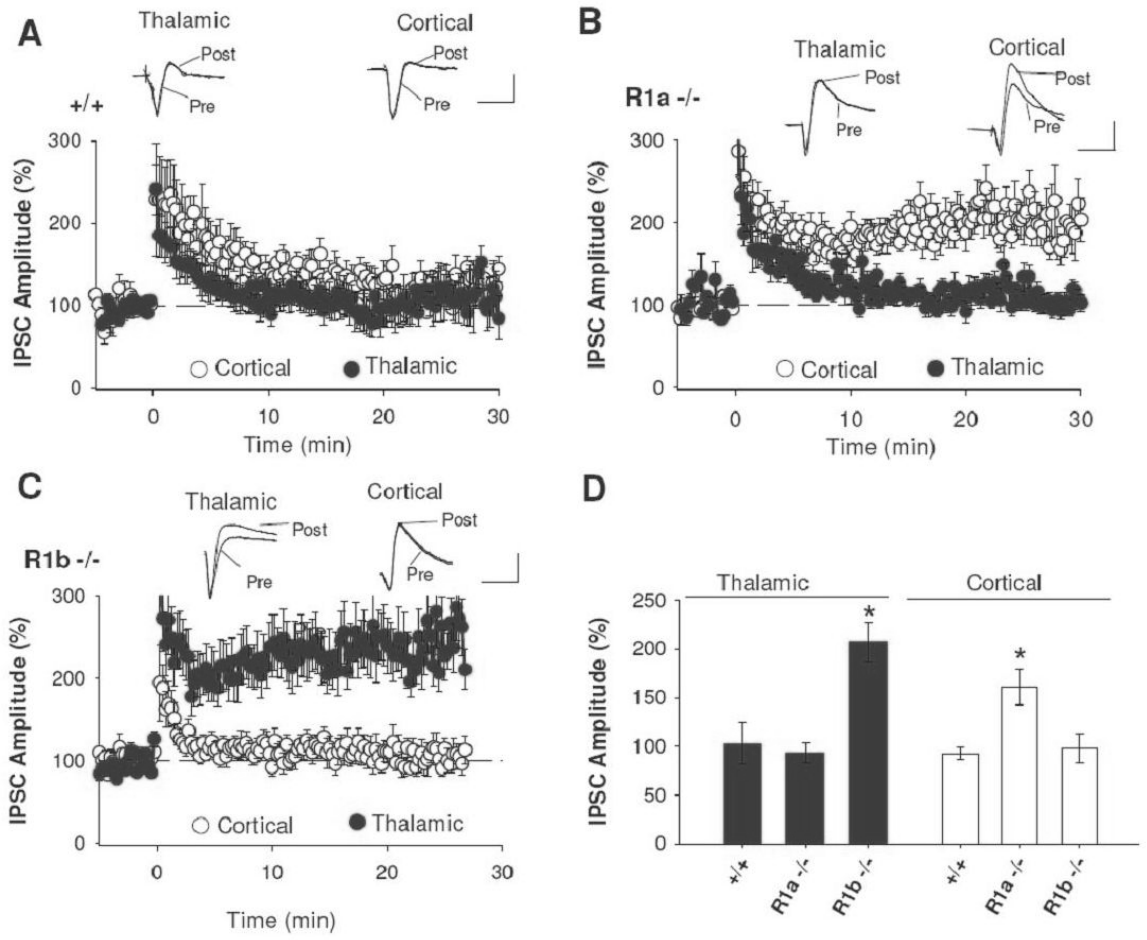

Figure 31) Disynaptic IPSCs input-specific facilitation of LTP induction in $GABA_{B(1a)}$-/- and $GABAB_{(1b)}$ -/- mice **A:** IPSCs are not potentiated in both afferents in wild type mice. **B:** LTP induction of IPSCs at cortical afferents is facilitated in $GABAB_{(1b)}$ -/- but not in $GABA_{B(1a)}$-/- animals in the absence of CGP55845A. **C:** LTP induction of IPSCs at thalamic afferents is facilitated in $GABAB_{(1b)}$ -/- but not in $GABA_{B(1a)}$ -/- mice in the absence of CGP55845A. **D: C:** Summary graph illustrating disynaptic input-specific facilitation of LTP induction in $GABA_{B(1a)}$-/- and $GABAB_{(1b)}$ -/- mice. scale bars: 5mv, 50 ms.

Deficits in cued fear conditioning in $GABA_{B(1b)}$ -/- mice

The above results suggest that in $GABA_{B(1b)}$ -/- mice, the thalamic inhibitory and excitatory synapses are specifically changed resulting in lower threshold for induction of LTP. To investigate the impact of this input-specific facilitation of LTP on emotional behavior, $GABA_{B(1b)}$ -/- mice were tested for auditory fear conditioning, a paradigm which widely used to test fear memory formation and the behavioral read-out of synaptic plasticity change. Animals were exposed either to tone (CS^+); paired with shock; or to different tone (CS^-); without footshock, 24 hours after training session mice were tested for fear memory formation (see Methods). In $GABA_{B(1b)}$ -/- mice we found a clear impairment in cued fear conditioning. Pairing of tone with footshock elicited fear

response in wild type mice that can be measured as percent of freezing. $GABA_{B(1b)}$ -/- mice showed less freezing behavior (n=9; $P<0.05$) relative to wild-type mice (Fig. 32A). To test whether $GABA_{B(1b)}$ -/- have different pain sensitivity than wild type, we repeated the whole set of experiments with a higher intensity footshock (0.9 mV). High intensity footshock, however, did not change the $GABA_{B(1)}$ -/- mice behavior in response to the CS^+ or CS^- (n=9, $P<0.001$) (Fig. 32B). Furthermore, we investigated the pain threshold of the mice by applying different footshock intensities and observed the first movement or the vocalization of the mice when reaching the pain threshold (Fig 32C). In our experiment, $GABA_{B(1b)}$ -/- and wild type mice showed similar pain sensitivity (Fig 32C).

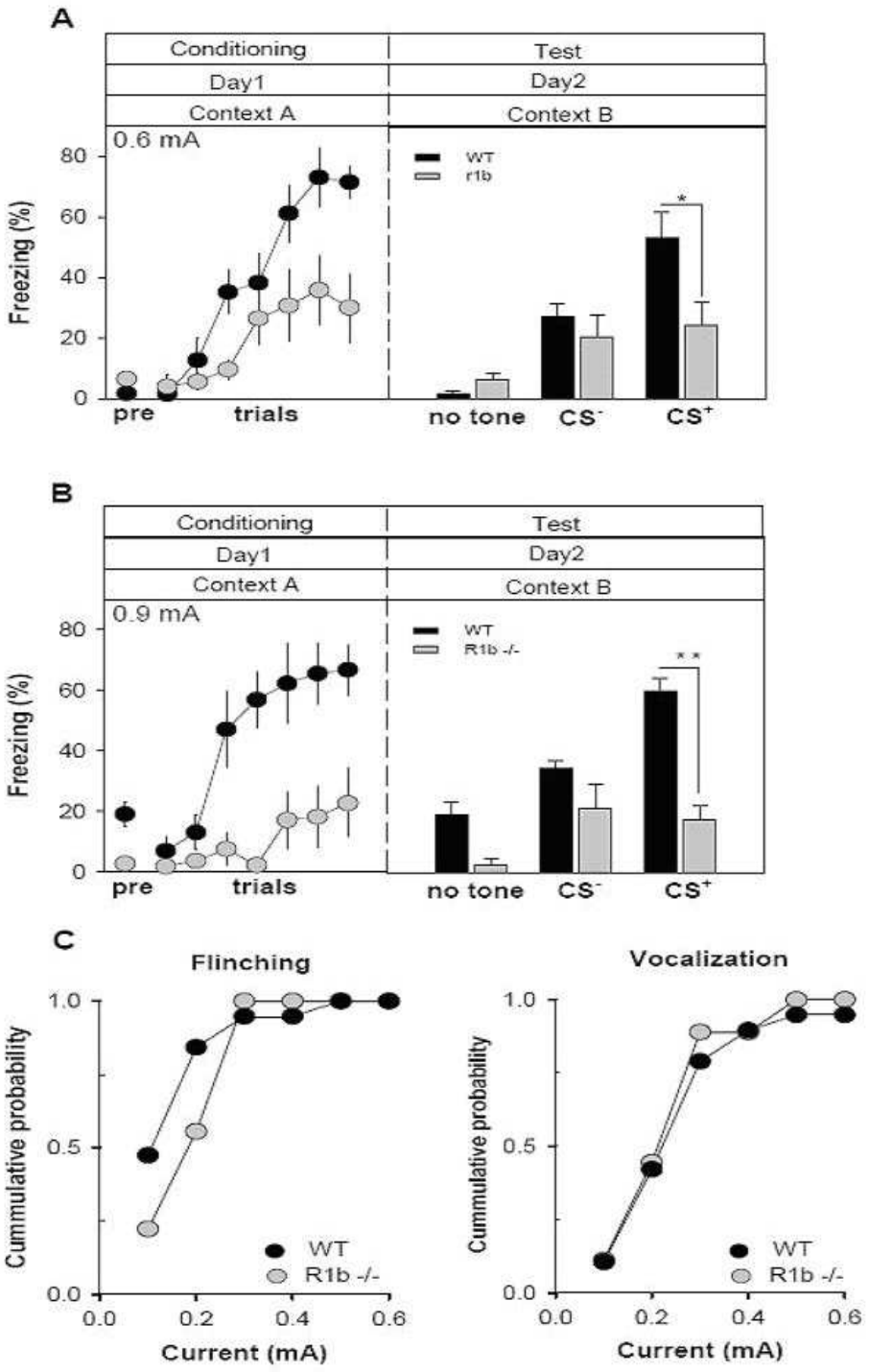

Figure 32) $GABA_{B(1b)}$ -/- mice exhibit specific deficits in fear learning A**:** Cued fear conditioning is impaired in $GABAB_{(1b)}$ -/- (n=9; P<0.05) mice relative to wild-type BALB/c animals (n=7). $GABA_{B(1b)}$ -/- mice cannot discriminate between the CS^+ (7.5 kHz, 80 dB tone) paired with the footshock) and the CS^- (3 kHz, 80 dB; unpaired). **B**: Higher shock intensity (0.9 mv) does not change the response to either CS^+ or CS-. **C:** Control of pain sensitivity of $GABA_{B(1b)}$ -/- mice. Right; flinching is similar to Wild type mice; Left; vocalization after footshock in $GABA_B$(1b) -/- mice is similar to wild-type mice.

5.3.4. Discussion

Pavlovian auditory fear conditioning results in long-lasting changes in synaptic transmission in the LA. In this experimental paradigm a neutral conditioned stimulus (CS) such as a tone is paired with an unconditioned stimulus (US), such as footshock (Quirk et al. 1995; McKernan and Shinnick-Gallagher 1997; Rogan et al. 1997; Collins and Pare 2000; Repa et al. 2001). Because inhibition was shown to play a critical role in fear memory formation (Li et al. 1996; Lang and Pare 1997), we focused on inhibition conducted by $GABA_B$ receptors. Many studies showed that $GABA_BR$-mediated inhibition regulates synaptic transmission in the LA (Asprodini et al. 1992; Li et al. 1996; McDonald et al. 2004). We first investigated the functionality of $GABA_BRs$ in the LA. Our results show that $GABA_BRs$ are functioning at thalamic and cortical synapses (Fig. 27). $GABA_BRs$ blockade facilitates the induction of LTP at thalamic afferents, although a weak subthreshold protocol was used, which does not induce LTP in control conditions (Fig. 28B). Thus, under physiological conditions $GABA_B$ receptors would increase the threshold for the induction of LTP at thalamic afferent.

Our results show that homosynaptic LTP at thalamic synapses was blocked by an NMDAR antagonist (Fig. 28D). Moreover, incorporation of Ca^{+2} chelator in the patch pipette prevents the induction of LTP, suggesting that elevation of postsynaptic Ca^{2+} concentration is involved in the induction mechanism. This indicates a postsynaptic induction mechanism. Furthermore, this LTP was associated with no change in paired-pulse ratio, indicating that a presynaptic mechanism is not involved.

Is this facilitation of thalamic LTP due to the blockade of pre- or postsynaptic $GABA_BRs$? Many studies showed a possible discrimination between pre- versus postsynaptic $GABA_BR$-mediated inhibition (Lambert and Wilson 1993; Phelan 1999; Yamada et al. 1999). In all these studies the specificity of the used pharmacological drugs, were questionable. Therefore, we used molecular tools to discriminate between pre- and postsynaptic $GABA_B$ receptor. Studies using Western blotting and immunohistochemistry with isoform-specific antisera showed that there is differential subcellular localization pointing at a pre- versus postsynaptic localization for $GABA_{B(1a)}$ and $GABAB_{(1b)}$, respectively (Kaupmann et al. 1998; Bischoff et al. 1999). In our previous study we showed that presynaptic $GABA_BR$- mediated inhibition at cortico-

amygdala afferents is specifically mediated by $GABA_B$ heteroreceptor composed of predominantly $GABA_{B(1a)}$ isoforms dimerized with $GABA_{B(2)}$ subunit. Here we showed that at thalamic synapses $GABA_{B(1a)}$ isoforms are predominantly presynaptically localized, whereas $GABA_{B(1b)}$ isoforms are mostly postsynaptically localized (Fig. 29). Our results show that presynaptic $GABA_BR$-mediated inhibition was strongly reduced in $GABA_{B(1a)}$ and postsynaptic inhibition was partially reduced in both genotypes (Fig. 29). This indicates some compensatory mechanism in the postsynaptic site. To explore the impact of this different inhibitory strength in knockout mice, we investigated the induction of LTP at thalamic afferents in both genotypes. $GABA_{B(1a)}$ -/- mice did not show facilitation of LTP induction in thalamic afferent, similar to control condition in the absence of CGP55845A (Fig. 30). This indicates that presynaptic inhibition at thalamic afferent is not affecting the induction of LTP in the projecting neurons, which is in agreement with result showed in (Fig. 28). Thus, thalamic LTP is most likely postsynaptically induced and expressed. Indeed, other studies showed that LTP at thalamic afferents is induced and expressed postsynaptically (Weisskopf et al. 1999; Bissiere et al. 2003; Humeau et al. 2005; Rumpel et al. 2005). This would suggest that any change in postsynaptic $GABA_B$ inhibitory input would specifically facilitate the induction of LTP only in thalamic afferents. Surprisingly, facilitation of the induction of LTP at thalamic afferents was specifically in $GABA_{B(1b)}$ -/- mice and not in $GABA_{B(1a)}$ -/- mice (Fig. 31). In our previous study we showed that postsynaptic $GABA_B$-mediated inhibition by the induction of outward K^+ current was equally reduced in $GABA_{B(1a)}$ -/- and $GABA_{B(1b)}$ -/- mice. The reason why this thalamic homosynaptic LTP was induced only in $GABA_{B(1b)}$ -/- and not $GABA_{B(1a)}$ -/- is not clear and needs more future investigations. A possible explanation to this paradoxical discrepancy is that $GABA_{B(1b)}$ subunit would be specifically coupled to other effectors (e.g., mGluR, ATF, PKA, PKC) (Calver et al. 2002; Bettler et al. 2004) than $GABA_{B(1a)}$ and by that specifically modulate postsynaptic LTP induction at thalamic afferents. Alternatively, $GABA_{B(1b)}$ receptors could be localized postsynaptically more close to the synaptic input than $GABA_{B(1a)}$ receptors so that they have stronger control of the synaptic inputs. This differential synaptic localization at the postsynaptic cell could not be observed in our experiments set

as we applied high concentration of baclofen (100μM) that activate all $GABA_BR$ subtypes irrespective of its location.

Homosynaptic LTP at thalamic afferents in $GABA_{B(1b)}$ -/- mice was abolished by incorporation of BAPTA in the postsynaptic neuron (Fig. 31). Similar to thalamic afferents LTP induced in wild type mice in the presence of CGP55485A. Further experiment would be needed to test if this LTP also needs postsynaptic NMDA receptor activation.

It is well established that stimulation of thalamic or cortical afferents produces an IPSP in excitatory cells mediated by $GABA_A$ and $GABA_B$ receptors (Sugita et al. 1993; Li et al. 1996). Thus, stimulation of either thalamic or cortical inputs would also stimulate inhibitory feed-forward inhibition. Recording disynaptic IPSCs from a projecting neuron showed a facilitation of LTP induction only at cortical afferents in $GABA_{B(1a)}$ -/- mice and specifically at thalamic afferents in $GABA_{B(1b)}$ -/- mice similar to the input specific facilitation at excitatory synapses (Fig. 30, 32). A previous study (Bissiere et al. 2003) reported a feed-forward inhibition at the thalamic afferents that can be modulated by dopamine receptors. Also, a recent study (Bauer and LeDoux 2004) reported that isolated GABAergic IPSPs between inhibitory and excitatory neurons could be potentiated. Additionally, endocannabinoid-dependent LTD was also reported in other previous study in isolated IPSPs (Marsicano et al. 2002). Whether potentiation of the disynaptic IPSCs occurs at excitatory inputs onto interneuron or at interneuron axons onto projecting neurons, is less clear. Further experiments would be required to investigate the site of synapses potentiation.

This input-specific facilitation of LTP induction in glutamatergic and GABAergic synapses would certainly affect the formation of fear memory. Behavioral analysis of $GABA_{B(1b)}$ -/- mice revealed impairment in associating CS with US (Fig. 32). Although we tried even stronger footshock intensity to examine if the $GABA_{B(1)}$ -/- mice have different pain perception, the results were similar with the lower intensity footshock (Fig. 32). Future experiments should reveal if $GABA_{B(1b)}$ -/- mice do freeze by US other than footshock (e.g., fox odour).

In our previous study we observed generalization of conditioned fear in $GABA_{B(1a)}$ -/- mice. Here, we observed fear learning deficit in $GABA_{B(1b)}$ -/- mice, presumably due to

the recorded invitro facilitation of postsynaptic LTP induction at thalamic afferents. The loss of postsynaptic $GABA_B$-mediated inhibition in the thalamic afferents would decrease the threshold for LTP induction in the postsynaptic cell. Induction of associative NMDA receptor-dependent LTP in the LA is thought to underlie the acquisition of classical fear conditioning in rodents (LeDoux 2000; Maren and Quirk 2004; Rumpel et al. 2005). Associative LTP induction protocol at thalamic afferents requires pairing presynaptic activation with postsynaptic depolarization (Humeau et al. 2002; Bissiere et al. 2003; Humeau et al. 2003). Furthermore, it was reported that $GABA_BR$-mediate inhibition of the NMDA component of synaptic transmission in the rat hippocampus (Morrisett et al. 1991) and rat amygdala (Huang and Gean 1994).

Here we report homosynaptic LTP at thalamic afferents that can be induced postsynaptically at lower LTP induction threshold. This lowering of LTP induction threshold would lead to wide-ranging activation in the whole LA network irrespective of the input afferents. This is possibly why $GABA_{B(1b)}$ -/- mice show impairment in fear learning as homosynaptic LTP would be unspecifically facilitated and occlude further induction of LTP during fear learning. However, we can not exclude the loss of $GABA_{B(1b)}R$-mediated inhibition in other amygdala nuclei than LA that would also lead to impairment in fear learning like the BL or the output nuclei CE. Moreover, other brain area up- and/or downstream from the amygdala could be also affected specifically in $GABA_{B(1b)}$ -/- mice that would affect the fear learning process.

The diversity of these memory effects makes it difficult to identify a common mechanism. The widespread distribution of $GABA_BRs$ in the brain and the numerous modulatory effects on various synapses leave ample room for speculations.

Taken together, here we show that $GABA_{B(1b)}$ receptors are mostly postsynaptically localized and mediate postsynaptic inhibition that keep NMDAR from the activation threshold and by that facilitate its function as associative detector of network activity. The loss of this postsynaptic $GABA_BR$-mediated inhibition leads to unspecific induction of LTP and impairment of fear learning.

Although GABA-mediated neurotransmission has long been known to have a crucial role in anxiety, data on the specific role of $GABA_B$ receptors are limited and variable (Millan

2003). A recent study showed that $GABA_{B(1)}$ -/- mice are more anxious than their wild type counterparts in several anxiety-related tests such as the light–dark box and staircase test (Mombereau et al. 2005). Interestingly, the anxiolytic-like effects of benzodiazepines are markedly diminished in $GABA_{B(1)}$-/- mice (Mombereau et al. 2004; Mombereau et al. 2004).

Our data indicate that activation of $GABA_B$ receptors might reduce anxiety. Indeed, the specific $GABA_BR$ agonist baclofen clinically demonstrated reversal effect on anxiety associated with alcohol withdrawal (Addolorato et al. 2002), post-traumatic stress (Drake et al. 2003), panic disorder (Breslow et al. 1989) and traumatic spinal-cord lesions (Hinderer 1990). More recently, the positive modulator GS39783 has been shown to be active in several animal models of anxiety (Cryan et al. 2004; Mombereau et al. 2004; Cryan and Kaupmann 2005). Therefore, more studies, focusing on behavioral and electrophysiological responses of $GABA_B$ receptor activation in LA, are needed.

Inhibitory inputs firmly control the activity of projecting LA principal cells (LeDoux 2000). The neuronal population in LA, however contains about 5-10 % interneurons, which have a lower spike threshold and show no spike frequency adaptation, so that strong stimulation of afferent inputs to LA produces a predominantly hyperpolarizing response in excitatory cells (Rainnie et al. 1991; Lang and Pare 1997). Moreover, IPSPs truncate evoked and spontaneous EPSPs in excitatory neurons. This suggests that IPSP potentiation would lead to change in the input–output functions of LA principal cells (Lang and Pare 1997; Gaiarsa et al. 2002).

In conclusion, we show that postsynaptic $GABA_B$ receptors control the threshold for the induction of LTP at thalamic afferents. This potentiation of synaptic transmission at thalamic afferents requires postsynaptic Ca^{2+} elevation and NMDA receptors activation. Finally we assume that localization of $GABA_{B(1b)}$ subunit isomer in the postsynaptic site would probably play a key role in regulating fear memory formation.

5.4. Redistribution of GABAB(1) Protein and Atypical GABAB Responses in GABAB(2)-Deficient Mice

Martin Gassmann,[1] * Hamdy Shaban,[2,3] * Réjan Vigot,[1] * Gilles Sansig,[4] Corinne Haller,[1] Samuel Barbieri,[1] Yann Humeau,[2,3,5] Valérie Schuler,[4] Matthias Müller,[4] Bernd Kinzel,[4] Klaus Klebs,[4] Markus Schmutz,[4] Wolfgang Froestl,[4] Jakob Heid,[4] Peter H. Kelly,[4] Clive Gentry,[6] Anne-Lise Jaton,[4] Herman Van der Putten,[4] Cédric Mombereau,[4] Lucas Lecourtier,[4] Johannes Mosbacher,[4] John F. Cryan,[4] Jean-Marc Fritschy,[7] Andreas Lüthi,[2,3] Klemens Kaupmann,[4] and Bernhard Bettler[1]

[1]Pharmazentrum, Department of Clinical-Biological Sciences, University of Basel, CH-4056 Basel, Switzerland, [2]Friedrich Miescher Institute, CH-4058
Basel, Switzerland, [3]Biozentrum, University of Basel, CH-4056 Basel, Switzerland, [4]Novartis Institutes for Biomedical Research, Novartis Pharma AG, CH-
4002 Basel, Switzerland, [5]Centre National de la Recherche Scientifique, Unite´ Propre de Recherche 2356, F-67084 Strasbourg, France, [6]Novartis Institute for
Medical Sciences, London WC1E 6B9, United Kingdom, and [7]Institute of Pharmacology and Toxicology, University of Zu¨rich, CH-8057 Zu¨rich, Switzerland

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In this paper I have done the electrophysiological investigations in the hippocampal slices. I could detect atypical electrophysiological $GABA_B$ responses in hippocampal slices of $GABA_{B(2)}^{-/-}$ mice (Fig 37). I have showed the loss of presynaptic $GABA_B$ functions in $GABA_{B(2)}^{-/-}$ mice (Fig37). Specifically, I have demonstrated that $GABA_{B(2)}^{-/-}$ mice lack functional $GABA_B$ heteroreceptors on Schaffer collateral terminals (Fig. 37). Moreover, I have showed that hippocampal interneurons lack $GABA_B$ autoreceptors in $GABA_{B(2)}^{-/-}$ mice (Fig. 37). I have investigated this atypical $GABA_BR$ response in $GABA_{B(2)}$ -/- mice and I have showed that $GABA_B$ receptors inhibit instead of activate K^+ channels in $GABA_{B(2)}^{-/-}$ mice (Fig. 38)

5.4.1. Summary

$GABA_B$ receptors mediate slow synaptic inhibition in the nervous system. In transfected cells, functional $GABA_B$ receptors are usually only observed after coexpression of $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits, which established the concept of heteromerization for G-protein-coupled receptors. In the heteromeric receptor, $GABA_{B(1)}$ is responsible for binding of GABA, whereas $GABA_{B(2)}$ is necessary for surface trafficking and G-protein

coupling. Consistent with these *in vitro* observations, the $GABA_{B(1)}$ subunit is also essential for all $GABA_B$ signaling *in vivo*. Mice lacking the $GABA_{B(1)}$ subunit do not exhibit detectable electrophysiological, biochemical, or behavioral responses to $GABA_B$ agonists. However, $GABA_{B(1)}$ exhibits a broader cellular expression pattern than $GABA_{B(2)}$, suggesting that $GABA_{B(1)}$ could be functional in the absence of $GABA_{B(2)}$. We now generated $GABA_{B(2)}$-deficient mice to analyze whether $GABA_{B(1)}$ has the potential to signal without $GABA_{B(2)}$ in neurons. We show that $GABA_{B(2)}^{-/-}$ mice suffer from spontaneous seizures, hyperalgesia, hyperlocomotor activity, and severe memory impairment, analogous to $GABA_{B(1)}^{-/-}$ mice. This clearly demonstrates that the lack of heteromeric $GABA_{B(1,2)}$ receptors underlies these phenotypes. To our surprise and in contrast to $GABA_{B(1)}^{-/-}$ mice, we still detect atypical electrophysiological $GABA_B$ responses in hippocampal slices of $GABA_{B(2)}^{-/-}$ mice. Furthermore, in the absence of $GABA_{B(2)}$, the $GABA_{B(1)}$ protein relocates from distal neuronal sites to the soma and proximal dendrites. Our data suggest that association of $GABA_{B(2)}$ with $GABA_{B(1)}$ is essential for receptor localization in distal processes but is not absolutely necessary for signaling. It is therefore possible that functional $GABA_B$ receptors exist in neurons that naturally lack $GABA_{B(2)}$ subunits.

5.4.2. Introduction

GABA, the predominant inhibitory neurotransmitter in the mammalian nervous system, signals through ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. $GABA_B$ receptors are coupled to G-proteins and modulate synaptic transmission by activating postsynaptic inwardly rectifying Kir3-type K^+ channels and by controlling neurotransmitter release (Bowery et al. 2002; Calver et al. 2002; Bettler et al. 2004) Molecular studies on $GABA_B$ receptors provide compelling evidence for heteromerization among G-protein-coupled receptors (GPCRs) (Marshall et al. 1999); (Mohler et al. 2001). Most experiments with cloned $GABA_{B(1)}$and $GABA_{B(2)}$ subunits expressed in heterologous cells and sympathetic neurons (Filippov et al. 2000) indicate that individual subunits are functionally inert unless they are co-expressed. $GABA_B$ receptors therefore appear different from other heterodimeric GPCRs in which individual

subunits are functional when expressed alone (Bouvier 2001). In the $GABA_B$ heteromer, the $GABA_{B(1)}$ $GABA_{B(1)}$ subunit binds GABA and all competitive $GABA_B$ ligands (Kaupmann et al. 1998), whereas the $GABA_{B(2)}$ subunit is responsible for escorting $GABA_{B(1)}$ to the cell surface and for activating the G-protein (Margeta-Mitrovic et al. 2000; Margeta-Mitrovic et al. 2001); (Calver et al. 2001); (Galvez et al. 2001); (Pagano et al. 2001); (Robbins et al. 2001). Two $GABA_{B(1)}$ isoforms, $GABA_{B(1a)}$ and $GABA_{B(1b)}$, arise by differential promoter usage (Kaupmann et al. 1997); (Bettler et al. 2004). Thus far, the data support the existence of two predominant, yet pharmacologically indistinguishable, $GABA_B$ receptors in the nervous system, the heteromeric $GABA_{B(1a),\ 2}$ and $GABA_{B(1b),\ 2}$ receptors $GABA_{B(1)}$-/- mice do not exhibit detectable $GABA_B$ responses in a variety of experimental paradigms, demonstrating that $GABA_{B(1)}$ is not only essential for $GABA_B$ signaling *in vitro* but also *in vivo* (Prosser et al. 2001); (Schuler et al. 2001); (Queva et al. 2003). However, no *in vivo* experiment addressed whether $GABA_{B(1)}$ can assemble functional $GABA_B$ receptors by itself or in association with a protein other than $GABA_{B(2)}$. In support of a separate role, GABAB(1) exhibits a more widespread cellular distribution than does $GABA_{B(2)}$ ((Kaupmann et al. 1998; Clark et al. 2000; Ng and Yung 2001; Burman et al. 2003; Kim et al. 2003; Kulik et al. 2003; Li et al. 2003). Furthermore, at odds with a strict requirement of GABAB(2) for plasma membrane delivery, $GABA_{B(1)}$ was originally cloned by surface expression in mammalian cells (Kaupmann et al. 1997). Additionally, $GABA_{B(2)}$ occasionally yields electrophysiological or biochemical responses when transfected alone into heterologous cells (Kaupmann et al. 1997; Kaupmann et al. 1998)It is therefore conceivable that $GABA_{B(1)}$ is functional either alone or in combination with an unknown protein. However, it remains unclear whether sporadic endogenous expression of $GABA_{B(2)}$ in heterologous cells is responsible for the surface expression and the responses that were seen when $GABA_{B(1)}$ was transfected alone. To clarify whether $GABA_{B(1)}$ can participate in functional $GABA_B$ receptors in the absence of $GABA_{B(2)}$ subunit-with collaboration with Bettler B. group-$GABA_{B(2)}$ knockout mice was generated.

Methods

Generation and analysis of $GABA_{B(2)}^{-/-}$ mice: $GABA_{B(2)}^{-/-}$ mice were generated in the BALB/c inbred strain using a newly established BALB/c embryonic stem (ES) cell line.

A targeting construct was designed containing a neomycin resistance cassette (pRay-2; GenBank accession number U63120) flanked by 4.5 and 1.8 kb of genomic *GABA*$_{B(2)}$ DNA that was amplified from a C57BL/6 bacterial artificial chromosome. Homologous recombination was confirmed by Southern blot analysis (Fig. 33*A, B*). Selected ES cell clones were microinjected into C57BL/6 blastocysts. Chimeric males were crossed with BALB/c females, resulting in an F1 generation of inbred BALB/c $GABA_{B(2)}^{+/-}$ mice. $GABA_{B(2)}^{+/-}$ mice were viable and fertile and allowed the generation of $GABA_{B(2)}^{-/-}$ mice in the F2 generation. The probes used in Northern blot analysis (Fig. 33*C*) hybridize to exons 4-8 and exons 11-15 upstream (5' probe) and downstream (3' probe) of the deletion, respectively (Martin et al. 2001). For *in situ* hybridization (Fig. 33*D*), antisense oligonucleotides corresponding to nucleotides 2039-79 and 1810-54 of the rat $GABA_{B(1a)}$ (GenBank accession number Y10369) and $GABA_{B(2)}$ (GenBank accession number AJ011318) cDNAs, respectively, were used. The probes were radiolabeled with [^{35}S]dATP (NEG0345H; NEN, Boston, MA) using terminal deoxynucleotidyl transferase (Promega, Madison, WI). For immunoblot analysis (Fig. 33*E, F*), polyclonal antibodies directed against the C terminus of $GABA_{B(2)}$ (AB5394; Chemicon, Temecula, CA), the C terminus of $GABA_{B(1)}$ (antibody 174.1) (Malitschek et al., 1998), the N terminus of $GABA_{B(2)}$ (antibody N22) (Kaupmann et al., 1998), and mouse calreticulin (ab4; Abcam, Cambridge, UK) were used. Monoclonal antibodies were used to detect PSD-95 (postsynaptic density protein-95) (MAB1598, Chemicon) and syntaxin (Sigma, St. Louis, MO). Blots were exposed to HRP-conjugated secondary antibodies [NA9340 (Amersham Biosciences, Little Chalfont, UK); A5545 and A0168 (Fluka, Buchs, Switzerland)] and developed using the ECL chemiluminescent detection system (RPN2016; Amersham Biosciences). Brain membrane preparations, ligand binding assays, and receptor autoradiography were performed as described previously (Olpe and Karlsson 1990; Kaupmann et al. 1997). Synaptic plasma membranes were isolated from the P2 pellets of brain lysates by combined flotation-sedimentation density-gradient centrifugation (Jones and Matus 1974). [^{3}H]CGP62349 (80 Ci/mmol), [^{3}H]CGP54626 (40 Ci/mmol), [^{125}I]CGP71872 (2000 Ci/mmol), and [^{125}I]CGP64213 (2000 Ci/mmol) were purchased from ANAWA (Wangen, Switzerland). [^{35}S]GTPγS (1000 Ci/mmol) was obtained from Amersham Biosciences. Nonradio-active $GABA_B$ receptor ligands were from Novartis

(Basel, Switzerland). [^{35}S]GTPγS binding was performed with 20 µg of membrane protein, 0.2 nM [^{35}S]GTPγS, and test compounds in 96-well Packard (Meridian, CT) Pico-Plates as described previously (Urwyler et al., 2001).

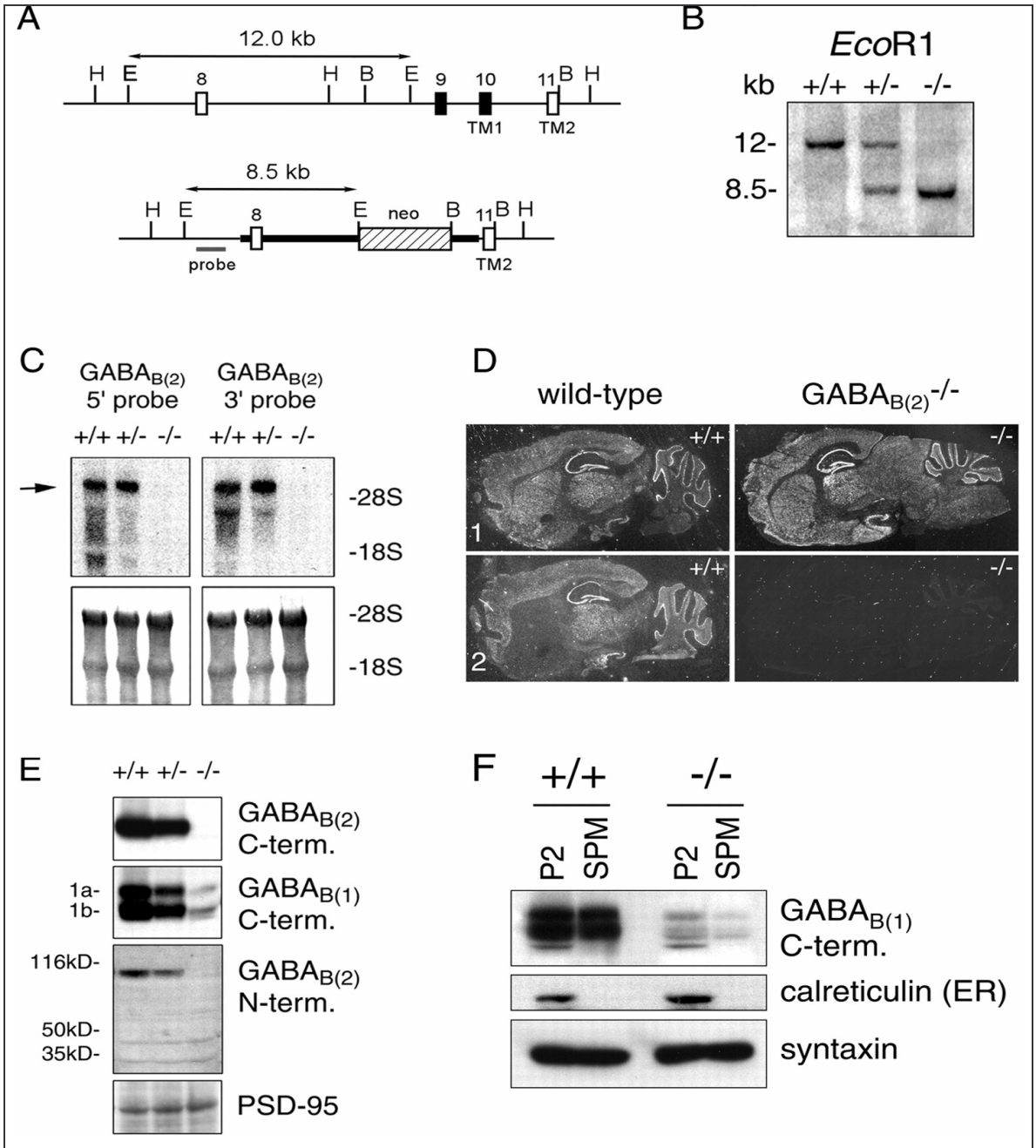

Figure 33). Characterization of GABA$_{B(2)}$$^{-/-}$ mice. *A*, Top, *GABA$_{B(2)}$* locus encompassing exons 8-11, encoding part of the N-terminal and the transmembrane (TM) domains 1 and 2. Bottom, *GABA$_{B(2)}$* allele after homologous recombination with a targeting construct containing a neomycin resistance cassette (neo) flanked by 4.5 and 1.8 kb of genomic DNA (bold lines). Exons 9 and 10 (3.5 kb, black boxes) are deleted. The Southern blot probe used in *B* is indicated. H, *Hind*III; E, *Eco*RI; B, *Bam*HI. *B*, Southern blot analysis of *Eco*RI cut genomic DNA from wild-type (+/+), GABA$_{B(2)}$$^{+/-}$ (+/-), and GABA$_{B(2)}$$^{-/-}$ (-/-) mice. *C*, Top,

Northern blot analysis of total brain RNA hybridized with $GABA_{B(2)}$ cDNA probes upstream (5' probe) and downstream of the deletion (3' probe). The probes hybridize to a band just above the 28 S ribosomal RNA (arrow) in wild-type and $GABA_{B(2)}^{+/-}$, but not in $GABA_{B(2)}^{-/-}$, mice. Bottom, Blots from top panels stained with methylene blue, demonstrating equal loading of RNA. Ribosomal RNA bands (18 and 28 S) are labeled. *D, In situ* hybridization analysis of $GABA_{B(1)}$ (1, top) and $GABA_{B(2)}$ (2, bottom) transcripts of sagittal sections from adult wild-type and $GABA_{B(2)}^{-/-}$ brains. *E*, Immunoblot analysis of brain extracts from adult mice using antibodies directed against C-terminal and N-terminal epitopes of $GABA_{B(2)}$ and $GABA_{B(1)}$. Antibodies to PSD-95 control for equal loading. $GABA_{B(1a)}$ (1a) and $GABA_{B(1b)}$ (1b) proteins are indicated. *F*, Immunoblot analysis demonstrating the presence of $GABA_{B(1)}$ protein in synaptic plasma membranes (SPM) purified from the P2 pellet (P2) of brain extracts of wild-type and $GABA_{B(2)}^{-/-}$ mice. Antibodies to calreticulin show that the synaptic plasma membrane fraction is free of ER proteins. Equal loading of samples was controlled with anti-syntaxin antibodies. To detect putative truncated $GABA_{B(2)}$ proteins, we used 15% SDS-PAGE and N-terminal $GABA_{B(2)}$ antibodies. In all other immunoblot experiments, we used 10% SDS-PAGE.

Immunohistochemistry: Immunoperoxidase staining was performed in brain sections of adult mice using guinea pig antisera against $GABA_B(2)$ (1:5000; AB5394; Chemicon) and $GABA_B(1)$ (1:3000; AB1531; Chemicon). Mice were deeply anesthetized with Nembutal (50 mg/kg) and perfused through the ascending aorta with 4% paraformaldehyde in 0.15 M phosphate buffer. Brains were postfixed for 3 hr, processed for antigen retrieval using microwave irradiation (Fritschy et al. 1998), cryoprotected in sucrose, and cut at 40 µm with a sliding microtome. The immunoperoxidase staining was performed using diaminobenzidine as chromogen (Fritschy et al. 1999). Tissue from different genotypes was processed together to minimize variability attributable to the staining procedure. Sections were analyzed by light microscopy (Axioskop; Zeiss, Jena, Germany) and photographed with a high-resolution digital camera.

Electrophysiology: Transverse hippocampal slices (350-µm-thick) from 3- to 4-week-old mice were prepared. Slices were maintained for 45 min at 35°C in an interface chamber containing saline equilibrated with 95% O_2 and 5% CO_2 and containing the following (in mM): 124 NaCl, 2.7 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 1.24 NaH_2PO_4, 18 glucose, and 2.25 ascorbate. Slices were then kept for at least 45 min at room temperature before being transferred to a superfusing recording chamber. Whole-cell recordings from CA1 pyramidal cells were performed at 30-32°C using infrared videomicroscopy to visualize cells. Patch electrodes (3-5 MΩ) were filled with a solution containing the following (in mM): 140 Cs-gluconate, 10 HEPES, pH 7.25, 10 phosphocreatine, 5 QX-314-Cl, 4 Mg-ATP, and 0.3 Na-GTP (295 mOsm). For measurements of postsynaptic holding currents (at -50 mV, in 0.5 µM TTX), Csgluconate was replaced by equimolar K-gluconate, and

QX-314 was omitted. Synaptic currents were elicited every 15 sec using a bipolar platinum-iridium electrode (diameter, 25 µm). EPSCs were measured at -70 mV in the presence of picrotoxin (100 µM). IPSCs were measured at 0 mV in the presence of kynurenic acid (2 mM). All experiments assessing presynaptic $GABA_B$ receptor function were performed in the presence of $BaCl_2$ (200 µM) to prevent the activation of postsynaptic Kir3 channels. $BaCl_2$ did not affect the EPSC or IPSC amplitudes. Current-voltage (*I-V*) relationships were assessed by ramp-command protocols (from -40 to -140 mV, 250 msec duration) before and after the application of agonists, and the agonist-induced *I-V* relationship was derived by subtraction. Data were recorded with an Axopatch 200B (Axon Instruments, Union City, CA), filtered at 2 kHz, and digitized at 10 kHz. Data were acquired and analyzed with the LTP Program (W. Anderson, University of Bristol, Bristol, UK) (Anderson and Collingridge, 2001) or with pClamp8.0 (Axon Instruments). All membrane potentials were corrected for the experimentally measured liquid junction potential of 11 mV for the internal K-gluconate solution. Slope conductance was determined between -140 mV and the reversal potential. Series resistance was monitored throughout the experiments by applying a hyperpolarizing pulse, and, if it changed >15%, the data were not included in the analysis. All values are expressed as means ± SEM. Statistical comparisons were done with paired or unpaired Student's *t* test as appropriate, at a significance level of 0.05. $GABA_B$ receptor ligands were from Novartis. Non-GABAergic drugs were from Fluka.

*Electroencephalogram measurements***:** Electroencephalogram (EEG) measurements were performed as described previously (Schuler et al. 2001; Kaupmann et al. 2003). The behavior of the mice, which were individually housed in wooden observation cages, was monitored with a video system. EEGs were amplified using an isolated four-channel bipolar EEG amplifier (EEG-2104; Spectralab, Maharashtra, India), recorded on a thermo recorder (MTK95; Astro-Med, West Warwick, RI), and stored on disk.

Measurement of core body temperature, locomotor activity, rotarod, and memory performance: Rectal temperature was determined to the nearest 0.1°C as described previously (Schuler et al. 2001; Kaupmann et al. 2003). Statistical analysis was

performed using repeated-measures ANOVA, followed by Fisher's least significant difference test when appropriate. Locomotor activity was recorded using a color video camera, surveying the open field, and analyzed using EthoVision 1.90 software (Noldus Information Technology, Wageningen, The Netherlands). To assess rotarod performance, mice were trained to stay on the rotarod (12 rpm) for 300 sec over two separate sessions the day before the experiment. During the test day, the length of time each mouse remained on the cylinder ("endurance time"; maximal score of 300 sec) was measured immediately before (time 0) and 1, 2, and 4 hr after the application of L-baclofen (12.5 mg/kg) or vehicle (0.5% methylcellulose). The dose of baclofen that shows maximal effects on rotarod performance was determined in previous studies (Schuler et al. 2001). Memory performance in the passive avoidance test was performed as described previously (Venable and Kelly 1990; Schuler et al. 2001).

Nociceptive tests: Heat or mechanical nociceptive stimuli were used in the antinociceptive tests as described previously (Schuler et al. 2001). The hotplate (Eddy and Leimbach 1953), tail-flick (D'Amour FE 1941), and the paw-pressure tests (Randall and Selitto 1957) are well established techniques to assess acute pain. The tail flick is a reflex response to a noxious thermal stimulus applied to the tail and is generally held to represent a spinal reflex response, whereas the hotplate response to a noxious thermal stimulus to the plantar surface of the paws is thought to involve supraspinal sites.

5.4.3. Results

Previous experiments showed that only $GABA_{B(1)}^{-/-}$ mice generated in the inbred BALB/c genetic background are viable (Prosser et al. 2001; Schuler et al. 2001; Queva et al. 2003). We therefore ablated the *GABA*$_{B(2)}$ gene in BALB/c ES cells (Fig. 33*A*). Southern blot analysis confirms deletion of exons 9 (81 bp) and 10 (151 bp), encoding part of the N-terminal extracellular and the first transmembrane domain of $GABA_{B(2)}$ (Fig. 33*B*). BALB/c $GABA_{B(2)}^{-/-}$ mice are viable, occur at a Mendelian ratio, and do not express detectable $GABA_{B(2)}$ mRNA, as shown by Northern blot analysis using hybridization probes flanking the *GABA*$_{B(2)}$ gene deletion (Fig. 33*C*). This demonstrates that any

truncated mRNA produced from the 5' part of the *GABA$_{B(2)}$* gene is highly unstable. The complete lack of $GABA_{B(2)}$ mRNA is confirmed by *in situ* hybridization (Fig. 33*D*). Hence, $GABA_{B(2)}^{-/-}$ mice do not express any full-length or truncated $GABA_{B(2)}$ protein, as shown by immunoblotting using antibodies directed against extreme C- or N-terminal epitopes (Fig. 33*E*). Immunoblot analysis further reveals that $GABA_{B(2)}^{+/-}$ mice express less $GABA_{B(2)}$ protein than wild-type mice. A densitometric analysis of *in situ* hybridizations from several brain sections reveals that $GABA_{B(1)}$ mRNA expression in $GABA_{B(2)}^{-/-}$ mice is not significantly changed when compared with wild-type littermates (Fig. 33*D*). However, immunoblot analysis indicates an ~50 and 90% reduction of $GABA_{B(1)}$ protein in $GABA_{B(2)}^{+/-}$ and $GABA_{B(2)}^{-/-}$ mice, respectively (Fig. 33*E*). This is reminiscent of the almost complete absence of $GABA_{B(2)}$ protein previously seen in $GABA_{B(1)}^{-/-}$ mice and yet again demonstrates that the two subunits cross-stabilize each other (Prosser et al. 2001; Schuler et al. 2001; Queva et al. 2003). Despite this considerable downregulation, we clearly detect $GABA_{B(1)}$ protein in synaptic plasma membrane preparations of $GABA_{B(2)}^{-/-}$ mice (Fig. 33*F*). This indicates that *in vivo* some $GABA_{B(1)}$ protein exits the endoplasmatic reticulum (ER) in the absence of the $GABA_{B(2)}$ subunit.

Redistribution of $GABA_{B(1)}$ in $GABA_{B(2)}^{-/-}$ neurons

The regional and cellular distribution of $GABA_B$ subunits was investigated using antibodies recognizing $GABA_{B(2)}$ or the common C terminus of $GABA_{B(1a)}$ and $GABA_{B(1b)}$ (Fig. 34). A comparison of $GABA_{B(2)}$-immunoreactivity (IR) and $GABA_{B(1)}$-IR in adjacent sections of wild-type mice reveals a mostly overlapping distribution throughout the brain, with strong staining in cerebellum, thalamus, and hippocampal formation (Fig. 34*A*). In $GABA_{B(2)}^{+/-}$ mice, $GABA_{B(2)}$-IR is reduced in all brain regions, whereas $GABA_{B(1)}$-IR remains similar to wild-type mice. In $GABA_{B(2)}^{-/-}$ mice, a partial expression of $GABA_{B(1)}$-IR is still seen in most brain regions, contrasting with the complete loss of $GABA_{B(2)}$ expression. At higher magnification, $GABA_{B(1)}$-IR in $GABA_{B(2)}^{-/-}$ mice exhibits a strikingly different cellular distribution than in wild-type mice, as illustrated for the hippocampal formation (Fig. 34*B*). The homogeneous, diffuse staining of the neuropil is almost reduced to background level, whereas the cell body layers, which normally are weakly labeled in wild-type mice, now appear very prominent.

In addition, some scattered hippocampal interneurons are more evident in $GABA_{B(2)}^{-/-}$ than in wild-type mice. The $GABA_{B(1)}$-IR prominently outlines the soma and the proximal dendrites of these isolated interneurons, shown at higher magnification in Figure 34*C*. Similar results were observed throughout the brain, with an apparent accumulation of $GABA_{B(1)}$-IR in the soma and proximal dendrites and a corresponding reduction of neuropil staining (data not shown). This staining was specific because no $GABA_{B(1)}$-IR was detected in brain sections from $GABA_{B(1)}^{-/-}$ mice, which were used as a control (data not shown). It is impossible to conclusively determine whether the $GABA_{B(1)}$-IR seen in $GABA_{B(2)}^{-/-}$ mice is partly associated with the plasma membrane or not. However, the strong $GABA_{B(1)}$-IR in proximal dendrites of scattered interneurons, as shown in the hippocampal formation (Fig. 34*B,C*) and the biochemical (Fig. 33*F*) and electrophysiological data (see below) (see Fig. 37) all suggest that this is the case. Altogether, our immunohistochemical analysis suggests that $GABA_{B(1)}$ fails to efficiently localize at distal neuronal sites in the absence of $GABA_{B(2)}$.

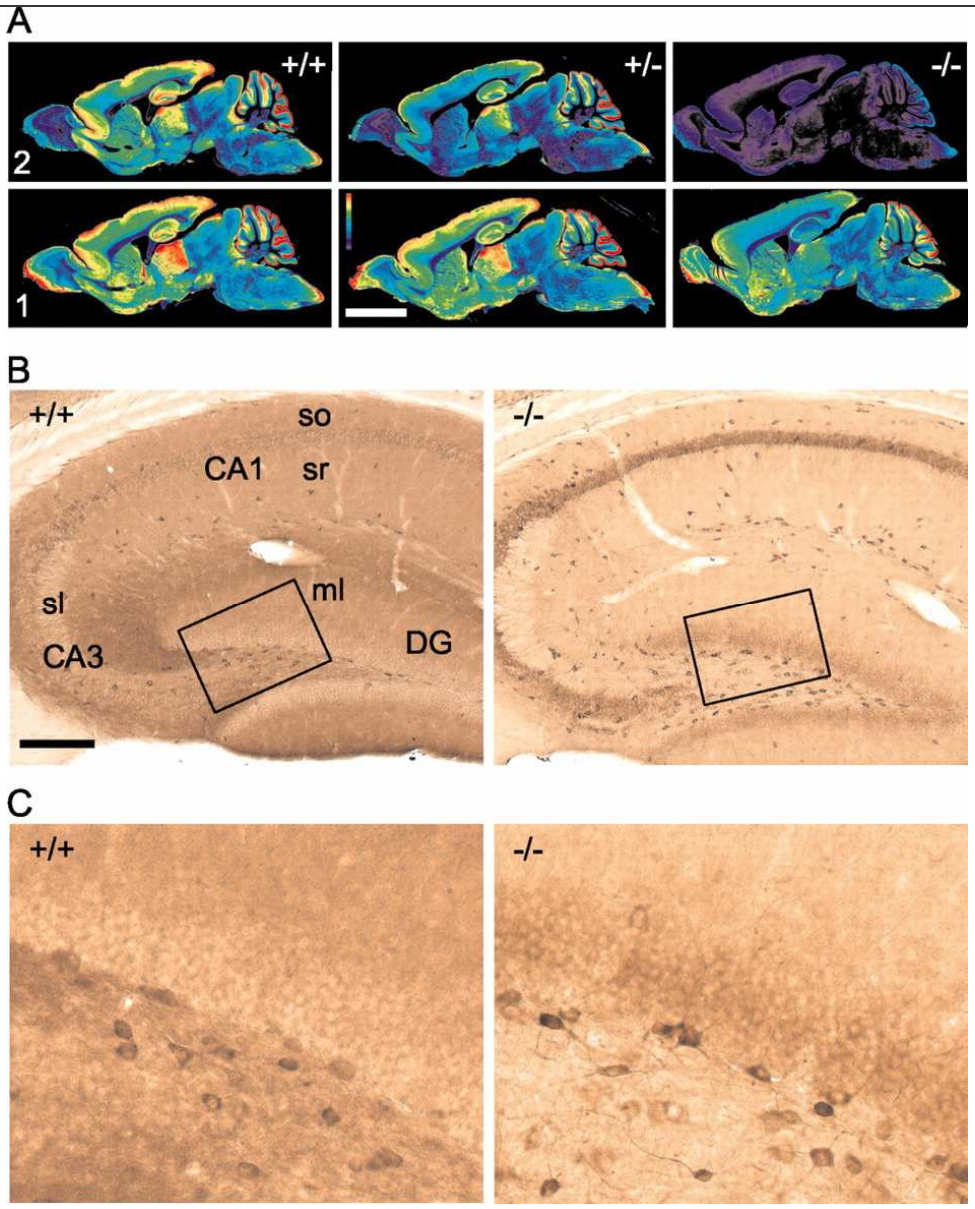

Figure 34) Alteration of $GABA_B$ receptor-IR in $GABA_{B(2)}^{-/-}$ brains. *A*, Effect of *$GABA_{B(2)}$* gene deletion on the distribution of $GABA_{B(2)}$-IR (2) and $GABA_{B(1)}$-IR (1), as visualized in color-coded parasagittal sections from adult wild-type (+/+), $GABA_{B(2)}^{+/-}$ (+/-), and $GABA_{B(2)}^{-/-}$ (-/-) mice. The color scale is indicated. The reduced expression of $GABA_{B(2)}$ in $GABA_{B(2)}^{+/-}$ mice and the complete loss of expression in $GABA_{B(2)}^{-/-}$ mice are evident throughout the brain (top). $GABA_{B(1)}$-IR is retained in $GABA_{B(2)}^{+/-}$ mice and partly reduced in $GABA_{B(2)}^{-/-}$ mice, in which it exhibits an altered cellular distribution, as seen in the hippocampus (bottom). The residual $GABA_{B(1)}$-IR in $GABA_{B(2)}^{-/-}$ mice is not caused by nonspecific binding of the secondary antibodies, which are the same for $GABA_{B(1)}$ and $GABA_{B(2)}$. The specificity of the $GABA_{B(1)}$ antiserum was also tested in $GABA_{B(1)}^{-/-}$ mice, in which no specific staining was observed (data not shown). *B*, Color photomicrographs of the hippocampal formation stained for $GABA_{B(1)}$ in adult wild-type and $GABA_{B(2)}^{-/-}$ mice. The pronounced increase of IR in the CA1-CA3 pyramidal cell layer and in the dentate gyrus granule cell layer (DG) contrasts with the strong reduction in the dendritic layers [stratum oriens (so), stratum radiatum (sr), stratum lucidum (sl), and molecular layer (ml)]. *C*, Enlargement of the framed areas in *B*. Numerous interneurons, which are primarily hidden in sections from wild-type mice because of the homogeneous staining, appear more strongly labeled in $GABA_{B(2)}^{-/-}$ mice but with a normal distribution and morphology. Scale bars: *A*, 2 mm; *B*, 200 µm.

Radioligand binding studies in $GABA_{B(2)}^{-/-}$ mice

All known competitive $GABA_B$ ligands bind exclusively to the $GABA_{B(1)}$ subunit (Kaupmann et al. 1998; Kniazeff et al. 2002). We therefore used antagonist radioligand binding to analyze $GABA_{B(1)}$ binding sites in $GABA_{B(2)}^{-/-}$ mice. Saturation binding experiments at brain membrane preparations with [^{125}I]CGP64213 failed to detect significant numbers of $GABA_{B(1)}$ binding sites in $GABA_{B(2)}^{-/-}$ mice (Fig. 35*A*). The failure to detect antagonist radioligand binding at neuronal membranes from $GABA_{B(2)}^{-/-}$ brains precludes agonist displacement studies. We were therefore unable to determine whether $GABA_B$ agonist affinity is lower in $GABA_{B(2)}^{-/-}$ mice, as one would expect from previous recombinant work showing that $GABA_{B(2)}$ increases agonist affinity at $GABA_{B(1)}$ by ~ 100-fold (Marshall et al. 1999). More sensitive detection systems, such as [^{125}I]CGP71872 photoaffinity labeling (Fig. 35*B*) and [^{3}H]CGP62349 autoradiography (Fig. 35*C,D*), reveal low but significant numbers of $GABA_{B(1)}$ binding sites in $GABA_{B(2)}^{-/-}$ mice. Photoaffinity labeling detects both $GABA_{B(1a)}$ and $GABA_{B(1b)}$ in $GABA_{B(2)}^{-/-}$ tissue (Fig. 35*B*), in agreement with the immunoblot analysis (Fig. 33*E*).

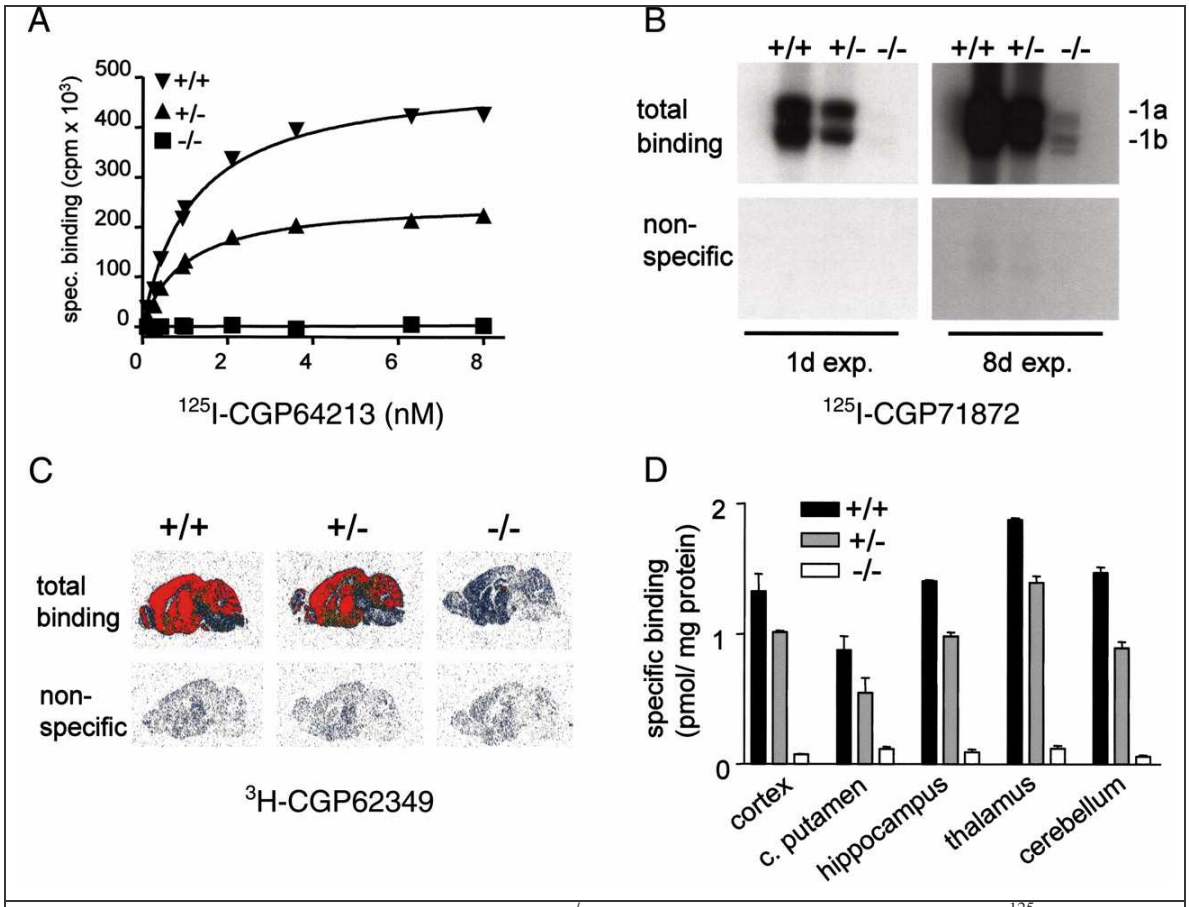

Figure 35) $GABA_{B(1)}$ binding sites in $GABA_{B(2)}^{-/-}$ brains. *A*, Saturation isotherms for [^{125}I]CGP64213 antagonist binding to cortex membranes. No significant binding is detected in membranes from $GABA_{B(2)}^{-/-}$ mice. The number of binding sites is reduced in $GABA_{B(2)}^{+/-}$ versus wild-type mice. The maximal number of binding sites (B_{max}) for wild-type and $GABA_{B(2)}^{+/-}$ mice are 1.4 ± 0.12 and 0.7 ± 0.05 pmol/mg protein, respectively; K_d values were 1.1 ± 0.06 and 0.9 ± 0.05 nM, respectively (mean ± SEM; n = 3). *B*, Autoradiograms of brain extracts from wild-type (+/+), $GABA_{B(2)}^{+/-}$ (+/-), and $GABA_{B(2)}^{-/-}$ (-/-) mice, labeled with the photoaffinity antagonist [^{125}I]CGP71872 (0.5 nM) and analyzed by SDS-PAGE. Exposure for 8 d (8d exp.) reveals low amounts of labeled $GABA_{B(1a)}$ (1a) and $GABA_{B(1b)}$ (1b) proteins in $GABA_{B(2)}^{-/-}$ brains. *C*, $GABA_{B(1)}$ subunit autoradiography. Sagittal cryostat sections were incubated with the $GABA_B$ antagonist [^{3}H]CGP62349. Nonspecific binding was determined in the presence of an excess of 100 µM unlabeled L-baclofen. Tritium-sensitive x-ray films were exposed for 24 hr and developed using a Cyclone Storage Phosphor screen (PerkinElmer Life Sciences, Boston, MA). *D*, Quantitative analysis of [^{3}H]CGP62349 receptor autoradiography. Individual brain regions (n = 3) were counted using the MCID software package (Imaging Research, St. Catharines, Ontario, Canada). The differences in radioligand binding between the three genotypes are significant (two-sided Dunnett test; $p < 0.001$ for combined analysis of all brain regions). *A-C*, Representative experiments, which were repeated three times.

We next used [^{35}S]GTPγS binding to investigate whether the residual $GABA_{B(1)}$ protein in $GABA_{B(2)}^{-/-}$ mice participates in functional receptors (Fig. 36). The [^{35}S]GTPγS binding assay preferentially detects receptors that are coupled to $G\alpha_{i/o}$-type G-proteins, the main effectors of native $GABA_B$ receptors. We did not detect any significant GABA- or baclofen-induced [^{35}S]GTPγS binding in $GABA_{B(2)}^{-/-}$ cortical (Fig. 36) or hippocampal

(data not shown) membrane preparations. This indicates that the $GABA_{B(1)}$ protein expressed in $GABA_{B(2)}^{-/-}$ mice is either not coupled to $G_{\alpha i/o}$ or not present in sufficient amounts to generate detectable [^{35}S]GTPγS binding. In $GABA_{B(2)}^{+/-}$ cortical membranes, baclofen and GABA elicit <50% of the [^{35}S]GTPγS binding seen with wild-type membrane preparations (Fig. 36), consistent with the reduced expression levels of $GABA_{B(1)}$ and $GABA_{B(2)}$ proteins (Figs.33E,35).

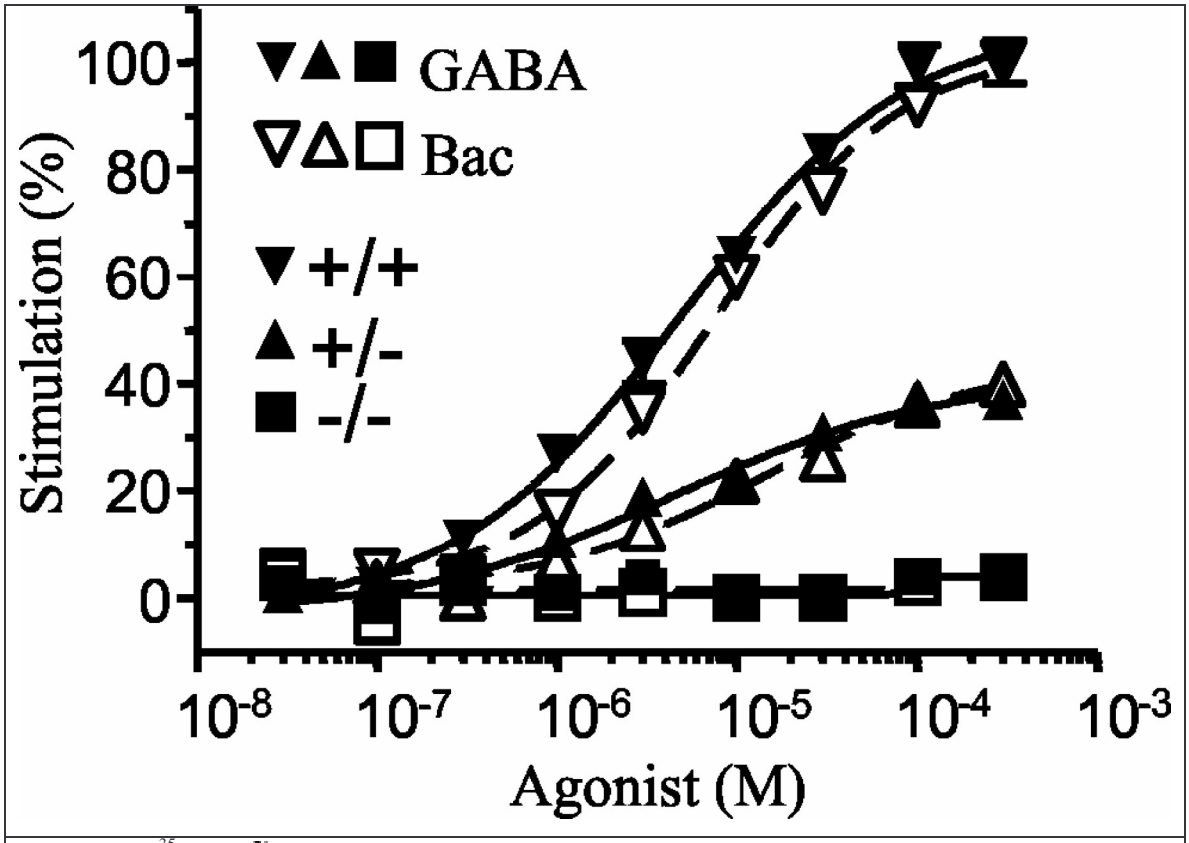

Figure 36) [^{35}S]GTPγS binding to cortex membranes. No significant GABA-stimulated (filled symbols, filled lines) or baclofen-stimulated (Bac; open symbols, dotted lines) [^{35}S] GTPγS binding is detected in $GABA_{B(2)}^{-/-}$ membranes. [^{35}S]GTPγS binding to membranes from $GABA_{B(2)}^{+/-}$ mice is significantly reduced compared with wild-type mice. Values are normalized to the maximal response obtained with wild-type mice.

Loss of presynaptic $GABA_B$ functions in $GABAB(2)^{-/-}$ mice

Electrophysiology provides a more sensitive means than [^{35}S]GTPβS binding for detecting functional $GABA_B$ receptors expressed by individual neurons. We therefore used whole-cell patch-clamp recording to examine $GABA_{B(2)}^{-/-}$ mice for the presence of $GABA_B$ heteroreceptors and autoreceptors on excitatory and inhibitory terminals, respectively. We first studied excitatory synaptic transmission in the hippocampus (Fig.

37A, B). Stimulation in the Schaffer collateral-commissural fibers induces EPSCs in CA1 pyramidal neurons. The amplitude of these EPSCs is reduced by the activation of $GABA_B$ heteroreceptors or A1 adenosine receptors that inhibit glutamate release (Schuler et al., 2001). Accordingly, in slices from wild-type mice, both baclofen and adenosine evoke the expected depression of the EPSCs (baclofen, 74.0 ± 3.2% inhibition, $n = 4$, $p < 0.01$; adenosine, 85.5 ± 5.3% inhibition, $n = 4$, $p < 0.01$). However, only adenosine has an effect in slices from $GABA_{B(2)}^{-/-}$ mice (baclofen, 0.9 ± 12.6% inhibition, $n = 8$; adenosine, 82.1 ± 7.3% inhibition, $n = 6$, $p < 0.001$).

This demonstrates that $GABA_{B(2)}^{-/-}$ mice lack functional $GABA_B$ heteroreceptors on Schaffer collateral terminals, whereas adenosine receptors are still operational and inhibit glutamate release. We next analyzed inhibitory synaptic transmission in the presence of ionotropic glutamate receptor antagonists (Fig. 37C, D). Activation of $GABA_B$ autoreceptors on interneurons attenuates IPSCs recorded from CA1 pyramidal neurons of wild-type mice (55.3 ± 5.8% inhibition; $n = 7$; $p < 0.001$). In contrast, baclofen is unable to inhibit IPSCs in $GABA_{B(2)}^{-/-}$ mice (-0.5 ± 3.9% inhibition; $n = 6$), although the μ-opioid receptor agonist [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) is still effective in both genotypes (wild-type, 46.5 ± 4.5% inhibition, $n = 7$, $p < 0.001$; $GABA_{B(2)}^{-/-}$ mice, 58.1 ± 3.7% inhibition, $n = 5$, $p < 0.001$). These latter experiments show that hippocampal interneurons lack $GABA_B$ autoreceptors in $GABA_{B(2)}^{-/-}$ mice.

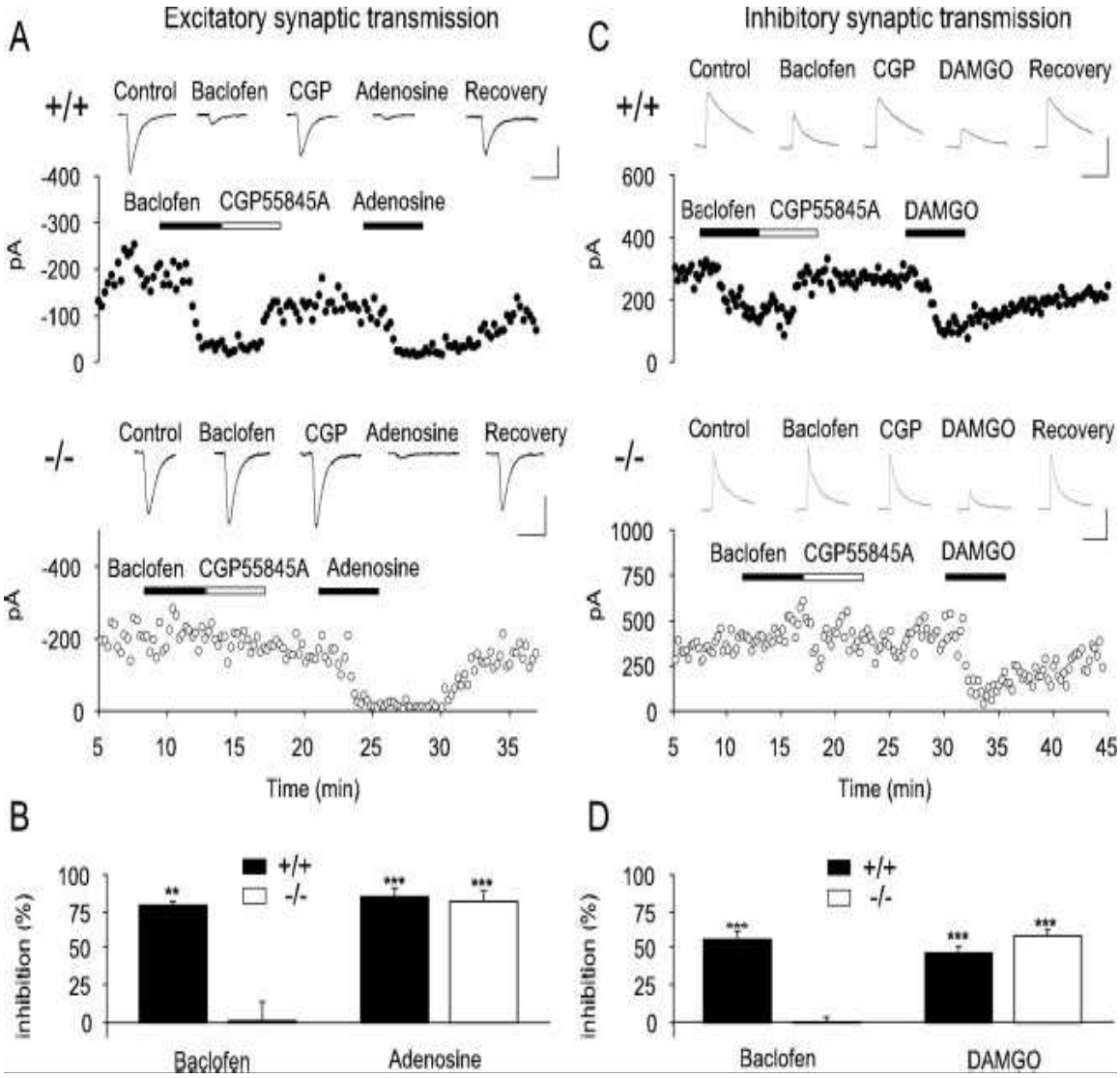

Figure 37) Lack of baclofen-induced presynaptic inhibition in CA1 pyramidal cells of $GABA_{B(2)}^{-/-}$ mice. *A*, Excitatory synaptic transmission. Monosynaptic EPSC peak amplitudes plotted versus time and representative traces from wild-type (top, filled circles) and $GABA_{B(2)}^{-/-}$ (bottom, open circles) mice. Both baclofen (50 µM) and adenosine (100 µM) significantly depress the EPSC amplitude in wild-type mice, whereas baclofen and CGP55845A (2 µM) have no effect on the EPSC amplitude in $GABA_{B(2)}^{-/-}$ mice. The effect of adenosine is similar in both genotypes. Traces are averages of 10 consecutive sweeps. Calibration: 40 msec, 100 pA. *B*, Summary graph showing the lack of baclofen-induced presynaptic inhibition of excitatory synaptic transmission in $GABA_{B(2)}^{-/-}$ mice (wild-type, $n = 4$; $GABA_{B(2)}^{-/-}$, $n = 8$). Adenosine-induced inhibition is similar in both genotypes (wild-type, $n = 4$; $GABA_{B(2)}^{-/-}$, $n = 6$). *C*, Inhibitory synaptic transmission. Monosynaptic IPSC peak amplitudes plotted versus time and representative traces from wild-type (top, filled circles) and $GABA_{B(2)}^{-/-}$ (bottom, open circles) mice. Both baclofen (50 µM) and the µ-opioid agonist DAMGO (1 µM) significantly depress the IPSC amplitude in wild-type mice, whereas baclofen and CGP55845A (2 µM) have no effect on the IPSC amplitude in $GABA_{B(2)}^{-/-}$ mice. The effect of DAMGO was similar in both genotypes. Traces are averages of 10 consecutive sweeps. Calibration: 100 msec, 200 pA. *D*, Summary graph showing the lack of baclofen-induced presynaptic inhibition of inhibitory synaptic transmission in $GABA_{B(2)}^{-/-}$ mice (wild-type, $n = 7$; $GABA_{B(2)}^{-/-}$, $n = 6$). DAMGO-induced inhibition was similar in both genotypes (wild-type, $n = 7$; $GABA_{B(2)}^{-/-}$, $n = 5$). $^{**}p < 0.01$; $^{***}p < 0.001$.

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$GABA_B$ receptors inhibit instead of activate K^+ channels in GABAB(2)$^{-/-}$ mice

Postsynaptic $GABA_B$ and adenosine receptors activate a Kir3-mediated K^+ conductance in CA1 pyramidal neurons (Luscher et al. 1997; Schuler et al. 2001). The $GABA_B$ receptor-activated K^+ conductance underlies the late IPSP (Luscher et al. 1997). Accordingly, at a holding potential of -50 mV and in physiological $[K^+]_{ext}$, baclofen elicits an outward current in CA1 pyramidal cells of wild-type mice (116.2 ± 26.7 pA; n = 5; $p < 0.05$) (Fig. 38A, B) that is blocked by the $GABA_BR$ antagonist CGP55845A (2 µM; 93.4 ± 12.5% inhibition; n = 4). Surprisingly, but consistent with the strong $GABA_{B(1)}$-IR observed on the soma and proximal dendrites, a baclofen-induced current is also seen in CA1 pyramidal neurons of $GABA_{B(2)}^{-/-}$ mice. However, baclofen elicits an inward instead of the typical outward current (-19.2 ± 4.5 pA; n = 9; $p < 0.01$) (Fig. 38A, B). This inward current can be blocked by the $GABA_BR$ antagonists CGP55845A (2 µM; 99.4 ± 2.7% inhibition; n = 8) (Fig. 38A) and CGP62349(4 µM; 87.5 ± 8.3% inhibition; n = 5), the ligand that was used for autoradiographic detection of $GABA_{B(1)}$. Whereas the baclofen-induced outward current in wild-type mice is associated with a decrease in the input resistance (-93.3 ± 25.8 MΩ; n = 5; $p < 0.05$), the inward current in $GABA_{B(2)}^{-/-}$ mice is associated with an increase in input resistance (35.2 ± 12.1 MΩ; n = 9; $p < 0.05$).

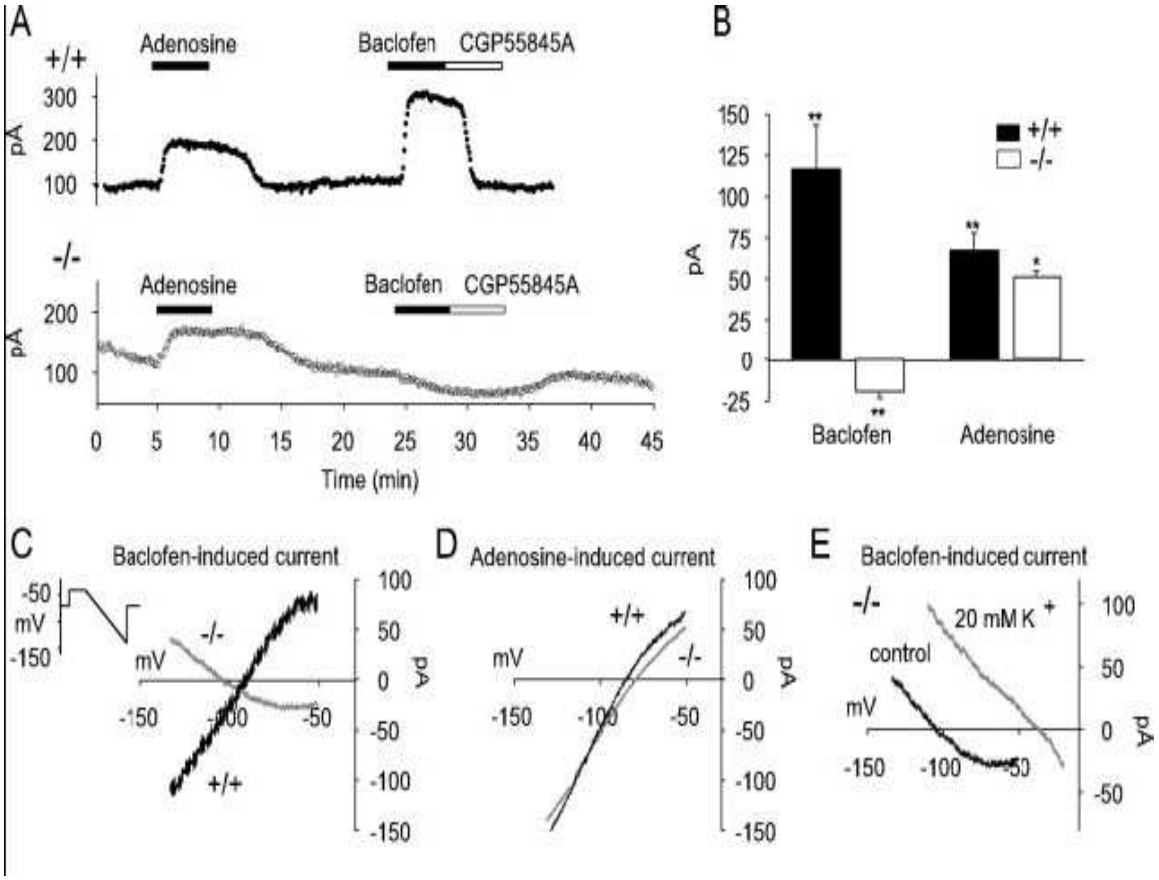

Figure 38a. Baclofen inhibits a postsynaptic K^+ conductance in CA1 pyramidal cells of $GABA_{B(2)}^{-/-}$ mice. *A*, Holding current (at -50 mV) plotted versus time for wild-type (top, filled circles) and $GABA_{B(2)}^{-/-}$ (bottom, open circles) mice. Whereas both baclofen (50 µM) and adenosine (100 µM) induce an outward current in wild-type mice, baclofen induces an inward current in $GABA_{B(2)}^{-/-}$ mice. Baclofen-induced effects were blocked by application of the $GABA_B$ receptor antagonist CGP55845A (2 µM) in wild-type as well as in $GABA_{B(2)}^{-/-}$ mice. *B*, Summary graph illustrating the baclofen-induced inward current at -50 mV in $GABA_{B(2)}^{-/-}$ mice. Baclofen-induced currents: wild-type, $n = 5$; $GABA_{B(2)}^{-/-}$, $n = 9$. Adenosine-induced currents: wild-type, $n = 5$; $GABA_{B(2)}^{-/-}$, $n = 4$. *C*, Current-voltage relationship of the baclofen-induced conductance in wild-type (black trace) and $GABA_{B(2)}^{-/-}$ (gray trace) mice. Currents were obtained by calculating the difference between the *I-V* curves before and after addition of baclofen. Whereas a current with a positive slope conductance is induced by baclofen in wild-type mice, a current with negative slope conductance is induced in $GABA_{B(2)}^{-/-}$ mice. *D*, Current-voltage relationship of the adenosine-induced conductance in wild-type mice (black trace) is not different from $GABA_{B(2)}^{-/-}$ mice (gray trace). *E*, Baclofen induces the closure of K^+ channels in $GABA_{B(2)}^{-/-}$ mice. Raising extracellular $[K^+]$ concentration shifts the reversal potential of the baclofen-induced current.

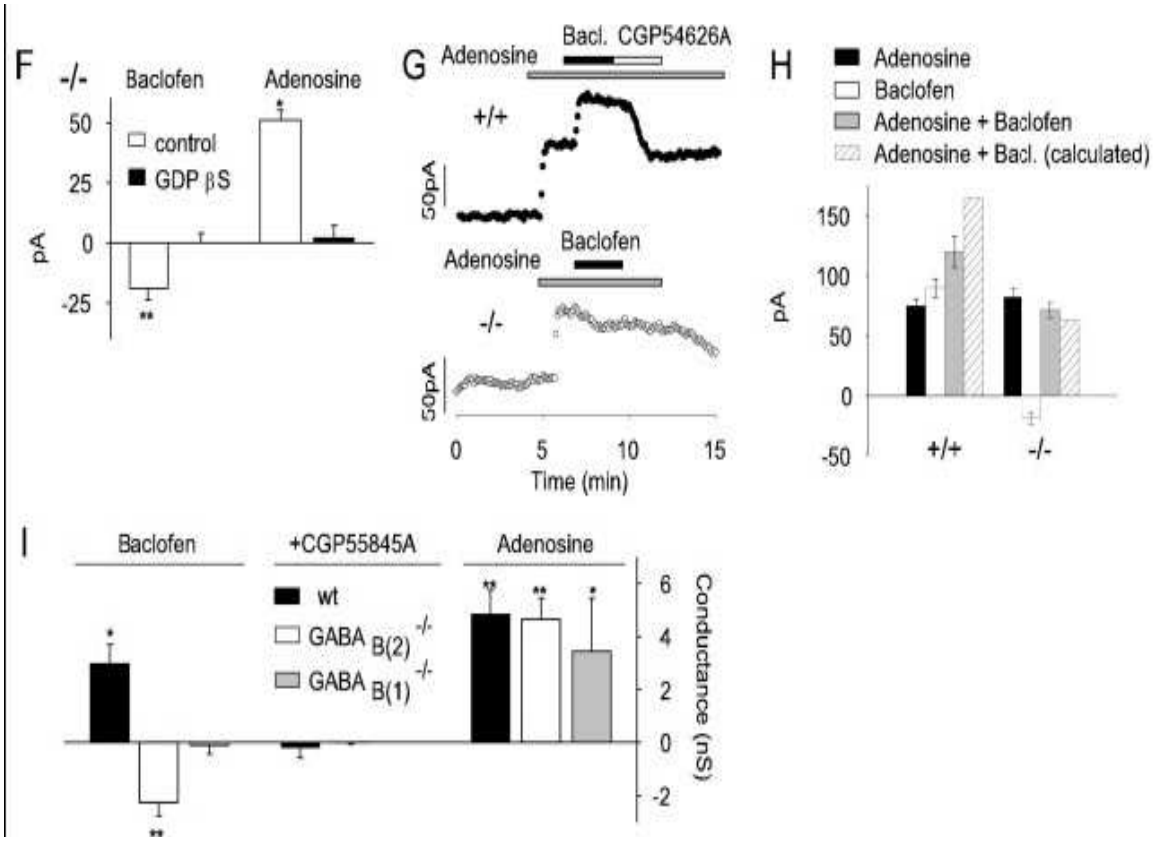

Figure 38b. Baclofen inhibits a postsynaptic K^+ conductance in CA1 pyramidal cells of $GABA_{B(2)}^{-/-}$ mice. *F*, The baclofen-induced conductance change is mediated by G-protein activation. In the presence of intracellular GDPβS (1 mM for 25 min), both the baclofen-induced (control, $n = 5$; GDPβS, $n = 5$) and adenosine-induced (control, $n = 4$; GDPβS, $n = 5$) currents are inhibited in $GABA_{B(2)}^{-/-}$ mice. *G*, Changes in the holding current (at -50 mV) in response to baclofen (Bacl.) after preincubation with adenosine. *H*, Summary graph illustrating that the effects of adenosine and baclofen are not fully additive. In wild-type neurons (+/+), the effect of a combined application of adenosine and baclofen is lower than the sum of the individual effects [Adenosine + Bacl. (calculated)]. In $GABA_{B(2)}^{-/-}$ (-/-) neurons, the effects of adenosine and baclofen are not fully additive. Application of baclofen does not obliterate the adenosine response. *I*, Left, Summary graph illustrating postsynaptic conductance changes induced by baclofen in wild-type ($n = 5$), $GABA_{B(2)}^{+/-}$ ($n = 10$), and $GABA_{B(1)}^{-/-}$ ($n = 4$) mice. The conductance changes were blocked by application of the GABAB(1) receptor antagonist CGP55845A (2 µM; wild-type, $n = 4$; $GABA_{B(2)}^{-/-}$, $n = 8$). Adenosine-induced conductance changes are not different between genotypes (wild-type, $n = 4$; $GABA_{B(2)}^{-/-}$, $n = 4$; $GABA_{B(1)}^{-/-}$, $n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$.

Adenosine-induced currents are similar in wild-type and $GABA_{B(2)}$ -/- mice (wild-type, 67.0 ± 11.1 pA, n = 5, p < 0.01; $GABA_{B(2)}$ -/-, 51.2 ± 3.8 pA, n = 4, p < 0.01) (Fig. 38A, B). The current-voltage relationship of baclofen-induced currents reveals a positive slope conductance in wild-type mice (3.0 ± 0.7 nS; n = 5; p < 0.05) (Fig. 38C, I), whereas a negative slope conductance is induced in $GABA_{B(2)}$ -/- mice (-2.3 ± 0.5 nS; n = 10; p < 0.01) (Fig. 38C, I). Consistent with the baclofen-induced increase in input resistance, a negative slope conductance indicates that baclofen application leads to the closure of ion channels in $GABA_{B(2)}$ -/- mice. The baclofen-induced conductance changes in wild-type and in $GABA_{B(2)}$ -/- mice are completely blocked by the $GABA_{B(1)}$ antagonist CGP55845A (2 µM; wild-type, -0.2 ± 0.4 nS, n = 4, p < 0.05; $GABA_{B(2)}$ -/-, -0.01 ± 0.06 nS, n = 8, p < 0.001) (Fig. 38I). Adenosine-induced conductance changes are similar in wild-type (conductance, 4.83 ± 0.91 nS; n = 4; p < 0.01; Vrev, -94.5 ± 1.2 mV; n = 4) and $GABA_{B(2)}$ -/- (4.63 ± 0.83 nS; n = 4; p < 0.01; Vrev, -93.5 ± 2.5 mV; n = 4) mice (Fig. 38D, I). The reversal potential of the baclofen-induced current in $GABA_{B(2)}$ -/- cells is shifted by raising the extracellular $[K^+]$ from 2.7 mM (V_{rev}, -96.7 ± 3.6 mV; *n* = 10; calculated V_{rev} for K^+, -99.5 mV) to 20 mM (V_{rev}, -47.6 ± 7.4 mV; *n* = 6; calculated V_{rev} for K^+, -45.8 mV) (Fig. 38E), indicating that a closure of K^+ channels underlies the baclofen-induced conductance change in $GABA_{B(2)}^{-/-}$ neurons. Barium at a concentration of 300 µM completely occludes the baclofen-induced channel closure in wild-type and $GABA_{B(2)}^{-/-}$ CA1 pyramidal cells (data not shown). It is therefore conceivable that the $GABA_B$ receptors in $GABA_{B(2)}^{-/-}$ CA1 neurons and the $GABA_{B(1,2)}$ receptors in wild-type CA1 neurons both couple to Kir3 channels but with opposite effects on channel activity. A large body of *in vitro* data supports that, within the heteromeric $GABA_{B(1,2)}$ receptor, the now-missing $GABA_{B(2)}$ subunit is absolutely necessary for G-protein coupling (Galvez et al. 2001; Margeta-Mitrovic et al. 2001; Robbins et al. 2001; Duthey et al. 2002; Havlickova et al. 2002). We therefore investigated whether the baclofen-induced closure of K^+ channels in $GABA_{B(2)}^{-/-}$ cells is mediated by G-proteins or not. We recorded postsynaptic responses in the presence of GDPβS, which prevents G-protein activation. Intracellular dialysis of CA1 pyramidal cells from $GABA_{B(2)}^{-/-}$ mice with βS (1 mM for at least 25 min) specifically blocks the induction of postsynaptic currents by

baclofen (control, -19.2 ± 4.5 pA, $n = 5$; GDPβS, 0.1 ± 3.9 pA, $n = 5$, $p < 0.01$) (Fig. 33F) or adenosine (control, 51.2 ± 3.8 pA, $n = 4$; GDPβS, 2.1 ± 4.9 pA, $n = 5$, $p < 0.05$) (Fig. 38F), demonstrating that the baclofen-induced conductance change in $GABA_{B(2)}^{-/-}$ CA1 pyramidal cells is G-protein mediated. It is conceivable that the baclofen-induced inhibition of a K^+ current is the consequence of a dominant-negative effect. For example, $GABA_{B(2)}$ activation in $GABA_{B(2)}^{-/-}$ neurons may sequester G-proteins that are normally associated with other GPCR-activating K^+ channels. Such a baclofen-dependent sequestering of G-proteins would reduce K^+ currents and could underlie the inward current observed in $GABA_{B(2)}^{-/-}$ neurons. We investigated whether baclofen can cross-inhibit the adenosine response by first applying adenosine to CA1 pyramidal cells, followed by a combined application of adenosine and baclofen (Fig. 38G, H). In both wild-type and $GABA_{B(2)}^{-/-}$ neurons, the effects of adenosine and baclofen are not fully additive, indicating that adenosine and $GABA_B$ receptors share G-proteins and/or effector K^+ channels. However, the cross-inhibitory effect was not larger in $GABA_{B(2)}^{-/-}$ than in wild-type neurons. Although the outcome of these experiments does not completely exclude sequestering, it clearly does not support it. The fact that the baclofen-induced current is blocked by GDPβS also argues against a passive sequestering of G-proteins and shows that activation of G-proteins is necessary to trigger the inward current (Fig. 38F). Others and we reported previously a complete loss of postsynaptic baclofen responses in $GABA_{B(2)}^{-/-}$ mice (Prosser et al. 2001; Schuler et al. 2001). We therefore reinvestigated $GABA_{B(2)}^{-/-}$ mice for baclofen-induced responses under identical experimental conditions as used for the analysis of $GABAB(2)^{-/-}$ mice (Fig. 38I). Consistent with our previous findings, we do not observe any postsynaptic conductance changes induced by baclofen in $GABA_{B(1)}^{-/-}$ mice. Therefore, exclusively $GABA_{B(2)}^{-/-}$ mice express residual functional $GABA_B$ receptors.

Lack of behavioral responses to baclofen in $GABA_{B(2)}^{-/-}$ mice

In addition to inducing electrophysiological responses, baclofen may also cause detectable behavioral responses in $GABA_{B(2)}^{-/-}$ mice. We therefore studied well known physiological responses to baclofen in $GABA_{B(2)}^{-/-}$ mice. First, we investigated whether baclofen still induces delta waves in the EEG, as shown previously for wild-type mice (Schuler et al. 2001; Kaupmann et al. 2003). Twenty minutes after baclofen application

(10 mg/kg, i.p.), delta waves appeared in the EEG of wild-type mice but not in the EEG of $GABA_{B(2)}^{-/-}$ mice (Fig. 39). Ten hours after baclofen administration, the EEG of wild-type mice reverted to normal. No significant EEG changes were observed in $GABA_{B(2)}^{-/-}$ mice during the entire duration of the experiment. This indicates that the baclofen-induced electrophysiological responses in $GABA_{B(2)}^{-/-}$ mice (Fig. 39) do not result in detectable changes of electrical activity at the network level.

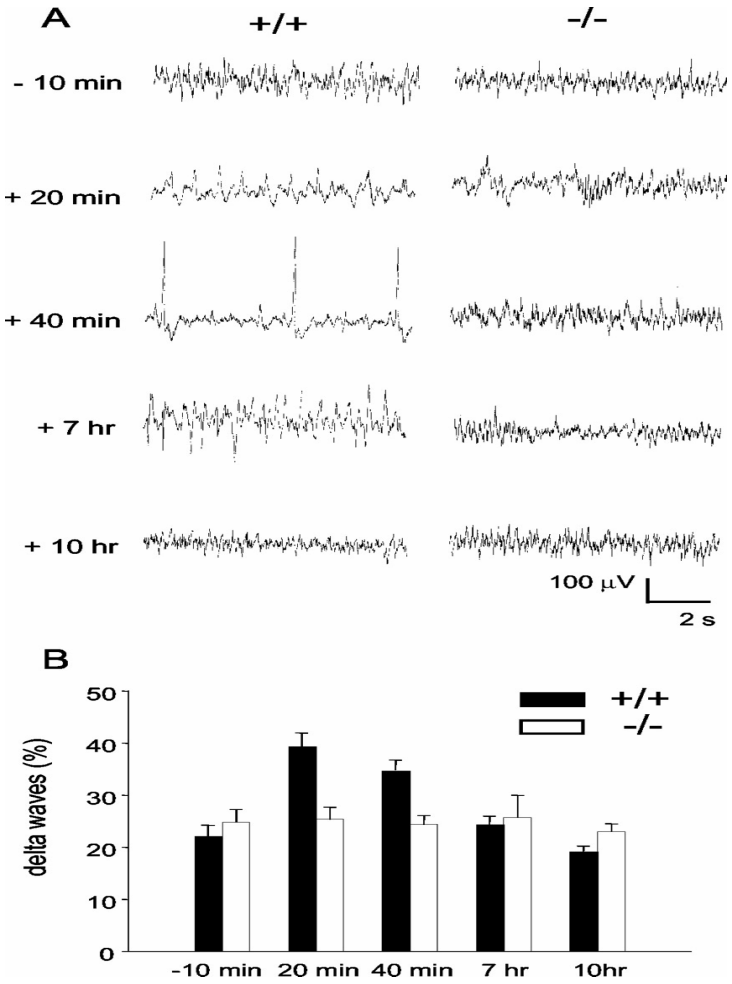

Figure 39). Lack of baclofen-induced delta waves in $GABA_{B(2)}^{-/-}$ mice. *A*, Effect of L-baclofen (10 mg/kg, i.p.) on the EEG of freely moving wild-type (+/+) and $GABA_{B(2)}^{-/-}$ (-/-) mice. The EEG of wild-type and $GABA_{B(2)}^{-/-}$ mice were similar 10 min before baclofen application (-10 min). Twenty minutes after baclofen application, delta waves were observed in the EEG of wild-type, but not of $GABA_{B(2)}^{-/-}$, mice (+20 min). Single spikes appeared sporadically in the EEG of wild-type mice (+40 min), followed by delta waves that lasted for several hours (+7 hr). Ten hours after baclofen application, the EEG traces of wild-type and $GABA_{B(2)}^{-/-}$ mice were again similar (+10 hr). *B*, Quantification of baclofen-induced delta waves in the EEG of wild-type and $GABA_{B(2)}^{-/-}$ mice. The percentage of delta waves of the total power amplitude was calculated over periods of 10 min. Three to four mice per genotype were analyzed.

We next investigated whether $GABA_B$ receptors in $GABA_{B(2)}^{-/-}$ mice are able to mediate the well known muscle-relaxant effect of baclofen. Baclofen induces muscle relaxation in wild-type but not in $GABA_{B(2)}^{-/-}$ mice, as shown by the inability or ability, respectively, of the mice to stay on the rotarod during a 5 min period (Fig. 40*A*). Similarly, $GABA_{B(2)}^{-/-}$ mice demonstrate a lack of baclofen-induced hypothermia (Fig. 40*B*). Together, these data indicate that residual $GABA_B$ receptors in $GABA_{B(2)}^{-/-}$ mice are unable to influence muscle relaxation or body temperature.

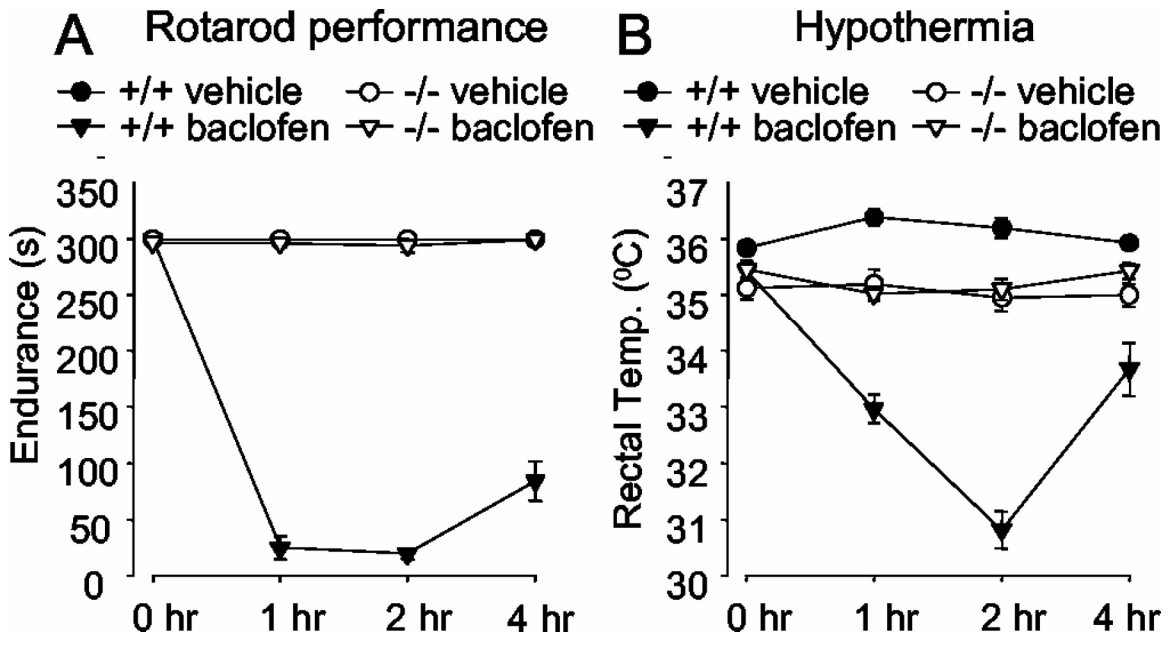

Figure 40) Lack of baclofen-induced motor impairment and hypothermia in $GABA_{B(2)}^{-/-}$ mice. *A*, No baclofen-induced impairment of rotarod endurance is observed in $GABA_{B(2)}^{-/-}$ mice (n = 7-10). In contrast, wild-type mice (+/+) show a marked reduction in rotarod performance after baclofen application ($p < 0.05$; Fisher's *post hoc* tests). The vehicle-treated control groups stayed on the rotarod during the entire experiment (300 sec) at all time points examined. Thus, in the graph, the data points for the wild-type vehicle control (black dots) are hidden behind the data points for the $GABA_{B(2)}^{-/-}$ vehicle control (white dots). At all time points after baclofen application (1, 2, and 4 hr), the $GABA_{B(2)}^{-/-}$ group (white triangles) differed significantly from the wild-type control group (black triangles) ($p < 0.05$; Fisher's *post hoc* tests). All data points represent mean ± SEM values. *B*, Baclofen induces a potent reduction in body temperature in wild-type mice (black triangles) compared with the vehicle control group (black dots) ($p < 0.05$; Fisher's *post hoc* tests), whereas it is without effect on basal temperature in $GABA_{B(2)}^{-/-}$ mice (n = 7-10). However, $GABA_{B(2)}^{-/-}$ mice (white dots) exhibit a slight but significantly reduced basal temperature compared with wild-type littermates (black dots) ($p < 0.05$; Fisher's *post hoc* tests). All data points represent mean ± SEM values.

$GABA_{B(2)}^{-/-}$ mice exhibit spontaneous epileptiform activity, hyperlocomotor activity, hyperalgesia, and impaired passive avoidance learning

We reported previously that adult $GABA_{B(1)}^{-/-}$ mice exhibit pronounced spontaneous epileptiform activity (Schuler et al. 2001). We therefore investigated whether adult $GABA_{B(2)}^{-/-}$ mice are epileptic and recorded continuous EEG in freely moving animals using implanted electrodes. $GABA_{B(2)}^{-/-}$ mice displayed several episodes of spontaneous seizures per day. The analysis of three $GABA_{B(2)}^{-/-}$ mice over a 96 hr period revealed an average of 3.75 (0, 11, 3, 1), 0.5 (1, 0, 0, 1), and 2.0 (3, 2, 2, 1) seizures per day. The recorded seizures were exclusively of the clonic type. This is in contrast to $GABA_{B(1)}^{-/-}$ mice, in which additionally absence-type and spontaneous tonic-clonic seizures occurred with low frequency (Schuler et al. 2001). Epileptiform activity was never observed in wild-type littermates (n = 3).

$GABA_{B(1)}^{-/-}$ mice exhibit a sporadic hyperlocomotor phenotype when exposed to a new environment (Schuler et al. 2001). We similarly studied the locomotor activity of $GABA_{B(2)}^{-/-}$ mice using the Ethovision recording system. During a 1 hr observation period, $GABA_{B(2)}^{-/-}$ mice moved over a significantly larger distance with significantly increased speed compared with wild-type and heterozygous littermates (Fig. 41A). These experiments demonstrate that functional $GABA_B$ receptors in $GABA_{B(2)}^{-/-}$ mice do not rescue the hyperlocomotor phenotype seen with $GABA_{B(1)}^{-/-}$ mice, which completely lack functional $GABA_B$ receptors.

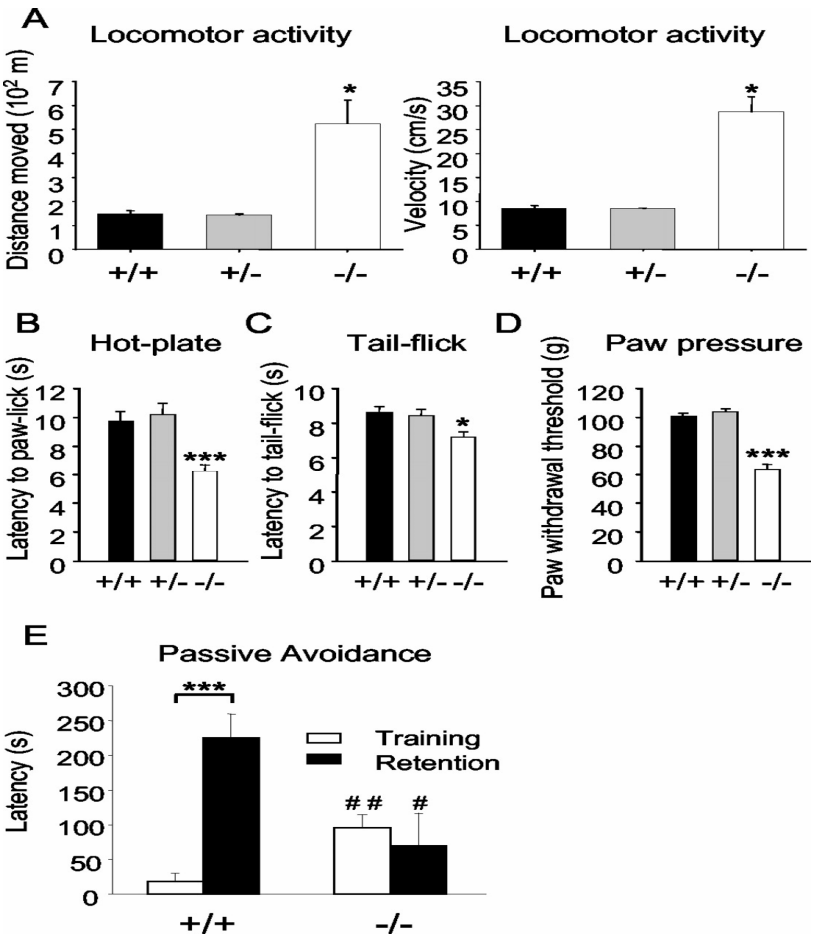

Figure 41. Behavioral analysis of $GABA_{B(2)}^{-/-}$ mice. *A*, Hypolocomotor activity in $GABA_{B(2)}^{-/-}$ mice. During a 1 hr observation period, $GABA_{B(2)}$ knock-out mice (-/-) moved over significantly longer distances (left histogram) with significantly greater speed (right histogram) than heterozygous (+/-) and wild-type (+/+) control mice. n = 7-8 per genotype; mean ± SEM; $^{*}p < 0.05$. *B*, Response latencies of wild-type (+/+), heterozygous (+/-), and $GABA_{B(2)}$ knock-out (-/-) mice in the hotplate test assessed at 55°C. $GABA_{B(2)}^{-/-}$ mice show significantly reduced paw-lick latencies compared with wild-type and heterozygous control groups. n = 19-20 per genotype; mean ± SEM; $^{***}p < 0.001$. *C*, Response latencies of wild-type (+/+), heterozygous (+/-), and $GABA_{B(2)}$ knock-out (-/-) mice in the tail-flick test assessed at infrared intensity 14. $GABA_{B(2)}^{-/-}$ mice show significantly reduced tail-flick latencies compared with wild-type and heterozygous control groups. n = 19-21 per group; mean ± SEM; $^{*}p < 0.05$. *D*, Paw-withdrawal thresholds for wild-type (+/+), heterozygous (+/-), and $GABA_{B(2)}$ knock-out (-/-) mice in response to a mechanical stimulus. Withdrawal thresholds of the left hindpaw were assessed for each genotype. $GABA_{B(2)}^{-/-}$ mice show a significantly reduced withdrawal threshold compared with wild-type and heterozygous control groups. n = 19-21 per group; $^{***}p < 0.001$. Nociception tests were analyzed with Tukey's honestly significant difference test. In all tests, there were no significant differences in the behavior of wild-type or heterozygous mice. *E*, Impaired passive avoidance learning in $GABA_{B(2)}^{-/-}$ mice. Step-through latencies of wild-type (+/+) and $GABA_{B(2)}$ knock-out (-/-) mice into the dark (shock) compartment on the training day (white bars) and in the retention test (black bars). $GABA_{B(2)}^{-/-}$ mice were slower to enter on training day but faster in the retention test compared with the wild-type control mice. Wild-type, but not $GABA_{B(2)}^{-/-}$, mice show significantly longer latencies to enter the dark compartment in the retention test compared with the training trial, which is taken as an index of memory of the initial experience. n = 6-11 per group; mean ± SEM; $^{***}p < 0.001$ versus training; $\#p < 0.05$ versus genotype; $\#\#p < 0.01$ versus genotype.

$GABA_B$ agonists exhibit antinociceptive properties in models of acute and chronic pain (Patel et al. 2001). These properties are likely mediated by supraspinal and spinal $GABA_B$ receptors (Malcangio et al. 1991). Consistent with these pharmacological findings, $GABA_{B(1)}^{-/-}$ mice exhibit pronounced hyperalgesia, suggesting that $GABA_B$ receptors exert a tonic control over nociceptive processes (Schuler et al. 2001). We used the hotplate (Fig. 41B), tail-flick (Fig. 41C), and paw-pressure (Fig. 41D) tests to measure acute pain behaviors in $GABA_{B(2)}^{-/-}$ mice. Similar to the $GABA_{B(1)}^{-/-}$ mice, $GABA_{B(2)}^{-/-}$ mice exhibit hyperalgesia in all three tests, showing significantly reduced response latencies or withdrawal thresholds when compared with wild-type or heterozygous littermate mice. In all three tests, we did not observe significant differences in the behavior of wild-type or heterozygous mice.

$GABA_B$ antagonists are reported to have profound effects on memory processing. They can either enhance (Getova and Bowery 1998; Nakagawa et al. 1999; Staubli et al. 1999) or attenuate (Brucato et al. 1996) cognitive performance in a variety of learning paradigms in mice and rats. We reported previously that $GABA_{B(1)}^{-/-}$ mice exhibit a severe impairment of passive avoidance learning (Schuler et al., 2001). We therefore investigated the memory performance of $GABA_{B(2)}^{-/-}$ mice (Fig. 41E). $GABA_{B(2)}$ -/- mice, in contrast to wild-type mice, show no increased latency in entering the darkened shock compartment in the retention trial that followed the training trial. This indicates that $GABA_{B(2)}^{-/-}$ mice exhibit an impairment of passive avoidance learning, similar to $GABA_{B(1)}^{-/-}$ mice. We further observed that $GABA_{B(2)}^{-/-}$ mice show increased latencies to enter the darkened shock compartment on the training trial compared with wild-type littermate mice ($p < 0.01$). This excludes the possibility that $GABA_{B(2)}^{-/-}$ mice have a tendency to enter the dark compartment more quickly, independent of the training experience.

5.4.4. Discussion

Pharmacological and behavioral analyses of $GABA_{B(2)}^{-/-}$ mice indicate that deletion of the $GABA_{B(2)}$ subunit is sufficient to abolish all well known responses to $GABA_B$ agonists, such as [^{35}S]GTPγS binding (Fig. 36), muscle relaxation (Fig. 40*A*), hypothermia (Fig. 40*B*), and EEG delta wave induction (Fig. 39). These findings are paralleled by a loss of typical electrophysiological $GABA_B$ responses in the $GABA_{B(2)}^{-/-}$ hippocampus (Figs. 37, 38). These results are analogous to the results obtained with $GABA_{B(1)}^{-/-}$ mice and suggest that all classical $GABA_B$ responses relate to heteromeric $GABA_{B(1,2)}$ receptors. The heteromeric nature of predominant native $GABA_B$ receptors is further emphasized by the substantial downregulation of $GABA_{B(1)}$ protein in $GABA_{B(2)}^{-/-}$ mice (Fig. 33*E*). An analogous requirement of $GABA_{B(1)}$ for stable expression of $GABA_{B(2)}$ was observed in $GABA_{B(1)}^{-/-}$ mice (Prosser et al. 2001; Schuler et al. 2001; Queva et al. 2003).

Strikingly, the remaining $GABA_{B(1)}$ protein in $GABA_{B(2)}^{-/-}$ neurons accumulates in distinct cellular compartments than in wild-type neurons. Throughout the $GABA_{B(2)}^{-/-}$ brain, we observed a redistribution of the $GABA_{B(1)}$-IR from the neuropil to the soma (Fig. 35 and data not shown). We also noticed some scattered hippocampal interneurons that are more evident in $GABA_{B(2)}^{-/-}$ than wild-type brains (Fig. 35*B,C*). The $GABA_{B(1)}$-IR prominently outlines the soma and proximal dendrites of these cells. This is reminiscent of the strong somatic $GABA_{B(1)}$-IR observed in a subset of GABAergic hippocampal interneurons lacking $GABA_{B(2)}$-IR (Fritschy et al. 1999; Sloviter et al. 1999; Kulik et al. 2002). Presumably, both a genetically induced and a natural lack of $GABA_{B(2)}$ expression leads to a relocation of $GABA_{B(1)}$ protein to the soma and proximal dendrites. Because $GABA_{B(2)}$ is important for exit of $GABA_{B(1)}$ from the ER, most of the somatic $GABA_{B(1)}$-IR likely reflects protein that fails to exit the ER. However, some of the $GABA_{B(1)}$-IR on the soma and proximal dendrites may also represent $GABA_{B(1)}$ protein at the cell surface. This is supported by biochemical (Fig. 33*F*) and electrophysiological (Fig. 38) data that reveal $GABA_{B(1)}$ expression in synaptic membranes and functional receptors in the somatodendritic compartment of CA1 pyramidal neurons, respectively. Besides being important for G-protein coupling and export from the ER (Margeta-Mitrovic et al. 2000; Calver et al. 2001; Galvez et al. 2001; Pagano et al. 2001; Robbins

et al. 2001), $GABA_{B(2)}$ may therefore also be necessary for the targeting of $GABA_{B(1,2)}$ receptors to the distal zones of neuronal processes.

Whether a physiologically relevant signaling underlies the electrophysiological $GABA_B$ responses that we observe in CA1 neurons of $GABA_{B(2)}^{-/-}$ mice is unclear. It is possible that these $GABA_B$ responses are a consequence of the knock-out situation, in which $GABA_{B(1)}$ is expressed in the absence of its usual dimerization partner. An abnormal intracellular accumulation of $GABA_{B(1)}$ protein in $GABA_{B(2)}^{-/-}$ pyramidal cells may overload the ER-retention machinery, thereby allowing some $GABA_{B(1)}$ to escape to the cell surface and to couple to G-proteins. Consistent with this possibility, $GABA_{B(1)}$ was originally expression cloned using [^{125}I]CGP64213 binding on the surface of live COS-1 cells (Kaupmann et al. 1997), showing that some $GABA_{B(1)}$ protein can overcome ER retention in the absence of $GABA_{B(2)}$. In further support of this possibility, no $GABA_B$ responses were detected in CA1 pyramidal neurons of mice expressing a C-terminally truncated version of the $GABA_{B(2)}$ protein (A. Calver, personal communication). Apparently, the truncated $GABA_{B(2)}$ protein dimerizes with $GABA_{B(1)}$ in the ER, generating a dominant-negative situation that impedes transit of $GABA_{B(1)}$ protein through intracellular compartments.

Normally, postsynaptic $GABA_B$ receptors activate a K^+ conductance underlying the late IPSP (Luscher et al. 1997). However, in $GABA_{B(2)}^{-/-}$ mice, baclofen induces a G-protein-dependent inward current instead of the expected outward current, most likely reflecting the closure of K^+ channels (Fig. 38). Barium at 300 µM occludes the baclofen-induced current inhibition seen in $GABA_{B(2)}^{-/-}$ mice (data not shown). Kir3 channels could therefore not only be the cause of the typical outward current seen in wild-type neurons (Luscher et al. 1997) but could also be responsible for the atypical inward current seen in $GABA_{B(2)}^{-/-}$ neurons. The $GABA_{B(1)}$ antagonists CGP55845A (Fig. 38*A,I*) and CGP62349 (see Results) block the baclofen-induced inward current seen in $GABA_{B(2)}^{-/-}$ CA1 pyramidal cells. A radioactive version of the antagonist used in electrophysiology, [^{3}H]CGP62349, specifically recognizes residual $GABA_{B(1)}$ protein in the $GABA_{B(2)}^{-/-}$ brain (Fig. 35*C*). Moreover, the inward current is not observed in $GABA_{B(1)}^{-/-}$ CA1 pyramidal cells (Fig. 38*I*). Together, this suggests that the baclofen-sensitive current is triggered by receptors incorporating $GABA_{B(1)}$. Baclofen-sensitive currents were seen in

the majority of $GABA_{B(2)}^{-/-}$ CA1 neurons analyzed, indicating that the neuronal environment reliably assists functioning of $GABA_{B(1)}$ in the absence of $GABA_{B(2)}$. It remains unclear why the $GABA_{B(1)}$-mediated electrophysiological responses in $GABA_{B(2)}^{-/-}$ CA1 neurons are opposite to those recorded in wild-type CA1 neurons. We addressed whether activation of $GABA_{B(1)}$ in $GABA_{B(2)}^{-/-}$ neurons takes on a dominant-negative effect by sequestering G-proteins that normally activate Kir channels. We did not observe increased cross-inhibition of the adenosine response by baclofen in $GABA_{B(2)}^{-/-}$ as opposed to wild-type neurons, rendering sequestering unlikely (Fig. 38*G,H*). Further arguing against a passive sequestering of G-proteins, the baclofen-induced inward current in $GABA_{B(2)}^{-/-}$ neurons is blocked by GDPβS (Fig. 38*F*). Some G-proteins are reported to inhibit rather than to activate Kir3 channels (e.g., by phospholipase C-mediated phosphatidylinositol-4, 5-biphosphate hydrolysis or PKC activation) (Schreibmayer et al. 1996; Sharon et al. 1997; Lei et al. 2000; Blanchet and Luscher 2002; Mao et al. 2004). Similar to what is now observed, metabotropic glutamate receptors not only activate but also inhibit K^+ channels, presumably by coupling to distinct G-proteins (Sharon et al., 1997). For example, they were shown to be able to suppress a barium-sensitive K^+ current in CA3 pyramidal cells (Lüthi et al. 1997) and to downregulate Kir3 channels in *Xenopus* oocytes (Sharon et al. 1997). It is therefore conceivable that the somatic redistribution of $GABA_B$ receptors (Fig. 35) in $GABA_{B(2)}^{-/-}$ neurons leads to a promiscuous coupling to G-proteins that are not normally associated with heteromeric $GABA_{B(1,2)}$ receptors. This would explain why no significant [^{35}S]GTPγS binding is detectable in neuronal membranes from $GABA_{B(2)}^{-/-}$ mice (Fig. 36) because this assay preferentially detects $G_{i/o}$-proteins that are typically associated with native $GABA_{B(1,2)}$ receptors. A promiscuous coupling to G-proteins in neurons may also explain why we never observed $GABA_B$ responses opposite to those of heteromeric $GABA_{B(1,2)}$ receptors when $GABA_{B(1)}$ was functional by itself in transfected cells (Kaupmann et al. 1997; Kaupmann et al. 1998). There is compelling *in vitro* evidence to show that, in the heteromer, the $GABA_{B(2)}$ subunit is necessary to engage and activate G-proteins (Galvez et al. 2001; Margeta-Mitrovic et al. 2001; Margeta-Mitrovic et al. 2001; Robbins et al. 2001; Duthey et al. 2002; Havlickova et al. 2002). $GABA_{B(1)}$ may therefore also function in association with another, yet unknown GPCR subunit, which couples to

G-proteins other than $GABA_{B(2)}$. In that respect, a $GABA_B$ receptor-related protein has been identified (Calver et al. 2003). However, in heterologous cells, this protein does not appear to participate in typical $GABA_B$ signaling. Furthermore, because "Family C" GPCRs preferentially assemble homodimers, the existence of homodimeric $GABA_{B(1)}$ receptors cannot be excluded (Bouvier 2001). It is possible that homodimeric $GABA_{B(1)}$ receptors couple to G-proteins other than heterodimeric $GABA_{B(1,2)}$ receptors. Moreover, they may exhibit a constitutive activity that can be inhibited by agonists. Of note, it was reported that chimeric $GABA_B$ receptors with two $GABA_{B(1)}$ extracellular domains exhibit an increased basal activity and, for unknown reasons, respond to GABA agonists with inhibition rather than activation of Kir3 channels (Margeta-Mitrovic et al. 2001). Similar observations were made in a related study (Galvez et al. 2001).

It is not ruled out that the baclofen-induced inward current is also present in wild-type CA1 pyramidal cells, in which it would be masked by simultaneous larger outward currents activated by heteromeric $GABA_{B(1,2)}$ receptors. Unfortunately, because we lack ligands that distinguish molecular subtypes of $GABA_B$ receptors, genetic manipulation is currently the only means to functionally dissociate native $GABA_B$ assemblies with and without a $GABA_{B(2)}$ subunit. Regardless of whether the baclofen-induced current seen in $GABA_{B(2)}^{-/-}$ CA1 neurons is a consequence of the knock-out situation or not, the observation of a functional $GABA_B$ receptor in the absence of $GABA_{B(2)}^{-/-}$ may be important. An increasing number of studies suggest that various cellular populations in the nervous system express $GABA_{B(1)}$ without $GABA_{B(2)}$ (Billinton et al. 2000; Calver et al. 2000; Clark et al. 2000; Ng and Yung 2001; Burman et al. 2003; Kim et al. 2003; Kulik et al. 2003; Li et al. 2003; Straessle et al. 2003). Our results imply that neurons that naturally lack a $GABA_{B(2)}$

6. Discussion

6.1. Pre- and postsynaptically $GABA_BR$-mediated inhibition in the LA

In this study we have investigated the $GABA_BR$-mediated inhibition at cortical and thalamic afferents converging on projection neuron in the LA. EPSC evoked by stimulating cortical or thalamic afferents were reduced by application of baclofen (Figs. 25, 27, 29). This baclofen-induced inhibition of glutamatergic transmission is thought to be mediated by activation of presynaptic $GABA_B$ receptors located on glutamatergic terminals (Kombian et al. 1996; Takahashi et al. 1998). Moreover this baclofen-induced inhibition was antagonized by the specific $GABA_B$ receptor antagonist CGP55845A. Interestingly, upon application of CGP55845A the EPSCs amplitude increased back to a level slightly higher than that of the baseline, which may indicate that $GABA_BRs$ were tonically activated (Fig. 27A). $GABA_BRs$ were also synaptically activated by three subsequent pulses at 20 Hz that activate $GABA_B$ receptor by stimulating feed forward inhibitory inputs. (Fig. 27D). Synaptic activation of $GABA_B$ receptors can be demonstrated by applying CGP55845A, which led to a decrease in the pair pulse ratio indicating presynaptic inhibition mechanisms. $GABA_B$ receptors are not only present presynaptically at thalamic and cortical afferents, but also postsynaptically in the LA projection neurons. Postsynaptic $GABA_B$ receptors can be activated pharmacologically by applying baclofen which leads to an increase in a K^+ conductance as seen by the recorded outward current (Fig. 27C). Moreover, synaptic activation of postsynaptic $GABA_BRs$ was also observed by stimulating the thalamic or cortical afferents with three pulses stimulation inducing outward current with slow kinetics typically for activation of $GABA_BRs$ (Fig. 27D).

Our results are consistent with previous investigation done in LA and other brain areas. It was showed that the responses of LA neurons are tightly regulated by inhibitory processes, which largely limit orthodromic spiking (Lang and Pare 1997) and may explain their virtual lack of spontaneous activity in unanesthesized cats (Pare and Gaudreau 1996). This inhibition is necessary to counterbalance strong excitatory inputs, which are revealed only when selectively activated with low intensity stimulation of LA afferents (Lang and Pare 1997).

In vitro studies have shed some light on the mechanisms underlying these inhibitory processes. LA neurons were demonstrated to have both $GABA_A$- and $GABA_B$-mediated IPSPs (Sugita et al. 1992; Sugita et al. 1993), which result primarily from the action of local interneurons (Le Gal LaSalle et al. 1978). $GABA_A$- and $GABA_B$-mediated IPSPs were shown to have distinct time courses and reversal potentials (Sugita et al. 1992; Sugita et al. 1993) that were similar to those found in other amygdaloid nuclei (Nose et al. 1991; Rainnie et al. 1991; Washburn and Moises 1992) and elsewhere in the nervous system (Hirsch and Burnod 1987; Dutar and Nicoll 1988; Soltesz et al. 1988; McCormick 1989). Thus, the $GABA_A$ IPSPs ($IPSP_B$) in the LA were shown to be mediated by a Cl^- conductance, reversed around −70 mV, had rapid times to peak (10-35 ms), and had short durations (50 ms). In contrast, $GABA_B$ IPSPs were found to be generated by a K^+ conductance, reversed around −90 mV, had long times to peak (120-170 ms), and had long durations (350-1,500 ms) (Sugita et al. 1993). Unlike $GABA_A$ receptor-mediated IPSPs, which at rest provide inhibition primarily via membrane shunting because of a 90–140 nS conductance change, $IPSP_B$ produces inhibition via a peak 10- to 20- mV membrane potential hyperpolarization that is accompanied by a macroscopic peak conductance change of only 13–19 nS (Deisz and Prince 1989) (single channel conductance of 5– 6 pS) (Premkumar et al. 1990). This hyperpolarization is substantial enough to significantly reduce the probability of action potential firing and restrict NMDA receptor-mediated glutamatergic synaptic transmission by maintaining the voltage-dependent magnesium block of the NMDA receptor channel (Morrisett et al. 1991; Davies and Collingridge 1996). It should be noted, however, that the effectiveness of $IPSP_B$ to inhibit strong synaptic inputs is far less than its ability to restrict weak synaptic inputs. Thus, in effect $IPSP_B$ enhance the neuronal signal to noise ratio by filtering out background noise.

In BLA neurons, $GABA_B$Rs have been shown to occur preferentially along the distal dendrites, whereas $GABA_A$R-mediated inhibition dominate somatically (Washburn and Moises 1992). Within the mammalian brain, the highest density of $GABA_B$ binding sites is in the thalamic nuclei, the molecular layer of the cerebellum, the cerebral cortex, the interpeduncular nucleus, and the dorsal horn of the spinal cord (Bowery et al. 1987; Chu et al. 1990). The BLA contains substantial amounts of $GABA_{B(1)}$ and $GABA_{B(2)}$ mRNA

(Kaupmann et al. 1997; Bischoff et al. 1999; Durkin et al. 1999; Clark et al. 2000) and exhibits signifiant $GABA_BR$ binding (Bowery et al. 1987; Bischoff et al. 1999).

Previous immunocytochemical studies of $GABA_BR$ localization in the brain have only briefly addressed the general levels of $GABA_BR$ immunoreactivity in the nuclei of the BLA (Margeta-Mitrovic et al. 1999). A recent study with an antibody that recognizes the main isoforms of $GABA_{B(1)}$ showed that many cell types in the BLA exhibit immunoreactivity for the $GABA_{B(1)}$ subunit. The highest concentration of perisomatic $GABA_{B(1)}$ is found in interneurons. Most of the large CCK interneurons express high levels of $GABA_{B(1)}$. Lower percentages of the three other interneuronal subpopulations (PV, SOM, and VIP cells) express $GABA_{B(1)}$, and the concentrations of $GABA_{B(1)}$ are apparently lower than in the CCK interneurons. In most pyramidal projection neurons, there is little or no $GABA_{B(1)}$ immunoreactivity in the perisomatic region, but ultrastructural observations indicate that there are many $GABA_{B(1)}$+ spiny dendrites in the neuropil, most of them probably belong to pyramidal cells. There is a high concentration of $GABA_B$ receptors along the dendrites of pyramidal neurons and particular subpopulations of interneurons in the BLA. Thus, the distal dendrites of pyramidal cells, and varying percentages of each of the four main subpopulations of interneurons in the BLA, express $GABA_BRs$ (McDonald et al. 2004).

This is in agreements with electrophysiological studies which have shown that $GABA_BRs$ presynaptically modulate glutamate and GABA release from axons in the BLA (Yamada et al. 1999; Szinyei et al. 2000) and postsynaptically mediate a slow, prolonged hyperpolarization of BL and LA neurons via activation of potassium channels (Rainnie et al. 1991; Washburn and Moises 1992; Sugita et al. 1993).

In our studies we have observed tonic activation of $GABA_BRs$ in both pre-and postsynaptic $GABA_BRs$ (Fig. 28). Previous studies showed that presynaptic $GABA_BRs$ which present on the terminals of the sensory afferents in the rat dorsal lateral geniculate nucleus and in the ventrobasal thalamus are tonically activated by endogenous GABA (Emri et al. 1996). In this study they showed that the endogenous GABA level is not sufficient for a tonic activation of postsynaptic $GABA_BRs$ (Emri et al. 1996). Moreover, other studies showed that $GABA_BR$ antagonist CGP55845A not only blocked the baclofen-mediated decrease in mIPSC frequency, but also produced an increase in the

mIPSC frequency compared with control (Le Feuvre et al. 1997). Application of CGP55845A alone produced large increase in the mIPSC frequency which indicates that $GABA_B$ autoreceptors are present on the GABAergic terminals within the thalamic sensory nuclei and that these receptors can be tonically activated by the ambient GABA (Le Feuvre et al. 1997). Furthermore, it was shown that $GABA_B$R-antagonist application elicited a marked potentiation of Ca^{2+} transients mediated by glutamatergic neurotransmission, suggesting that tonic synaptic GABA release exerts an inhibitory tone on glutamate receptor-mediated Ca^{2+} transients via $GABA_B$Rs activation (Obrietan and van den Pol 1999). In addition, in the presence of TTX to block action potential-mediated neurotransmitter release, stimulation with exogenously applied glutamate triggered a robust postsynaptic Ca^{2+} rise that was dramatically depressed (>70% in cortical neurons, >40% in hypothalamic neurons) by baclofen. This suggests both a pre- and postsynaptic component for the modulatory actions of the $GABA_B$Rs. These results indicate an important role for the $GABA_B$Rs as a modulator of the excitatory actions of glutamate (Obrietan and van den Pol 1999). A similar study also showed that Adenosine receptor 1 (A1) and $GABA_B$ antagonists increased the amplitude of evoked IPSCS (eIPSCs) in a supra-additive manner, suggesting a tonic activation of these receptors by ambient adenosine and GABA (Hugel and Schlichter 2003). A more recent study suggested that signaling pathways that regulate cAMP levels in neurons may have profound effects on the tonic synaptic inhibition by modulating the availability of $GABA_B$Rs (Fairfax et al. 2004).

In summary, we showed that $GABA_B$Rs are present in the pre- and postsynaptic cells at thalamic and cortical afferents in the LA. Moreover, we noticed that endogenous ambient GABA possibly activates $GABA_B$ receptors. At the presynaptic glutamatergic terminal $GABA_B$ receptors are inhibiting glutamate release acting as heteroreceptors. At presynaptic GABAergic terminals, $GABA_B$Rs inhibit GABA neurotransmitter release acting as autoreceptors. At the postsynaptic site $GABA_B$ receptor activation evokes an outward current by modulating K^+ conductance. Taking together, it appears that $GABA_B$ receptors play an important role in the synaptic transmission in the LA, by exerting pre- and postsynaptic inhibition at the thalamic and cortical afferent synapses in the LA

6.2. $GABA_B$ heteroreceptor-mediated inhibition at thalamic and cortical afferents is impaired in $GABA_{B(1a)}$ -/- mice

In this study, we show that baclofen-induced inhibition of excitatory synaptic transmission is strongly reduced in $GABA_{B(1a)}$ -/- mice whereas it is only slightly reduced in $GABA_{B(1b)}$ -/- mice (Figs.25, 29). Moreover, baclofen-induced inhibition of excitatory synaptic transmission is completely vanished in $GABA_{B(1)}$ -/- mice suggesting that baclofen does not effect synaptic transmission in $GABA_{B(1)}$ -/- mice (Figs. 25, 29). In all genotypes presynaptic inhibition induced by activation of adenosine receptors is not affected (Figs. 25, 29). This demonstrates that $GABA_{B(1a)}$ -/- mice largely lack functional $GABA_B$ heteroreceptors on thalamic and cortical afferent terminals.

Interestingly, while activation of $GABA_B$ autoreceptors on interneurons is completely abolished in $GABA_{B(1)}$ -/- mice, presynaptic inhibition on interneurons is not different from wild-type animals in $GABA_{B(1a)}$ -/-and in $GABA_{B(1b)}$ -/- mice (Figs. 25, 29). This suggests that $GABA_{B(1b)}$ -/- mediated presynaptic inhibition on GABAergic terminals is either redundant, or that $GABA_{B(1a)}$ subunits are able to compensate for the loss $GABA_{B(1b)}$ subunits and vice versa. Postsynaptic inhibition mediated by the induction of an outward current (at -50 mV) via activation of GIRK-type K^+ channels is equally reduced in $GABA_{B(1a)}$ -/- and $GABA_{B(1b)}$ -/- mice (Fig. 25). However, adenosine-induced postsynaptic activation of GIRK currents was not affected in all genotypes (Figs. 25, 29). This suggests that postsynaptic $GABA_B$ receptors are containing both $GABA_{B(1a)}$ and $GABA_{B(1b)}$ isomers. Thus, the two $GABA_B$ subunits isoforms R1a and R1b are differently distributed in the subcellular synapses so that $GABA_{B(1a)}$ is predominantly localized in the presynaptic site and $GABA_{B(1b)}$ is postsynaptically localized.

Our results are in agreement with a recent study done in the hippocampus (Vigot 2005). Many studies investigated $GABA_{B(1)}$ splice variants and identified different splice variants of the $GABA_{B(1)}$ subunit (Kaupmann et al. 1997; Isomoto et al. 1998; Pfaff et al. 1999; Calver et al. 2000; Schwarz et al. 2000; Wei et al. 2001; Wei et al. 2001). Studies using Western blotting and immunohistochemistry with isoform-specific antisera showed that there is differential subcellular localization pointing at a pre- versus postsynaptic

localization for $GABA_{B(1a)}$ and $GABA_{B(1b)}$, respectively (Kaupmann et al. 1998; Bischoff et al. 1999). During postnatal maturation, the expression of the two splice variants is differentially regulated with $GABA_{B(1a)}$ being preponderant at birth (Fritschy et al. 1999). In adult brain, $GABA_{B(1b)}$ immunoreactivity is predominant, and the two isoforms largely accounted for the pattern of $GABA_BR$ binding sites in the brain (Fritschy et al. 1999). Another, in situ hybridization studies of mRNA for the $GABA_{B(1a)}$ and $GABA_{B(1b)}$ splice variants revealed that they are distributed differentially in brain (Liang et al. 2000). Studies with rat and human cerebellum and spinal cord indicate that $GABA_{B(1a)}$ is associated with presynaptic receptors, whereas $GABA_{B(1b)}$ is located predominantly at postsynaptic sites, at least in cerebellum (Kaupmann et al. 1998; Billinton et al. 1999; Bischoff et al. 1999; Princivalle et al. 2000; Towers et al. 2000). Elsewhere in the brain, however, the $GABA_{B(1b)}$ protein is in presynaptic terminals and the $GABA_{B(1a)}$ at postsynaptic sites (Benke et al. 1999; Princivalle et al. 2001). In the dorsal horn of the rat spinal cord, the density of $GABA_{B(1a)}$ is low, whereas in the dorsal root ganglia, which contain cell bodies of the primary afferent fibres, $GABA_{B(1a)}$ is the predominant proportions and $GABA_{B(1b)}$ is much less expressed (Towers et al. 2000). Similarly, in rat and human cerebellum, $GABA_{B(1a)}$ mRNA is detected over the granule cells, which send their excitatory fibres into the molecular layer to innervate the Purkinje cell dendrites (Kaupmann et al. 1998; Billinton et al. 1999; Bischoff et al. 1999). In contrast, $GABA_{B(1b)}$ mRNA is associated with the Purkinje cell bodies, which express $GABA_B$ receptors on their dendrites in the molecular layer postsynaptic to the GABAergic stellate cells. However, the contrary arrangement has also been observed elsewhere in the brain. For example, $GABA_{B(1a)}$ subunits appear to be postsynaptic on cell bodies in the thalamocortical circuits (Princivalle et al. 2001). Thus, it is not possible to generally assign a functional role or cellular location to specific $GABA_B$ receptor subunit splice variants (Poorkhalkali et al. 2000; Princivalle et al. 2001). In this study we observed that presynaptic inhibition at both cortical and thalmic afferents is specifically mediated by $GABA_B$ receptors containing the R1a subunit. This in agreement with previous immunohistochemical studies (Fritschy et al. 1999; Poorkhalkali et al. 2000; Fritschy et al. 2004), and recent experiments carried out in the CA1 area of the hippocampus revealed that presynaptic heteroreceptors on glutamatergic terminals are exclusively

comprised of $GABA_{B(1a)}$ and $GABA_{B(2)}$ subunits (Gassmann et al., 2004; Vigot et al., 2005) (Fig. 42).

Our data indicate that presynaptic heteroreceptors on glutamatergic terminals are comprised of $GABA_{B(1a)}$ and $GABA_{B(2)}$ subunits. In contrast, presynaptic autoreceptors on GABAergic terminal are seem to be comprised of $GABA_{B(1b)}$ containing $GABA_BRs$ and/or $GABA_{B(1a)}$ containing $GABA_BRs$. Additionally, postsynaptic $GABA_BRs$ are also comprised of $GABA_{B(1b)}$ containing $GABA_BRs$ and/or $GABA_{B(1a)}$ containing $GABA_BRs$.

6.3 $GABA_B$-mediated modulation.of homosynaptic and heterosynaptic LTP at cortical afferents

At cortical afferents, presynaptic NMDAR-dependent LTP can be induced by associative co-activation of thalamic and cortical afferents (Humeau et al. 2003). This form of associative heterosynaptic LTP is presynaptically expressed and depends on presynaptic NMDAR activation and subsequent Ca^{2+} influx. Upon stimulation of cortical or thalamic afferents indvidually, homosynaptic LTP can not be induced in the presence of intact $GABA_BR$-mediated inhibition (Figs. 22, 28). This suggests that $GABA_BR$-mediated inhibition suppress the induction of homosynyaptic LTP. We also show that $GABA_{B(1a)}$-/- , but not $GABA_{B(1b)}$-/- mice, exhibit facilitated induction of cortical afferent LTP (Fig. 25). This non-associative homosynaptic LTP at cortical afferents is completely presynaptic, as the induction is independent of postsynaptic activity and the expression is accompanied by decrease in the PPF (Figs. 22, 24). Thus, presynaptic LTP at cortical afferents can be induced by either stimulation of both converging afferents leading to heterosynaptic associative LTP expression, or by blocking $GABA_BRs$ and stimulation of cortical afferents leading to homosynaptic nonassociative LTP expression (Fig. 22). In both forms, LTP induction would lead to Ca^{2+} entry in the presynapses or Postsynaptic cells. Thus, Ca^{2+} in turn might activate Ca^{2+} sensitive adenyle cyclase that trigger cAMP, PKA signal cascade leading to phosphorylation process that act for the expression of LTP (Linden and Ahn 1999; Huang et al. 2000; Schafe and LeDoux 2000)(Fig. 42).

Our data suggest that homosynpatic LTP has a higher threshold for induction than heterosynaptic LTP, due to a presynaptic $GABA_BR$-mediated inhibition.

Associative learning is thought to be mediated mostly by NMDA receptors as detectors of associativity (Tang et al. 1999; Blair et al. 2001). Homosynpatic LTP at cortical afferents seems to be nonassociative since it is NMDAR-independent. In contrast, associative heterosynaptic LTP at cortical afferents is NMDAR-dependent. Both forms of LTP need Ca^{2+} entry through either NMDARs (heterosynaptic) and/or L-VDCC (homosynaptic). L-VDCC could be activated due to depolarization of the presynapses or inhibited by $GABA_B$ receptor direct modulation. It has been reported that $GABA_BRs$ can regulate an L-type calcium channel, and thus neurotransmitter release, in the axon terminals of tiger salamander bipolar cells (Maguire et al. 1989). Likewise, $GABA_B$ receptors inhibit N-, L-, P- and Q-type high voltage-activated Ca^{2+} currents (Dolphin et al. 1990; Scholz and Miller 1991; Mintz and Bean 1993) through a direct membrane-delimited mechanism thought to involve the interaction of Go α subunits with the Ca^{2+} channel itself (Campbell et al. 1993). The role of ßγ subunits, if any, in this inhibitory effect is unclear.

The facilitation of homosynaptic LTP at cortical afferents in $GABA_{B(1a)}$ -/- mice could be due to the loss of $GABA_B$-mediated presynaptic inhibition at cortical afferents, which would lead to decrease in the LTP induction threshold at cortical terminals. Interestingly, presynaptic LTP at cortical afferents requires the activation of cAMP/PKA-dependent signaling similar to the mechanism modulating hippocampal mossy fibers LTP (Linden and Ahn; Schmitz et al.).

At the behavioral level, $GABA_{B(1a)}$ -/- mice showed generalized fear response to CS^- (Fig. 26). Behavioral analysis showed that $GABA_{B(1a)}$ -/- mice exhibit indistinguishable freezing behavior in response to the pairing of cued audio stimulus CS^+ with the foot shock and the unpaired CS^-. While these data clearly show that $GABA_{B(1a)}$-/- mice perform poorly in a discriminatory fear conditioning task, we cannot exclude that $GABA_{B(1a)}$-/- containing receptors play an important role for stimulus discrimination in a brain area upstream from the amygdala such as the auditory cortex (for review see: Weinberger, 2004).

Our invitro investigation showed that homosynaptic LTP at cortical afferents is associated with a loss of associativity between thalamic and cortical afferent activation.

This loss of associativity could be the reason why $GABA_{B(1a)}$-/- mice exhibit this striking deficit in associative stimulus discrimination at the behavioral level. The loss of heterosynaptic $GABA_BR$-mediated inhibition leads to a decrease in the LTP induction threshold at the cortical afferents. This in turn would lead to unspecific potentiation of all signals conveyed to the LA through the cortical afferents. Indeed, the cortico-amygdala pathway was suggested to be implicated the in stimulus discrimination and generalization of conditioned fear (Jarrell et al. 1987, Saha et al. 1993, LeDoux 1994; but see: Armony et al. 1997).

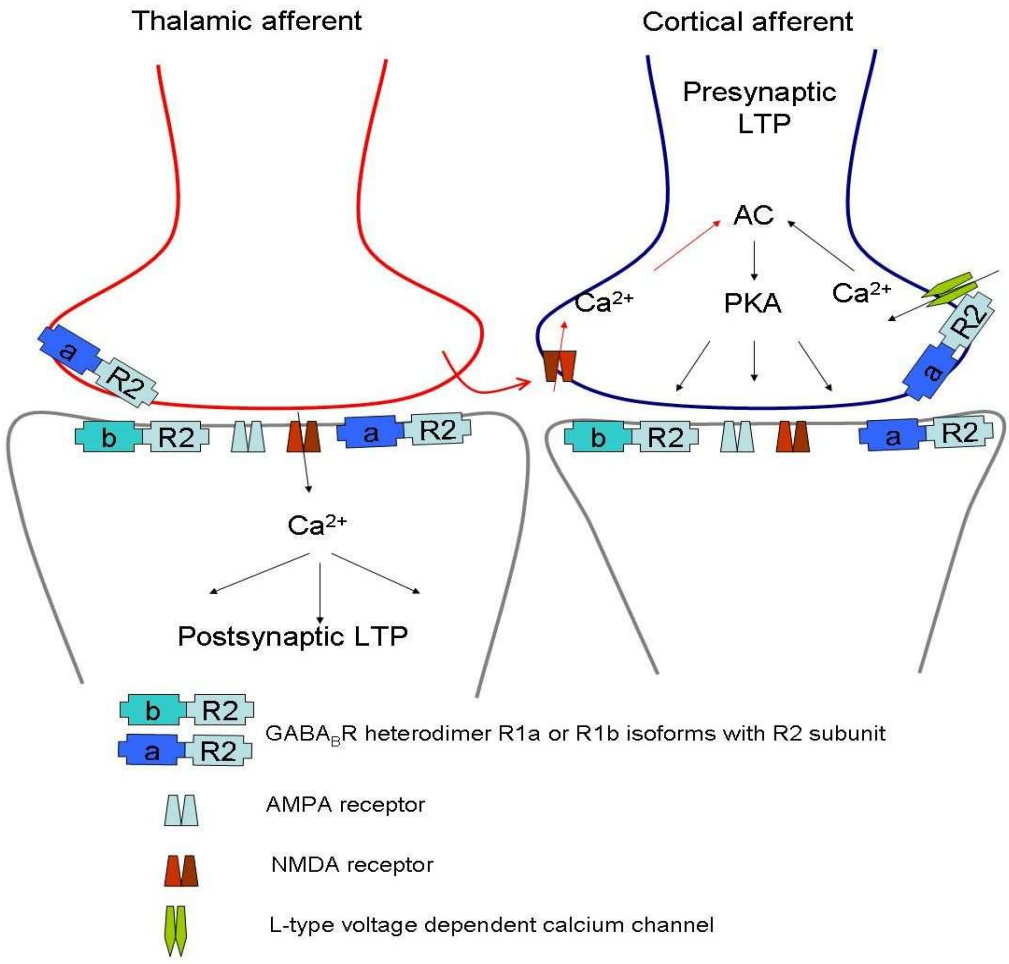

Figure 42) $GABA_BR$ cellular distribution and different modulation of synaptic LTP, $GABA_B$ (see text above)

6.4. Homosynaptic LTP at thalamic afferents is postsynaptically induced

We showed that LTP induction at thalamic afferents is facilitated by blockade of $GABA_BRs$ (Fig. 28B). In contrast to cortical LTP, thalamic LTP requires NMDAR activation and postsynaptic Ca^{2+} entry (Fig. 28D, E). We did not observe any change in PPR after induction of thalamic LTP (Fig. 28C). This suggests that a presynaptic mechanism is not involved in thalamic LTP. In agreement with previous results (Bissiere et al. 2003), we did not notice any facilitation of LTP in the presence of intact $GABA_AR$-mediated inhibition when $GABA_BRs$ were blocked (Fig. 28A). This suggests that $GABA_AR$-mediated inhibition is strongly regulating the induction of postsynaptic LTP at thalamic afferents. In contrast to $GABA_{B(1a)}$ -/- mice, $GABA_{B(1b)}$ -/- mice showed facilitation of homosynaptic LTP only at thalamic afferent and not at cortical afferent (Fig. 30). Facilitation of thalamic LTP was observed not only at the glutamatergic synapses, but also at disynaptic GABAergic inputs onto projection neurons (Fig. 31). The reasons why blockade of $GABA_BRs$ or knocking out $GABA_{B(1b)}$ but not $GABA_{B(1a)}$ subunit facilitates postsynaptic LTP induction only at thalamic afferent is an open question. The possible reasons for postynaptic facilitation of homosynaptic LTP at thalamic afferents could be due to the observation that dendritic spines contacted by thalamic and cortical afferents exhibit different morphologies (Humeau et al. 2005). In contrast to cortical afferents, thalamic afferents have significantly larger spine heads. Moreover, α-1E containing R-VDCCS are preferentially located at thalamic afferent synapses. Thus, postsynaptic LTP would be favored at thalamic synapses whereas presynaptic LTP is favored at cortical afferents. Therefore, upon the loss of $GABA_BR$-mediated inhibition, either by pharmacological blockade or genetic modification of $GABA_{B(1b)}$ isoforms, elevation of postsynaptic excitability would facilitate the induction of postsynaptic LTP selectively at thalamic afferetns. The reason why this homosynaptic LTP was induced at thalamic afferents specifically in $GABA_{B(1b)}$ -/- and not $GABA_{B(1a)}$ -/- is not clear and needs more future investigations. A possible explanation to this paradoxical discrepancy is that $GABA_{B(1b)}$ subunit would be specifically coupled to other effectors (e.g., mGluR, ATF, PKA, PKC) (Calver et al. 2002; Bettler et al. 2004) than $GABA_{B(1a)}$ and by that specifically modulate postsynaptic LTP induction at thalamic

afferents. Alternatively, $GABA_{B(1b)}$ containing $GABA_BRs$ could be localized postsynaptically more close to the synaptic input than $GABA_{B(1a)}$ receptors so that they have stronger control of the synaptic inputs. This differential synaptic localization at the postsynaptic cell could not be observed in our experimental set as we applied high concentration of baclofen (100µM) that acitvates all $GABA_BR$ subtypes irrespective of their synaptic location.

At the behavioral level, $GABA_{B(1b)}$ -/- mice showed deficit in fear learning (Fig32), presumably due to the unspecific facilitation of LTP induction at thalamic afferents. The loss of postsynaptic $GABA_BR$-mediated inhibition at the thalamic afferents could lead to imbalance between postsynaptic inhibition and excitation. This would postsynaptically facilitate the induction of LTP at thalamic afferents.

According to the Hebb rule, the efficacy of the synaptic transmission would be increased with the co-activation of pre- and postsynaptic elements. NMDAR-dependent LTP needs the coincident activation of NMDAR by presynaptically released glutamate and postsynaptic depolarization. NMDAR-mediated coincidence detection is an attractive model for Pavlovian conditioning because a CS-generated glutamatergic input that weakly activates a synapse will be potentiated if the US causes the cell to fire within a temporally limited window. Thus, the cells that participate in this plasticity must receive both CS and US inputs. Our data suggest that facilitated LTP at thalamic afferents in $GABA_{B(1b)}$ -/- mice a would occlude further induction of LTP during fear learning. Thus, the facilitated potentiation of synaptic transmission at thalamic afferents could impair fear memory formation.

In other studies it was shown that baclofen induces EEG slow waves that have been associated with reduced memory performance (Jones-Gotman et al. 1994; Schuler et al. 2001). In contrast, $GABA_BR$ antagonists exhibit a wide range of memory-enhancing effects in a variety of learning situations (Mondadori et al. 1996). It was proposed that $GABA_B$ receptor antagonists facilitate cholinergic transmission, given the known memory-enhancing effects of cholinergic substances in animals (Mondadori et al. 1996). Likewise, it was stressed that the known modulatory effects of $GABA_B$ antagonists on glutamatergic synapses could produce similar effects (Mondadori et al. 1996). On the

other hand, memory-impairing effects of $GABA_B$ antagonists are also reported (Castellano et al. 1993; Brucato et al.), as well as memory-improving effects of baclofen (Castellano et al.; Saha et al.). The diversity of these memory effects makes it difficult to identify a common mechanism. The widespread distribution of $GABA_BRs$ in the brain and the numerous modulatory effects on various synapses leave ample room for speculations.

Taken together, here we show that $GABA_{B(1b)}$ receptors are mostly postsynaptically localized and mediate postsynaptic inhibition keeping NMDAR far from the activation threshold and by that facilitate its function as associative detector of the network activity. The loss of this postsynaptic $GABA_BR$-mediated inhibition leads to unspecific induction of LTP and consequently deficit in fear learning.

6.5. Intrinsic properties of $GABA_B$ receptors

We have also investigated $GABA_{B(2)}$-/- mice for $GABA_B$ function via electrophysiological invistigations and found a clear abolition of all well known responses to $GABA_B$ agonists except for an atypical inward rectifying K^+ current. Pharmacological and behavioral analyses of $GABA_{B(2)}$ -/- mice indicate that deletion of the $GABA_{B(2)}$ subunit is sufficient to impair $GABA_B$ functions such as [35S]GTPγS binding, muscle relaxation, hypothermia, and EEG delta wave induction (Figs. 36,39,40). These findings are paralleled by a loss of typical electrophysiological $GABA_B$ responses in the $GABA_{B(2)}$ -/- hippocampal neurons (Figs. 37, 38). These results are analogous to the results obtained with $GABA_{B(1)}$ -/- mice suggesting that $GABA_BRs$ are mainly comprised of heteromeric $GABA_{B(1)}$ and $GABA_{B(2)}$ subunits. Our results imply that neurons that naturally lack a $GABA_{B(2)}$ subunit nevertheless have the potential to express functional $GABA_BRs$. Unfortunately, it is currently impossible to identify such cells for electrophysiological recordings. This, together with the finding that the $GABA_B$ receptors seen in $GABA_{B(2)}$ -/- mice do not appear to be involved in classical $GABA_B$ functions, makes it currently difficult to address the possible physiological role of such receptors. $GABA_B$ receptors mediate slow synaptic inhibition in the nervous system, via G-proteins signal transduction cascade, modulating postsynaptic inwardly rectifying

Kir3-type K^+ (Bowery et al. 2002; Calver et al. 2002; Bettler et al. 2004). Molecular studies on $GABA_BRs$ provided many evidences for heteromerization (Marshall et al. 1999); (Mohler et al. 2001). In the $GABA_B$ heteromer, the $GABA_{B(1)}$ subunit binds GABA and all competitive $GABA_B$ ligands (Kaupmann et al. 1998), whereas the $GABA_{B(2)}$ subunit is responsible for escorting $GABA_{B(1)}$ to the cell surface and for activating the G-protein (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). The regional distribution of individual $GABA_{B(1)}$ and $GABA_{B(2)}$ protein subunits is similar to that of the wild-type receptor, but in some brain areas such as the caudate-putamen, $GABA_{B(2)}$ is not detectable, even though $GABA_{B(1)}$ and the native receptor are present (Durkin et al. 1999; Margeta-Mitrovic et al. 1999; Clark et al. 2000). In addition, there appears to be very little $GABA_{B(2)}$ mRNA, relative to $GABA_{B(1)}$ mRNA, in the hypothalamus (Jones and Westbrook 1996; Clark et al. 2000). These findings, along with those suggesting that $GABA_{B(1)}$ and $GABA_{B(2)}$ subunit expression is not regulated in tandem (McCarson and Enna 1999), support the existence of additional, yet unidentified, $GABA_B$ receptor subunits.

Taken together, here we show that loss of the R2 subunit would lead to loss of all typical $GABA_BR$ functions. Nevertheless, we recorded an atypical K^+ current in the $GABA_{B(2)}$ -/- hippocampal slices. We conclude that association of $GABA_{B(2)}$ with $GABA_{B(1)}$ is essential for receptor localization and distal processes but is not absolutely necessary for signalling, so that there is a possible existence of functional $GABA_BR$ in neurons lacking $GABA_{B(2)}$ subunits

6.6. Relevance of the $GABA_BR$-modulation of synaptic plasticity in LA in anxiety treatment

Fear conditioning is associated with increases in the coherent oscillations in the BLA and the rhinal/hippocampal region at the theta frequency (Pare et al. 2002; Chapman et al. 2003; Pape and Stork 2003). This rhythmic activity in the amygdalohippocampal network appears to be responsible for facilitating NMDA-dependent LTP in the BLA and the hippocampus (Maren and Fanselow 1995; Pape and Stork 2003). The depression of NMDA-mediated EPSPs by $GABA_BR$-mediated inhibition in the BLA, during fear

conditioning-induced theta freequency, may act as a filter to ensure that only strong, emotionally salient stimuli produce synaptic plasticity.

By increasing inhibition on inputs not actively involved in CS-US integration, plasticity of interneurons may therefore serve to reduce activity and plasticity at those synapses and thus increase the signal-to-noise ratio of CS processing. Inhibitory transmission in LA may also play a role in the extinction of fear memories. The medial prefrontal cortex (mPFC) is thought to inhibit amygdala output during fear extinction (Morgan et al. 1993; Milad and Quirk 2002; Sotres-Bayon et al. 2004). Thus, modulation of the inhibitory network in the amygdala affects not only fear learning but also extinction (Marsicano et al. 2002; Shumyatsky et al. 2002). The impact of $GABA_BR$-mediated inhibition was also illustrated in the epilepsy research field where it was shown that blockade of $GABA_B$ receptors accelerates amygdala kindling development (Karlsson et al. 1992).

Most of pharmacological treatment for anxiety disorders involves activation of inhibition either by targeting $GABA_A$ or 5-HT1A receptors (Nemeroff 2003). Recent study showed that $GABA_{B(1)}$ -/- mice are more anxious than their wild type counterparts in several anxiety-related tests such as the light–dark box and staircase test (Mombereau et al. 2005). Moreover, it was shown that the anxiolytic-like effects of benzodiazepines are markedly diminished in $GABA_{B(1)}$-/- mice (Mombereau et al. 2004; Mombereau et al. 2004). Furthermore, more recently, the positive modulator GS39783 has been shown to be active in several animal models of anxiety (Cryan et al. 2004; Mombereau et al. 2004; Cryan and Kaupmann 2005).

It is noteworthy that despite $GABA_BRs$ inhibitory role, it is also clear that the membrane hyperpolarization afforded by $IPSP_B$ can be ‘excitatory’ in certain brain regions (Crunelli and Leresche 1991). Thus, in the thalamus, for example, $IPSP_B$ provides a mechanism for deinactivating T-type Ca^{2+} channels in GABAergic thalamocortical neurones. Following activation of an $IPSP_B$, the inactivation of T-type Ca^{2+} channels that exists at resting membrane potentials is removed such that when the $IPSP_B$ terminates and the membrane potential returns to resting levels or just depolarized to these levels, a T-type Ca^{2+} current is generated that causes a burst of action potentials (Crunelli and Leresche 1991). Furthermore, there is increasing evidence for multiple protein ‘signalosome’ complexes centred around the $GABA_B$ receptor, possibly linking the receptor subunits with

components of the relevant signalling pathways, structural and transport elements of the cellular architecture, and even other related receptors and/or ion channels (Calver et al. 2002). Consistent with this, it has recently been shown that in rat cerebellum the $GABA_B$ receptor is associated with membrane lipid rafts, specific cholesterol-rich microdomains situated within the plasma membrane that have been suggested to act as assembly platforms for cell signalling complexes (Becher et al. 2001). Therefore, it is entirely conceivable that the receptor diversity observed for the $GABA_B$ receptor in invivo could be explained not only by the existence of novel receptor subunits, but by differences in the receptor environment and the associated proteins available to interact with the known receptor subunits.

Studies in the cortex suggest that $GABA_B$Rs on pyramidal cells and interneurons are mainly activated when many GABAergic neurons fire simultaneously, in association with rhythmic oscillations, including theta oscillations (Mott et al. 1999; Scanziani 2000). This results in spillover of GABA into the extracellular space where it may activate extrasynaptic receptors. Interestingly, it was established that bursts of stimulation at the theta rhythm (4–8 Hz) are optimal for the induction of NMDAR-mediated LTP in the hippocampus (Larson et al. 1986). Moreover, the slow inhibition mediated by dendritic $GABA_B$Rs is optimally timed to suppress NMDAR-mediated EPSPs in both pyramidal neurons and interneurons in this region (Morrisett et al. 1991; Mott et al. 1999). Similar modulation of NMDAR-mediated EPSPs by $GABA_B$R-mediated inhibition has also been seen in the BLA (Huang and Gean 1994), where it was found that maximal depression was observed at a stimulus interval of 200 ms (i.e., the interval associated with the theta rhythm).

In vivo data demonstrated a powerful control through GABAergic inhibition over the activity of projecting principal cells (Lang and Pare 1997; Pape et al. 1998) which renders the role to the GABAergic interneurons in the control of excitation in this region GABAergic activity was shown to be modulated by different neuromodulatory system. For example, gastrin-releasing peptide (GRP) and its receptor (GRPR) was shown to be specifically expressed in the LA and in regions sending synaptic projections to the LA, whereas GRP receptors are expressed by a subset of GABAergic interneurons in the LA. (Shumyatsky et al. 2002). Application of GRP in vitro excites interneurons and increases

GABA release onto pyramidal cells (Fig. 43). Another system modulating GABAergic inputs in the amygdala is conducted by dopamine. The LA receives massive dopaminergic projections from the ventral tegmental area (VTA) (Nestler 2001). Application of dopamine in in vitro slices was shown to suppress feed forward inhibition of principal cells and facilitate inhibition of interneurons via activation of D2 receptors (Bissiere et al. 2003) (Fig. 43). Additionally, endocannabinoid-dependent LTD of isolated IPSPs was also reported in another previous study in isolated IPSPs (Marsicano et al. 2002). Here we add modulation of the feed forward inhibition by presynaptic $GABA_BR$ of cortical afferents to LA. Whether potentiation of the disynaptic inhibition occurs at inhibitory synapses onto principle cell, or at excitatory inputs onto interneurons (Fig. 43) is still not clear. Further experiments would be required to investigate the site of synaptic plasticity.

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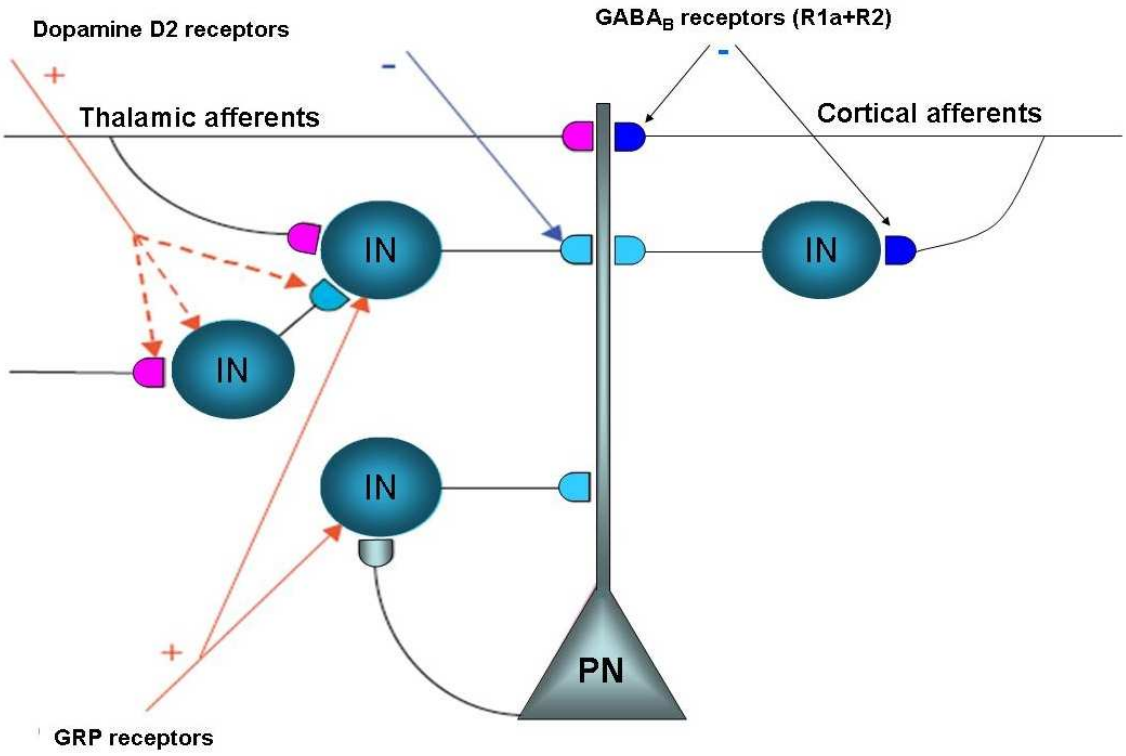

Figure 43) Modulation of GABAergic inhibitory input by different modulatory system, Dopamine modulates feed forward inhibition in the thalamic afferents, GRP modulates interneurons activity, $GABA_BR$ modulates excitatory inputs onto interneurons

6.7. Outlook and future experiments

Individuals suffering from anxiety experience an unpleasant emotional state defined by psychological and physiological responses to the anticipation of real or imagined danger. The treatment for anxiety disorders involves pharmacological targeting of $GABA_AR$ using benzodiazepines or allosteric modulators of $GABA_ARs$, or 5-HT systems using 5-HT1A receptor agonists and selective 5-HT reuptake inhibitors (SSRIs) (Nemeroff 2003). All these approaches have drawbacks because they have many unwanted side-effects, including tolerance, sedation, cognitive impairments and ethanol interactions, and, generally, besides the 5-HT receptor ligands onset of action is slow (Nemeroff 2003). Additional classes of $GABA_ARs$ modulators, the neurosteroids, have been limited use as anesthetics, and have been proposed as potential therapeutic agents for anxiety disorders. However, the poor bioavailability, solubility, and side effect profiles of these compounds have limited their application in humans.

In this study, we show that $GABA_B$ play key role in modulating synaptic plasticity in the LA. It is thought that fear emotion is associated with synaptic plasticity in the LA, so that would suggest $GABA_BR$ as a target for treatment of anxiety disorder. Further experiments would be required to explore the tonic activation of $GABA_BRs$ in the LA. Moreover, further studies are needed to characterize the anxiolytic potential of positive modulators of $GABA_BRs$ which would have potential advantages like not having side-effects as benzodiazepines.

7. List of abbreviations

-/+	Heterozygote-knockout
-/-	Homozygote-knockout
A	Amper
5-HT	5-hydroxytryptamine (serotonin)
AC	Adenylyl Cyclase
ACSF	Artificial Cerebrospinal Fluid
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPAR	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor
APV	DL-2-amino-5-phosphononalerate
ATP	Adenosine Triphosphate
BaCl	Barium Chloride
Baclofen	L- Lioresal (generic name)
BAPTA	1,2-bis-(o-aminophenoxy)ethane-N,N,N ,N -tetraacetic acid
BAPTA-AM	1,2-bis-(o-aminophenoxy)ethane-N,N,N ,N -tetraacetic acid-acetomethoxyester
BL	Basal Nucleus of the Amygdala
BLA	Basolateral Amygdala
Bpc	Basal Nucleus Magnocellular Subdivision
$CaCl_2$	Calcium Dichloride
CaMKII	Calcium/ Calmodulin Dependent Protein Kinase II
CAMP	cyclic 3',5'-adenosine monophosphate
CeA	Central Nucleus of the Amygdala
Cl^-	Chloride ione
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione sodium salt
CO_2	Carbondioxide
CPP	(R)-4-(3-phosphonopropyl)piperazine-2-carboxylic acid
CPPene	3-(2-carboxypiperazin- 4-yl)-1-propenyl-1-phosphonic acid
CS	Conditioned Stimulus
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DRIP	DA Receptor Interacting Proteins
e.c.	External Capsule
GABA	Gamma-aminobutyric acid
$GABA_BR$	Gamma-aminobutyric acid receptor subtype B
GDPβS	guanosine 5'-2-O-(thio)diphosphate
G_i	Inhibitory Guanine nucleotide binding Protein
G-protein	Guanine nucleotide binding proteins
G_s	Stimulatory Guanine nucleotide binding Protein
GTP	Guanosine Triphosphate
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonate)
IP_3	Inositol triphosphate
IPSC	Inhibitory Postsynaptic Current
LA	Lateral Amygdala
Ladl	Lateral Amygdale Dorsolateral Subdivision

Lam	Lateral Amygdale Medial Subdivision
Lavl	Lateral Amygdale Ventrolateral Subdivision
L-VDCC	Low Voltage Depndent Calsium Channel
LTD	Long term depression
LTP	Long term Potentiation
Mcd	Lateral Amygdale Dorsal Subdivision
Mcv	Lateral Amygdale Ventral Subdivision
mIPSC	Miniature Inhibitory Postsynaptic Current
mPFC	Medial Prefrontal Cortex
NaH_2PO_4	Monobasic Sodium Phosphate
$NaHCO_3$	Sodium Bicarbonate
NMDA	N-Methyl-D-Aspartate
NMDAR	N-Methyl-D-Aspartate Receptor
Osm	Osmolar
PIP_2	Phosphoinositol bi-Phosphate
Pir	Piriform Cortex
PKA	Protein Kinase A
PKC	Protein Kinase C
PP1	4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]-pyrimidine
PTK	Protein Tyrosine Kinase
PTX	Picrotoxin
Rp-cAMPs	Adenosine- 3', 5'- cyclic monophosphorothioate
s.t.	Stria Terminalis
sIPSC	Spontaneous Inhibitory Postsynaptic Current
Src	Rous Sarcoma Virus Transforming Oncogene
STP	Short term potentiation
STD	Short term depression
TTX	Tetrodotoxin
US	Unconditioned Stimulus
VGCC	Voltage Gated Calcium Channels
VTA	Ventral Tegmental Area

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Curriculum Vitae

Persönliche Daten:

Name	Shaban Hamdy
Geburtsdatum	6. Juni 1969
Nationalität	Schweizer/Aegypter
Zivilstand	Verheiratet
Addresse	Ahornstrasse 3 4055 Basel
Private	P: +41-061 301 73 76
Mobile	P: +41-078 768 37 24
Email:	Hamdy.shaban@fmi.ch

Ausbildung:

Mai 2002-Juni 2005	Ph.D thesis, Friedrich Miescher Institut, Novartis Forschungsstiftung, Basel, unter der Leitung von Prof. Dr. Andreas Lüthi: *"GABAB receptor-mediated modulation of synaptic plasticity in lateral amygdala"*
Feb. 2001-Apr. 2002	Diplomarbeit, ETH Zürich, Abteilung Biochemie, unter der Leitung von Prof. Dr. Christoph Richter: *" Apoptosis induced by inhibtion of cytochrom c oxidase with A2E N-retinyl-N-retinylidene ethanolamine"*
Okt. 1999-Apr. 2002	Master Biochemie, ETH Zürich
Okt. 1987-Okt. 1991	Bsc. Biochemie, Universität Alexandria, Aegypten

Referenzen:

Leiter der Doktorarbeit	Prof. Dr. Andreas Lüthi Friedrich Miescher Institut Maulbeerstrasse 66 4058 Basel 061 697 82 71 andreas.Luthi@fmi.ch

Korreferate	Prof. Dr. Bernhard Bettler Physiologisches Institut Pharmazentrum Klingelbergstrasse 50 4056 Basel 061 267 16 32 Bernhard.Bettler@unibas.ch
Leiter der Diplomarbeit	Prof. Dr. Christoph Richter ETH Zurich 8001 Zurich Abteilung Biochemie 0044 1844 344 292 ch.richter@btconnect.com

Konferenzen und Präsentationen:

Teilnahme:

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Juli 2004	Neurex Interneuron Meeting, Freiburg, Germany
Nov. 2003	33rd Annual Meeting of the Society for Neuroscience, New Orleans, USA
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