Brain circuit dynamics related to extremes in trait anxiety in mice

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“Men ought to know that from nothing else but the brain come joys, delights, laughter and sports, and sorrows, griefs, despondency, and lamentations. And by this, in an especial manner, we acquire wisdom and knowledge, and see and hear and know what are foul and what are bad and what are good, what are sweet and what are unsavoury….And by the same organ we become mad and delirious, and fear and terrors assail us.…All these things we endure from the brain when it is not healthy.…In these ways I am of the opinion that the brain exercises the greatest power in the man.”

-Hippocrates, on the Sacred Disease (Fourth century B.C.)

.....Dedicated to my family (Eleni, Sofia and Christodoulos)

.....and Mrs. Loukia Christodoulou

Abstract

Anxiety disorders are among the most common psychiatric diseases and contribute to the development of other psychiatric conditions, such as major depression, leading to a high impairment of daily life quality. Although it is obvious that the physiological architecture of neuronal networks and its modifications are essential for the ability of the brain to process incoming information and to control highly organized behaviour, the mechanisms underlying anxiety disorders still remain poorly understood.

We focused our attention on two brain structures, which are strongly involved in emotional responses of mammals, namely the hippocampus and the amygdala. Both structures belong to the limbic system and play fundamental roles in information processing. Recent findings indicate that alterations in neuronal network properties of these two brain areas critically contribute to the development of such disorders. To potentially uncover changes in neuronal network features associated with abnormal anxiety, we performed experiments in a well-established animal model of extremes in trait anxiety, the high vs. low anxiety-related behaviour (HAB/LAB) mice. HAB mice exposed to an enriched environment (HAB E.E.) and stressed LAB mice (LAB Str.) were also used in the present study. HAB E.E. animals showed decreased anxiety compared to standard HABs, whereas LAB Str. animals displayed an increase in anxiety levels compared to standard LABs. Anxiety levels were measured by means of the elevated plus maze. For our investigations, we employed classical electrophysiological techniques and high-speed voltage-sensitive dye imaging (VSDI) in acute hippocampal (dorsal & ventral) and amygdalar brain slices.

Field potential recordings revealed that HAB animals exhibit weaker long-term potentiation at CA3-CA1 synapses (CA1 LTP) in the dorsal hippocampus and an increased LTP in the ventral hippocampus compared to LAB and control CD1 mice. These observations could support the idea of an exacerbated activation of the “emotional” (ventral) hippocampus concomitantly with a decreased activity in the “cognitive” (dorsal) hippocampus, findings that have also been made in patients suffering from anxiety disorders.

To examine whether neuronal activity propagation through the amygdala differs between HAB, HAB E.E., LAB and LAB Str. mice, we used a quantitative VSDI approach. Our results demonstrate that HAB animals exhibit stronger neuronal

activity propagation through the amygdala compared to LAB mice. This indicates that differences in anxiety levels may correlate with the effectiveness of neuronal activity flow through the amygdalar network. Our study also provides strong evidence that environmentally induced shifts in trait anxiety are associated with changes in intrinsic amygdalar network properties.

To summarize, HAB animals showed increased “excitability” in the ventral hippocampus and in the amygdalar network, both structures known to be involved in the control of emotional states and in the stress response in mammals. In addition, the differences in amygdalar network activity were rescued by environmental conditions (enriched environment). Dysregulation of these structures could lead to the “pathologic anxiety-like” behaviour, which can be observed in HAB animals.

1 Introduction

1.1 Anxiety: physiology and pathology

1.1.1 From emotion to pathology

What are emotions? Why do we need emotions? How can they influence and contribute to the way an animal or a person acts in his environment? How can emotions motivate an individual to choose a certain pattern of behaviour to avoid a dangerous situation or to get a reward? What are the brain mechanisms underlying emotions and motivation? All these questions and many more are the main interest of researchers all over the world in the field of neuroscience and psychopharmacology. A major motivation to answer these questions is not only to understand how the brain works, but also to contribute to the understanding and treatment of mood / mental disorders (Rolls, 2000).

It is important to note that fear and anxiety by themselves are not only of pathological relevance, but are Nature's first defence reaction, as the ability to sense a potential danger before it strikes. In the natural environment, organisms are exposed to a large number of events that might have potentially grave consequences, including death. The risk management, for example to escape from predators or other dangerous situations, is a key problem of survival. Because such risks have very large consequences for the reproductive fitness, it is likely that evolution created a well conserved neurobiological system directed toward them (Woody and Szechtman, 2011). Even if fear and anxiety are closely related to each other, there is no interchangeable relationship between them. It is also important to distinguish between these two reactions: Fear is a reaction to a external stimulation and characterized through episodic appearance and autonomic hyper-arousal when the individual is exposed to a threatening stimulus (Pavuluri et al., 2002). Anxiety, in contrast, is characterized by a chronic or continuous appearance, not related to an external stimulus. Persons which experience anxiety, tend to be hyper-aroused even in on an ongoing basis (Pavuluri et al., 2002).

Unfortunately, this "surviving" program, which serves as a protection from a potential danger, has a price. It is also the basis for human suffering and psychological indisposition. In the past two decades, interest has strongly increased in the pathophysiology of anxiety disorders because they appear to be the most

prevalent mental disorders in the world. A systematic review on behalf of the European College of Neuropsychopharmacology (ECNP) Task Force on “Size and Burden of Mental Disorders in Europe” reported that anxiety disorders had 12-month and lifetime prevalence rates of approximately 12.0% and 21.1% respectively (Wittchen et al., 2005). These disorders are not only characterized by a massive reduction in life quality, but also by an early age of onset and prolonged course with a marked impairment of social and occupational functions and a strong tendency to develop psychiatric comorbidity (such as depression and substance abuse) (Wittchen and Jacobi, 2005). Furthermore, patients suffering from anxiety disorders have an increased risk of attempted and completed suicide (Sareen et al., 2005; Hawgood and De Leo, 2008). As already mentioned, there is a high level of comorbidity between anxiety disorders and major depression (Wittchen and Jacobi, 2005), bipolar disorders (Henry et al., 2003; Gaudiano and Miller, 2005), schizophrenia (Buckley et al., 2009), substance misuse (Castle, 2008; Ziedonis et al., 2008; Crippa et al., 2009; Robinson et al., 2009), and physical illness (Davies et al., 2007; Roy-Byrne et al., 2008).

The standard of health care appears in many cases to be suboptimal and the effectiveness of pharmacological and psychological treatment interventions can be disappointing (Baldwin et al., 2010). In the field of psychopharmacological treatment, the 20th century showed a major development in available drugs. Many patients do not respond or tolerate common pharmacological approaches (antidepressants) and psychological interventions (Garner et al., 2009). Although recently developed medications are better tolerated, they are not necessarily more effective than the previous generations of psychotropic drugs (Durham et al., 2004). Despite some advances in the scientific understanding of the basic neurobiology of anxiety and its cognitive and behavioural characteristics, the causes of anxiety disorders remain largely unknown.

Importantly, while many of the behavioural and physiological features of fear resemble those found under stressful conditions, stress is usually considered to be causal to anxiety and anxious subjects usually show a hyperresponse to stress.

1.1.2 Neurobiology of stress

1.1.2.1 Definition and overview

All organisms constantly interact with their external environment, receiving information through their sensory systems. Anxiety and fear serve as a physiological "surviving" process, by setting an organism under alarm when a potential threat appears. Environmental or physical changes, independent on their negative (threat/punishment) or positive (reward) nature, produce a spectrum of physiological responses to allow the organisms to adapt to the special needs of every situation. In face of a potential danger, these adaptations maximize the chance for an organism to survive from a frightening situation. The stereotypic, relatively nonspecific adaptive response of our body to any demand has been referred to in 1936 by Hans Selye under the term "stress". According to Selye, stress is defined as the interaction between a deforming force and the resistance of the organism to this force (Selye, 1973; Selye, 1998). Conditions that challenge homeostasis (the complex dynamic body equilibrium = set point) by intrinsic or extrinsic adverse forces are referred as "stressors". These stressors can be separated into three major categories according to their different nature (psychological or physiological) (Van de Kar and Blair, 1999):

1) Stressors based on learned response from the threat of prospective hostile conditions (like fear after exposure to a novel uncontrollable environment) can be defined as psychological stressors.
2) Stressors like pain, foot shock or immobilization represent in a physical stimulus and have a strong psychological component.
3) Stressors threatening cardiovascular homeostasis like haemorrhage, orthostatic stress, body exercise or heat exposure.

In spite of these differences, a common property of all these stressors is that they lead to adaptive responses to bring the homeostasis back to the initial set point. Under unexpected and potentially dangerous situations, animals exhibit an alarm reaction, mainly considered as non-specific and immediate behavioural response (such as startle), followed by a specific pattern of behavioural responses (e.g. flight and fight). These responses are mediated by the neuroendocrine systems (Tsigos and Chrousos, 2002; Engelmann et al., 2004; Ulrich-Lai and Herman, 2009).

1) The **sympatho-adrenergic system** (SAS), a part of the autonomic nervous system, provides a very fast response to a threatening situation and prepares the animal, through changes of physiological parameters and activation of behavioural responses, for a successful escape from the threat by active coping. The SAS activates brainstem nuclei, the vagal nerve (nerve X) and the medulla of the surrenal glands, leading to the release of adrenaline and noradrenaline into the bloodstream. This is followed by adaption on energy metabolism (e.g., direction of oxygen and nutrients to the brain, gluconeogenesis, lipolysis, inhibition of growth, and reproductive systems, containment of inflammatory responses) and behaviour pattern (e.g., increased arousal, vigilance and cognition, suppression of feeding and reproductive behaviour) (Engelmann et al., 2004).

2) The **hypothalamic-pituitary-adrenocortical (HPA) axis**

In contrast to the activation of the SAS, whenever an encounter is perceived to be aversive and cannot be controlled via a fight/flight reaction, passive coping occurs. This is associated with the activation of the HPA axis and leads to hormonal changes influencing both energy balance and behaviour (Koolhaas et al., 1999; Engelmann et al., 2004). Since the HPA axis plays a major role in the control and the regulation of stress and anxiety, it is important to describe it in detail.

1.1.2.2 The HPA axis

The HPA axis regulates the physiological and behavioural response to stress. Neurons of the medial parvocellular subdivision of the paraventricular nucleus (mpPVN) of the hypothalamus synthesize corticotropin-releasing hormone (CRH) and send projections to the median eminence, where they release CRH into the hypophysial portal blood vessels. The mpPVN receives synaptic innervation from neurons known to receive and process first- or second-order inputs from somatic nociceptors, visceral afferences or humoral sensory pathways. Once released, CRH acts upon specific cells of the anterior pituitary: the corticotrophs. CRH binds to G protein-coupled receptor (CRHR1/CRHR2) on the cell membrane of corticotrophs and activates an intracellular cascade, in turn leading to the synthesis and the release of the adrenocorticotropic hormone/corticotrophin (ACTH) into the general circulation. ACTH stimulates the synthesis and the secretion of glucocorticoids

(corticosterone in rodents and cortisol in humans) from the cortex of the adrenal glands, which act as the final effectors of the HPA axis (Chang et al., 1993; Chen et al., 1993; Vita et al., 1993; Chalmers et al., 1995; Perrin et al., 1995; Herman et al., 2002; Reul and Holsboer, 2002; Tsigos and Chrousos, 2002; Charmandari et al., 2005) (**Figure 1-1)**.

The mpPVN neurons also co-express a number of other peptides such as arginin-vasopressin (AVP) (Herman et al., 2002). AVP also exerts a modulatory effect on ACTH release from corticotroph cells, by acting in synergy with CRH. However, CRH remains the main ACTH secretion factor (Herman et al., 2002).

Activation of the HPA axis is generally characterized by a short release of ACTH, followed by a turn-off signal generated by the negative feedback of glucocorticoids on ACTH and CRH release (Herman et al., 2003).

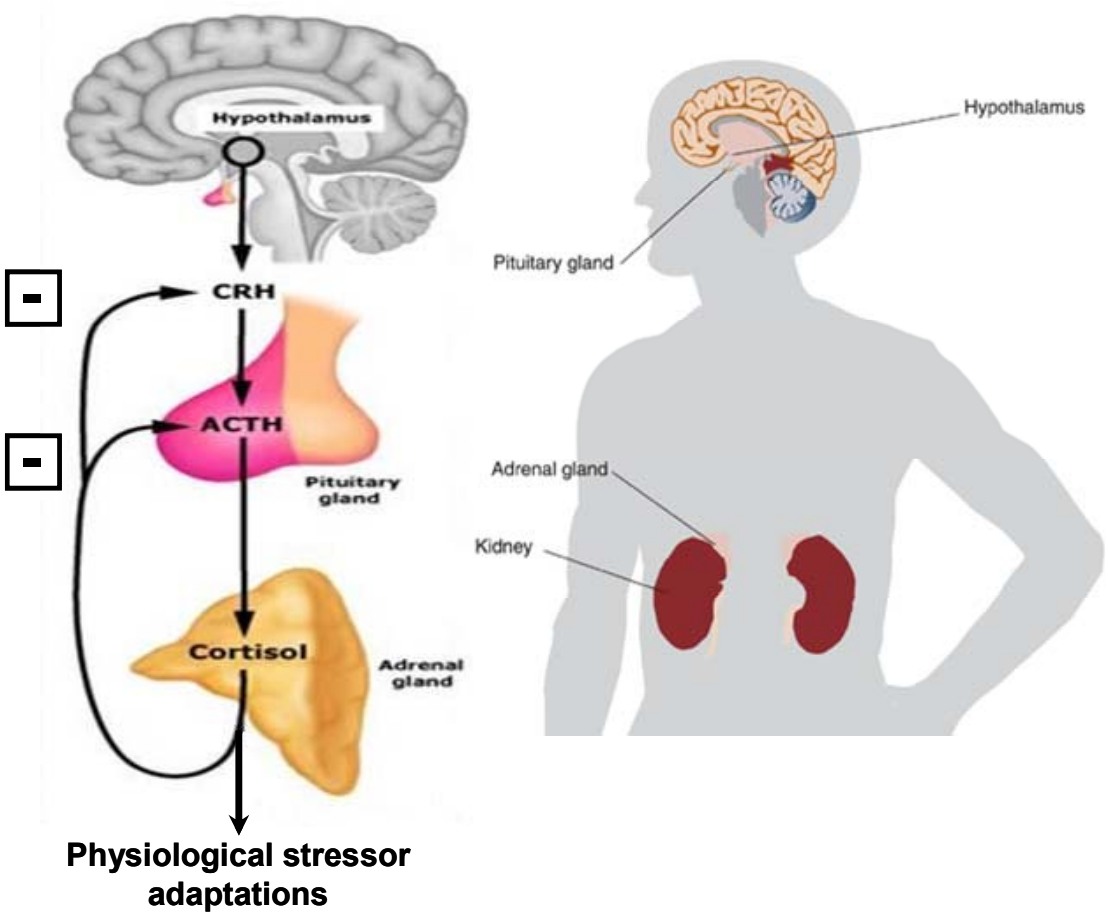


Figure 1-1: **The HPA axis**
Simplified schematic illustration of the central components of the HPA axis. Paraventricular nucleus (PVN) of the hypothalamus: Corticotropin-relasing hormone (CRH); Adrenocorticotropic hormone (ACTH).
Adapted from http://www.richslatcher.com/research/health.html

1.1.2.3 Glucocorticoids: action and effects

Glucocorticoids are the final effectors of the HPA axis and participate in the adaptation to stress and restore homeostasis, by enhancing emotional arousal, energy balance and promoting motivational and cognitive processes (Habib et al., 2001; Carrasco and Van de Kar, 2003; Fink, 2007; de Kloet et al., 2008). Under normal conditions, glucocorticoid release follows a circadian rhythm, with a peak at the initiation of the awaking cycle. This seems to be critical to organize the functional tone of sleep- and daily-related events (Herman et al., 2003; Hermann et al., 2003; de Kloet et al., 2008).

The effect of glucocorticoids is mediated through two receptor subtypes: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). In rodents, it has been shown that the MR has a higher affinity for corticosterone (Kd ≈ 1 nM) and plays a well-established role in synchronizing limbic circuits to maintain homeostasis. The GR has a lower affinity (Kd ≈ 5 nM) for corticosterone and only becomes active after stress exposure or during the ultradian cycles of the circadian rhythm. The activation of GR contributes to the stress response by facilitating recovery of brain activity, coordinating energy distribution and readjusting corticosterone baseline levels through a retrograde inhibitory loop of the HPA axis (De Kloet and Reul, 1987). MR is mostly localized on neurons of the limbic system, namely, the medial prefrontal cortex (mPFC), the amygdala and the hippocampus. GRs are ubiquitously expressed throughout the brain in neurons but also in glial cells; they are expressed at particularly high levels in the PVN and in the hippocampus (Reul and de Kloet, 1985).

Both receptors act as ligand-dependent transcription factors, i.e. induce or repress gene expression (Carrasco and Van de Kar, 2003; de Kloet et al., 2008). In the unbound form, they are located in the cytoplasma. The binding of corticosterone leads to a translocation of the ligand-receptor complex to the nucleus. Once in the nucleus, gene transcription is activated through the binding of this complex to specific DNA sequences, or through indirect interactions with other transcription factors (Beato and Sanchez-Pacheco, 1996). MR and GR share almost identical genome-binding sequences but they do not bind to the same sets of genes, therefore, activation of each receptor can lead to distinct cellular responses.

For many years, glucocorticoids were believed to exclusively produce a delayed and long-lasting response after stress exposure. However, recent studies

show that corticosteroids influence a wide range of behaviours and responses of the endocrine system within minutes, on a timescale which is too short to be associated with genomic effects (de Kloet et al., 1999; Haller et al., 2008; Riedemann et al., 2010). The cellular basis of these rapid effects remains poorly understood and controversial (Riedemann et al., 2010; Groeneweg et al., 2011). In general, fast non-genomic responses are those that occur within the first 20 minutes of increased steroid secretion, a much shorter timeframe than required for gene regulated and protein synthesis effects (Riedemann et al., 2010).

1.1.2.4 Regulation and termination of the HPA axis response

Generally, the stress-induced activation of the HPA axis is designed to be acute and limited to a short period. This short-term activation ensures that the concomitant anti-reproductive, anti-growth, catabolic and immunosuppressive effects do not injure the body state (Tsigos and Chrousos, 2002). As important it is to understand how the HPA axis is activated during stress, as important it is to understand what mechanisms are responsible for the limitation and the termination of this response since, depending on the quality, intensity and duration of the stress, the organism may not be able to adapt sufficiently, resulting in chronically elevated levels of glucocorticoids; the latter are known to be damaging at both the peripheral and brain levels.

An important modulation of the HPA axis activity occurs through activation of γ-aminobutyric acid (GABA) inhibitory neurocircuits. It has been shown that lesions in the bed nucleus of the stria terminalis (BNST), the medial preoptic area (MPOA) and the hypothalamus induce an increased ACTH and corticosterone secretion under basal or stress conditions. All these areas contain remarkable populations of GABAergic neurons. It has also been found that, after stress exposure, these areas show increased mRNA levels of glutamic acid decarboxylase (GAD), the GABA synthesis enzyme. Studies with targeted lesions also point to the regulatory function of the lateral septum, the mPFC and the hippocampus on the HPA axis. Indeed, lesions within these brain regions can increase corticosterone secretion in response to stress. One possible explanation could be that excitatory projecting neurons from the mPFC and the hippocampus excite GABAergic neurons of the BNST, the MPOA

and the hypothalamus, and thus decrease the activity of the HPA axis (Herman et al., 2003; Herman et al., 2005; Baumann and Turpin, 2010).

Regulation of the HPA axis is also directly mediated by corticosterone at different levels: PVN, pituitary and higher brain areas, but also on different timescales according to the fast non-genomic or slow genomic effects. Thus, corticosterone release can lead to a down-regulation of the transcription of CRH and AVP genes in the hypothalamus. The mechanisms underlying fast non-genomic feedback inhibition is poorly understood until now (Groeneweg et al., 2011).

At the hypothalamic level, the excitability of PVN neurons was reduced by application of corticostreroids in a rapid but prolonged manner (Groeneweg et al., 2011). Tasker *et al.* were able to show that application of high doses of corticosterone (100 nM - 1 µM) reduce the frequency of miniature excitatory postsynaptic currents (mEPSCs) in PVN neurons (Tasker, 2006; Tasker et al., 2006). Fast non-genomic effects of corticosteroids have also been described at the level of the anterior pituitary gland (Groeneweg et al., 2011). Inhibition of ACTH release was seen 1 min after corticosteroid administration. This action was insensitive to protein synthesis inhibitors and therefore, is believed to be mediated by non-genomic pathways (Keller-Wood and Dallman, 1984). Rapid non-genomic effects of corticosterone release have also been observed in the hippocampus and the amygdala (Groeneweg et al., 2011). Administration of corticosterone to hippocampal neurons for 5 min significantly enhanced the frequency of mEPSCs, i.e. the opposite effect as observed in the PVN (Karst et al., 2005; Olijslagers et al., 2008). This effect was found in wild-type and GR-knockout mice, but not in MR-knockout mice, suggesting that rapid corticosterone actions in the hippocampus are mediated by MRs (Karst et al., 2005). Stressful experiences also activate the amygdala, which can be seen as the emotional centre of the brain (Roozendaal et al., 2009). Genomic effects of corticosterone in the amygdala occur in an opposite direction to those observed in the hippocampus, i.e. they enhance neuronal activity in the amygdala (Duvarci and Pare, 2007; Mitra and Sapolsky, 2008) and reduce activity and plasticity in the hippocampus (Alfarez et al., 2002; Alfarez et al., 2009). An interesting finding with respect to the non-genomic actions of corticosterone in the amygdala is the demonstration that MR and GR are located in the plasma membrane of amygdalar neurons (Johnson et al., 2005; Prager et al., 2010). In basolateral amygdalar (BLA)

neurons, corticosterone induces a marked enhancement in mEPSC frequency, comparable to the effects observed in the hippocampus. This enhancement of mEPSC frequency after corticosterone administration was MR-dependent and nongenomic in nature (Karst et al., 2010).

Corticosterone also downregulates the activity of the HPA axis through negative feedback by higher brain areas. Lesions of the hippocampus lead to elevated CRH and AVP mRNA levels in mpPVN neurons. This effect occurs not only during stress, but can also be observed under basal conditions, suggesting that corticosterone-mediated negative feedback is reduced after damage of the hippocampus (Mizoguchi et al., 2003). Similar to this regulatory effect, administration of glucocorticoids into the mPFC or MPOA produced a blockade of stress hormone secretion under restraint stress conditions. The exact mechanisms underlying this inhibitory feedback are still not known. However, all of the observed findings indicate that corticosterone interacts with neuronal relay stations to increase inhibitory control of HPA axis activity (Fink, 2007).

1.1.2.5 Dysregulation of the HPA axis and mental disorders

Under stressful and basal conditions, the neuronal control of the HPA axis activity, through the regulation of the mpPVN activity and glucocorticoid secretion, appears to be crucial for the well-being and health of the organism. Stress-induced alterations in neuroendocrine control are likely to be associated with the pathogenesis of depression and anxiety disorders. Several reports demonstrated that dysregulation of the HPA axis is observed in a large percentage of patients suffering from depression (Carroll et al., 1981; Holsboer, 1983; Arana et al., 1985; Holsboer, 1999b). Severe anxiety and depression are stress-related disorders and have been hypothesised to result from an exaggerated stimulation of one or more of the CRH-modulated pathways mediating the stress responses. Patients suffering from strong melancholic depression show physiological and behavioural symptoms, associated with an enhanced activation of the CRH system, including comorbid anxiety and agitation (Gold et al., 1996; Holsboer, 1999b). The assumption that chronic CRH hypersecretion could play a crucial role in the aetiology of major depression has been

supported by post-mortem measurements of very high concentrations of CRH in the cerebrospinal fluid of severely depressed suicide victims (Gold et al., 1996; Hucks et al., 1997).

The anxiogenic effect of CRH is mediated via the activation of CRHR1 (Holsboer, 1999; Dautzenberg and Hauger, 2002). CRHR1 knock-out mice show reduced anxiety-related behaviour on the elevated plus maze (EPM) and in the light-dark box test. Both behavioural paradigms measure the anxiety level of the tested animals (Smith et al., 1998; Timpl et al., 1998). Inhibition of CRHR1 expression by central administration of CRHR1 antisense RNA or treatment of rats with non-peptidergic antagonists that selectively block CRHR1 also produce anxiolytic-like effects (Liebsch et al., 1995; Schulz et al., 1996; Heinrichs et al., 1997; Skutella et al., 1998; Deak et al., 1999; Liebsch et al., 1999; Okuyama et al., 1999).

Neuroendocrine alterations could also be caused by dysfunctions of the limbic regions involved in feedback inhibition, such as the hippocampus, the mPFC and the amygdala. Imaging studies link changes in the activity or in the volume of these brain structures with the development of mood disorders, such as major depression and pathological states of anxiety (Drevets et al., 1992a; Drevets et al., 1992b; Sapolsky, 1996; Herman and Cullinan, 1997; Mataix-Cols and Phillips, 2004; Freitas-Ferrari et al., 2010). Corticosteroid receptors (MR and GR) are also likely to play a critical role in maintaining homeostasis. They are highly expressed in the hippocampus and amygdala and genetically- or stress-induced disturbances in the expression of these receptors lead to changes in the activation patterns and the feedback loops, which in turn modulate neuronal excitability, stress responsiveness and behavioural adaptation to situations of enhanced susceptibility to disease (Tronche et al., 1999; Wei et al., 2012).

To summarise, the activation of the HPA axis maximises the survival rate of mammals under dangerous conditions, by acute alterations in cardiovascular activity, glucose, protein and fat metabolism, but also by influencing cognitive processes, to maintain internal homeostasis. The neuronal and endocrine control of the HPA activity appears crucial for the health of an individual, whereas its dysregulation can lead to mental and metabolic disorders or cardiovascular diseases. Since the HPA axis seems to be strongly involved in the pathophysiology of stress-related diseases, intensive research is needed in order to identify central mechanisms underlying the neuroendocrine stress responses and how alterations contribute to the pathogenesis

of such disorders. Regulatory neuronal circuits could serve as targets for future therapeutic approaches, providing a better treatment of patients suffering from stress-related pathologies. The limbic-hypothalamic system appears to be a good target for such approaches.

1.2 The limbic system

The limbic system, a part of the telencephalon, regroups allocortical areas (i.e. olfactory cortex, amygdala, and hippocampal formation, including the subicular cortices) and transitional areas between the allocortex and isocortex (i.e. nearly all parahippocampal and cingulate cortices, but also caudal orbital and medial prefrontal cortex, part of the temporal polar cortex, the ventral part of the agranular and dysgranular insular cortex, and also the mammillary bodies, the anterior thalamic nuclei and their connections) (Groen et al., 2008).

These limbic areas are functionally diverse, but the high degree of connectivity between them suggests that they comprise an underlying unity. The limbic system is strongly involved in emotional responses on the one hand and learning and memory on the other hand. Given the link between limbic regions, stress and stress-related disorders, it is important to understand the role played by these structures in stress integration, memory consolidation and emotional interpretation of sensory stimuli.

Based on the fact that the hippocampus and the amygdala are strongly involved in the regulation of the HPA axis activity, and, therefore, most likely also in the pathogenesis of stress-related disorders, the present study mainly focused on these two limbic structures.

1.2.1 The hippocampus

The hippocampus is located in the temporal lobe of the cerebral cortex and is composed of four distinct regions: the dentate gyrus (DG), the cornu ammonis 3 (CA3), the cornu ammonis 1 (CA1), and the subiculum (Andersen et al., 2007) (**Figure 1-2**).

1.2.1.1 Hippocampal anatomy

a- The dentate gyrus

The DG is composed of three layers. The most superficial one, the molecular layer, is a relatively cell-free layer. Deeper to the molecular layer lies the granular cell layer. Between the granular cell layer and the proximal end of the cornu ammonis is the polymorphic layer located. The granular cell layer contains the principle cells of the DG, namely the granule cells which send their axonal projections, the so-called mossy fibers, and make synapses on the dendrites of the principle cells of the CA3 region. The DG also contains GABAergic interneurons, such as basket cells (Ribak and Seress, 1983).

b- The CA3 subfield

The CA3 subfield is composed of four layers. The most superficial one is called the stratum radiatum. Below this layer lies the stratum lucidum. These two layers consist of the mossy fibers coming from the DG and of the dendrites of the pyramidal cells which lie in the third layer, the stratum pyramidale. The deepest layer, the stratum oriens, can be defined as an infrapyramidal region containing the axonal projections of the pyramidal neurons, the so-called Schaffer collaterals (Andersen et al., 2007). The pyramidal cells are excitatory neurons and are the most prominent cell type in the CA3 subfield. As in the DG, the CA3 region also contains a fairly heterogenous population of interneurons (Freund and Buzsaki, 1996).

c- The CA1 subfield

The CA1 subfield is also composed of four layers. The most superficial one is the stratum lacunosum moleculare, which contains fibers coming from cortical regions (ie the enthorhinal cortex). Below this layer lies the stratum radiatum containing the Schaffer collaterals which make synapses on the apical dendrites of the principle cells, the pyramidal cells, which lie in the third layer, the pyramidal layer. As in the CA3 subfield, the deepest layer is called the stratum oriens and contains the axonal projections of the CA1 pyramidal cells to the subiculum. The CA1 pyramidal cells are excitatory neurons that are slightly smaller than CA3 pyramidal neurons (Andersen et al., 2007). Finally, the CA1 subfield also contains a heterogenous population of inhibitory interneurons (Freund and Buzsaki, 1996).

d- The subiculum

The CA1/subiculum border is abruptly marked by a widening of the pyramidal cell layer. The CA1 stratum radiatum also ends at this border and is replaced by the molecular layer of the subiculum. The deepest portion of this layer receives axonal projections from CA1 and contains the dendrites of the principal cells of the subicular pyramidal layer, whereas the superficial portion receives direct innervation from the enthorhinal cortex. The stratum oriens is no longer present in the subiculum (Greene and Totterdell, 1997; Andersen et al., 2007). The principal cell layer of the subiculum contains large pyramidal cells, defined according to their electrical properties as regular firing and bursting pyramidal cells (Greene and Totterdell, 1997). Intermingled among the pyramidal cells are many smaller neurons representing the subicular interneurons.

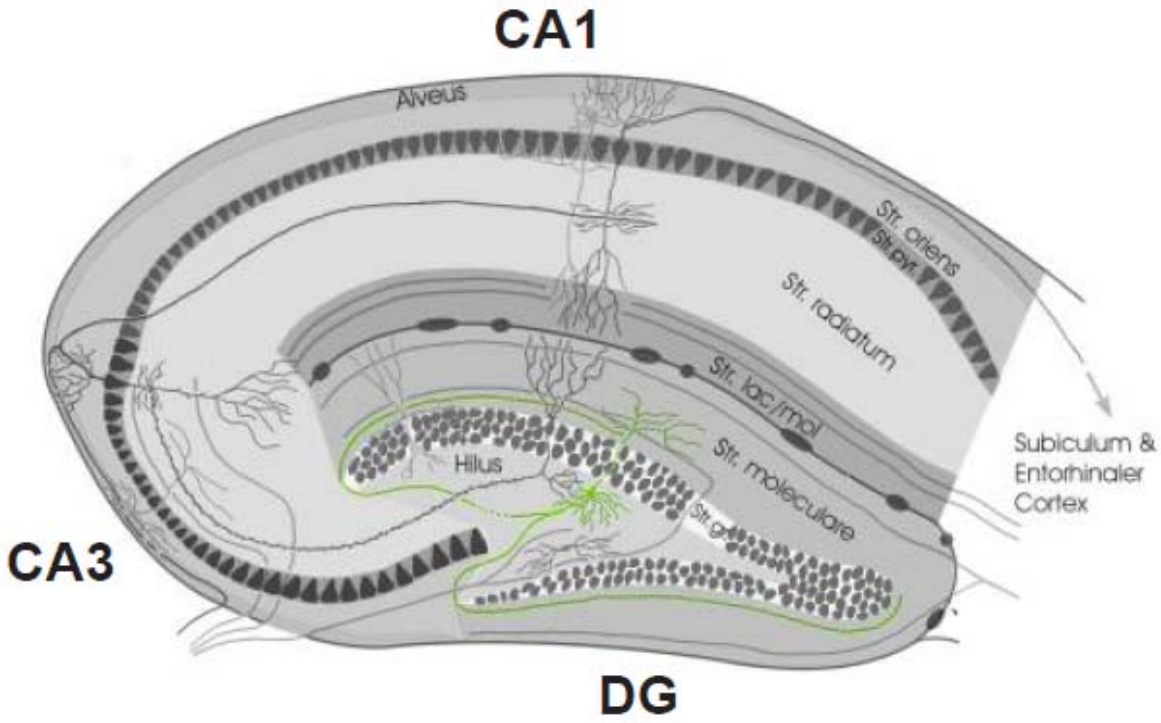


Figure 1-2: **Summary of the organisation of hippocampal pyramidal cells**
Dentate Gyrus (DG); Cornus ammoni 3 area (CA3); Cornus ammoni 1 area (CA1). Adapted from http://anatomie.vetmed.uni-leipzig.de/external/hippocampus/hippocampus_moos.html

1.2.1.2 Hippocampal afferences

a- To the dentate gyrus

The most prominent subcortical input to the DG is the projections from the septal nuclei. Septal fibers strongly innervate cells of the polymorphic layer, mostly in a region subadjacent to the granular cell layer (Mosko et al., 1973; Swanson et al., 1978; Baisden et al., 1984). The DG is also innervated by the hypothalamus, the strongest projections coming from the supramammillary nucleus which terminate in a narrow zone of the molecular layer, located superficially to the granular cell layer (Wyss et al., 1979; Vertes, 1992; Magloczky et al., 1994). In addition, the DG also receives prominent input from the locus coeruleus. These fibres mainly target the polymorphic layer of the DG and extend into the stratum lucidum of the CA3 subfield (Pickel et al., 1974; Swanson and Hartman, 1975; Loughlin et al., 1986).

However, the major input to the DG arises from the entorhinal cortex via the perforant pathway (Ramòn y Cajal, 1893). Cells located in layer II of the entorhinal cortex project to the DG, although a minor part of these projections arise from layer IV (Steward and Scoville, 1976). Projections from the entorhinal cortex are only restricted to the superficial two-thirds of the molecular layer of the DG (Nafstad, 1967; Hjorth-Simonsen and Jeune, 1972). The perforant path can be divided into two parts, the lateral and medial perforant path, according to the region of origin and pattern of termination (Andersen et al., 2007). Fibres coming from the lateral entorhinal area terminate in the most superficial third of the molecular layer and fibres originating from the medial entorhinal area terminate in the middle third of the molecular layer.

b- To the CA3 area

Like the DG, the CA3 subfield receives projections from the entorhinal cortex. The origin and laminar terminal distribution of the perforant path projections to the CA3 are similar to those of the DG (Witter, 1993). On one hand, projections from the lateral entorhinal cortex terminate superficially in the stratum lacunosum-moleculare. On the other hand, those from the medial entorhinal area terminate in the deep half of this layer. One remarkable feature of intra-hippocampal connectivity is that the majority of its synaptic input arises from within its own boundaries. Thus, the CA3 pyramidal cells are heavily innervated by collaterals from their own axons (called

associational connections) and from axons of the contralateral CA3 subfield (Andersen et al., 2007). The CA3 is the only brain region which receives direct projections from the DG and the mossy fibres projecting to CA3 arise exclusively from the granular cells and terminate in a relatively narrow zone, mainly located just above the CA3 pyramidal cell layer (Blackstad, 1956; Gaarskjaer, 1978; Swanson et al., 1978; Claiborne et al., 1986).

The major subcortical input to the CA3 subfield is provided by the septal projections, arising primarily from the medial septal nucleus and the nucleus of the diagonal band of Broca. These projections terminate most heavily in the stratum oriens and to a lesser extent in the stratum radiatum (Andersen et al., 2007). Tracing studies recently showed that CA3, especially its temporal parts, receives a direct input from the amygdaloid complex (Pikkarainen et al., 1999; Pitkanen et al., 2000). These projections arise from the caudomedial portion of the parvocellular division of the basal nucleus and terminate in the stratum oriens and stratum radiatum.

c- To the CA1 area

Similar to the DG and the CA3 area, the CA1 subfield is also directly innervated by the entorhinal cortex. However, the organisation of these projections is fundamentally different compared to the projections to the CA3 area (Andersen et al., 2007). First, the afferences arise from cells in layer III (and not layer II). Secondly, the distribution of terminals is not laminar but topographically organised. According to this organisation, fibres arising from the lateral entorhinal area terminate in the distal portion of the CA1, whereas fibres originating from the medial entorhinal area terminate in the proximal portion of the CA1 area, close to the CA3 border. Consequently, depending on where a CA1 pyramidal cell is located in the transverse axis of the hippocampus, it receives inputs from different parts of the entorhinal cortex.

Tracing experiments have shown that CA1 receives inputs from the amygdaloid complex (Pikkarainen et al., 1999; Pitkanen et al., 2000). This input originates mainly from the caudomedial part of the parvocellular division of the basal nucleus and terminates heavily in the stratum oriens and stratum radiatum. Like the CA3 area, CA1 also receives substantial septal projections, but weaker as compared to CA3. The septal fibres are most densely distributed in the stratum oriens (Andersen et al., 2007).

Pyramidal neurons of the CA1 area receive their strongest innervation from pyramidal cells of the CA3 subfield. The distribution pattern of these terminals strongly depends on the location of the CA3 cells from which they arise. Each CA3 pyramidal neuron gives rise to highly collateralised axons that follow both a transverse and oblique orientation through CA1 (Ishizuka et al., 1990). These projections terminate on the basal dendrites in the stratum oriens and the apical dendrites in the stratum radiatum. The probability for a CA1 neuron to be innervated by a particular CA3 cell depends on its transverse position and septotemporal level. Distal CA3 pyramidal neurons are more likely to innervate proximal CA1 pyramidal cells whereas proximal CA3 cells are more likely to innervate distal CA1 neurons.

d- To the subiculum

White *et al.* showed in a combined neuroanatomical and electrophysiological study, that projections from the anterior cingulate cortex reach the subiculum (White et al., 1990). However, this finding is still under debate. Other projections to the subiculum arise from the perirhinal cortex, but terminate only in its proximal third (Andersen et al., 2007).

The proximal subfield of the subiculum is also innervated by projections from the parvocellular portion of the basal nucleus and the posterior cortical nucleus of the amygdala, and the adjacent amygdalo-hippocampal area (Pitkanen et al., 2000). Another source of afferences to the subiculum arises from the supramammillary region, which strongly projects to its temporal part. This portion of the subiculum also receives an input from the premammillary nucleus.

Thalamic projections mainly arise from the nucleus reuniens, the paraventricular nucleus, and the parataenial nucleus. The midline thalamic projections appear to be largely restricted to the molecular layer of the subiculum. The projections to the subiculum arise from various intermingled populations of neurons in the nucleus reuniens (Andersen et al., 2007).

Another source of afferent projections to the subiculum arises from the brainstem, namely the noradrenergic locus coeruleus, the dopaminergic ventral tegmental area, and the serotoninergic median and dorsal raphe nuclei (Andersen et al., 2007).

1.2.1.3 Hippocampal efferences

The hippocampus sends projections to numerous other brain regions. For example, CA1 pyramidal cells give rise to reciprocal projections to the entorhinal cortex (Naber et al., 2001). The projection cells send most of their axons to the same region of the entorhinal cortex from which they receive their inputs. Another major connection is with the neocortex. However, only selected parts of the hippocampus form direct connections with the neocortex. CA1 pyramidal neurons give rise to return projections to the perirhinal cortex. CA1 cells located in the septal part of the hippocampus also project to the retrosplenial cortex, and those located at mid-septotemporal levels provide substantial projections to the medial frontal lobe. The temporal two-thirds of the distal part of CA1 is reciprocally connected with the amygdaloid complex (Andersen et al., 2007).

The subiculum appears to be a major source of efferent projections from the hippocampus. After the discovery by Swanson and Cowan that the subiculum, rather than the cornu ammonis, is the origin of the major subcortical connections to the diencephalon and brainstem, it was widely accepted that the subiculum is one of the two primary output structures of the hippocampus (Swanson and Cowan, 1975; Swanson et al., 1981; Ishizuka, 2001; Kloosterman et al., 2003a). The subiculum projects to the presubiculum, as a series of pathways, distributing information that has been processed in the DG, the cornus ammoni and the subiculum to a series of cortical and subcortical structures. Therefore, the subiculum can be seen as the last relay station in the entorhinal-hippocampal loop (Andersen et al., 2007). The subiculum also sends strong projections to the medial and ventral orbitofrontal cortices, the prelimbic and infralimbic cortices and the anterior cingulate cortex (Verwer et al., 1997).

The initial temporal third of the subiculum also gives rise to reciprocal projections to the amygdaloid complex. The majority of these projections terminate in the accessory basal nucleus and a few of them reach several other nuclei other than the lateral nucleus. The ventral subiculum sends projections to the BNST and to the ventral part of the claustrum or endopiriform nucleus (Andersen et al., 2007).

Finally, the strongest subcortical connections of the subiculum are to the nucleus accumbens, the mammillary nuclei, and the hypothalamus. Subicular fibers terminate throughout the nucleus accumbens, but most densely in its caudomedial

part. A major feature of these connections is their unidirectional projection. The subicular projections to the mammillary nuclei consist of fibers originating mainly from the septal two-thirds of the subiculum and form the main input to this structure. Fibers of the subiculum also project to the lateral hypothalamic region, adjacent to the lateral mammillary nucleus.

1.2.1.4 Synaptic plasticity in the hippocampal CA1 area

a- Definition

For more than a century, it has been suggested that the ability of the brain to translate and accumulate experiences into various forms of long-lasting memories, can be attributed to activity-dependent changes in the synaptic communication between neurons (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). Such plasticity has been extensively studied in the hippocampus. Experimental evidence for this hypothesis first came in 1973, when Bliss and Lomo showed that repetitive activation of excitatory synapses in the hippocampus caused a robust and persistent enhancement in synaptic strength (Bliss and Lomo, 1973). This phenomenon was referred as long-term potentiation (LTP) and, since then, has been the subject of intense investigations. It is commonly accepted, that experimentally induced LTP serves as a powerful tool to elucidate the cellular and molecular mechanisms by which the brain can store information (Malenka and Nicoll, 1999). LTP can be elicited at all excitatory synapses in the hippocampus, and can also be observed in other brain regions. Growing experimental evidence confirms the assumption that it underlies at least certain forms of memory (Morris et al., 1990; Doyere and Laroche, 1992).

LTP can be induced by a number of different experimental protocols, the most common is tetanic stimulation of afferents (typically a train of 100 stimuli at 100 Hz). LTP has three basic characteristics: input-specificity, associativity and cooperativity. Input-specificity means that, when LTP is triggered at one set of synapses at a particular cell, the evoked increase in synaptic transmission normally does not occur at other synapses on this cell (Andersen et al., 1977). This property is a major advantage because it increases the storage capacity of individual neurons. Cooperativity means that there is an intensity threshold for induction of LTP; “weak”

tetanic stimulation, which activates relatively few afferent fibres, does not trigger LTP. The threshold which has to be overstepped in order to produce LTP is a complex function of the intensity and pattern of tetanic stimulation. LTP is associative in the sense that a “weak” input can be potentiated if it is active at the same time as a strong tetanus takes place in a separate but convergent input (McNaughton et al., 1978; Levy and Steward, 1979).

b- Mechanisms

The contribution of several excitatory amino-acid receptor subtypes to LTP induction has been widely reviewed in the literature. It has been shown that CA1-LTP requires the activation of postsynaptic *N*-methyl-D-aspartate (NMDA) receptors. The important role of the NMDA receptor is based on its specific properties, especially the voltage-dependent block of its pore by Mg^{2+}. In order to open the NMDA channel and, thus, to induce LTP, it is necessary that two events occur simultaneously: 1) the membrane has to be sufficiently depolarised to release the Mg^{2+} block from the pore; 2) L-glutamate has to bind to the NMDA receptor, which in turn promotes the opening of the channel.

Depolarisation of the postsynaptic membrane is achieved by activation of a second type of glutamate receptor, i.e. the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor. The AMPA receptor forms a channel permeable to monovalent cations (Na^+ and K^+) and is responsible for basal synaptic transmission. In case of a LTP induction, the tetanic stimulation leads to the release of high amounts of glutamate from the presynaptic bouton, which binds to AMPA receptors thereby depolarising the postsynaptic membrane. This depolarisation entails Mg^{2+} dissociation from its binding site within the NMDA receptor pore. Glutamate binds to the NMDA receptor allowing the opening of the channel and Ca^{2+} and Na^+ influx in to the dendritic spine. The rise of intracellular Ca^{2+} concentration is the critical trigger for LTP because it activates signal transduction pathways, leading finally to the increase in synaptic strength (Collingridge, 1987; Bashir et al., 1991; Bliss and Collingridge, 1993; Malenka and Nicoll, 1999; Lu et al., 2001; Malenka and Bear, 2004). Several molecules have been shown to play a key role in LTP induction, with α-calcium-calmodulin-dependent protein kinase II (CaMKII) being perhaps the most important (Teyler and DiScenna, 1987; Gustafsson and Wigstrom, 1988; Bliss and Collingridge, 1993; Larkman and Jack, 1995; Nicoll and Malenka, 1995; Lisman

et al., 2002). The final step of LTP induction is the phosphorylation of AMPA receptors by CaMKII, which in turn leads to the stabilisation of these receptors in the plasma membrane and to the insertion of new AMPA receptors (Barria et al., 1997) (**Figure 1-3**).

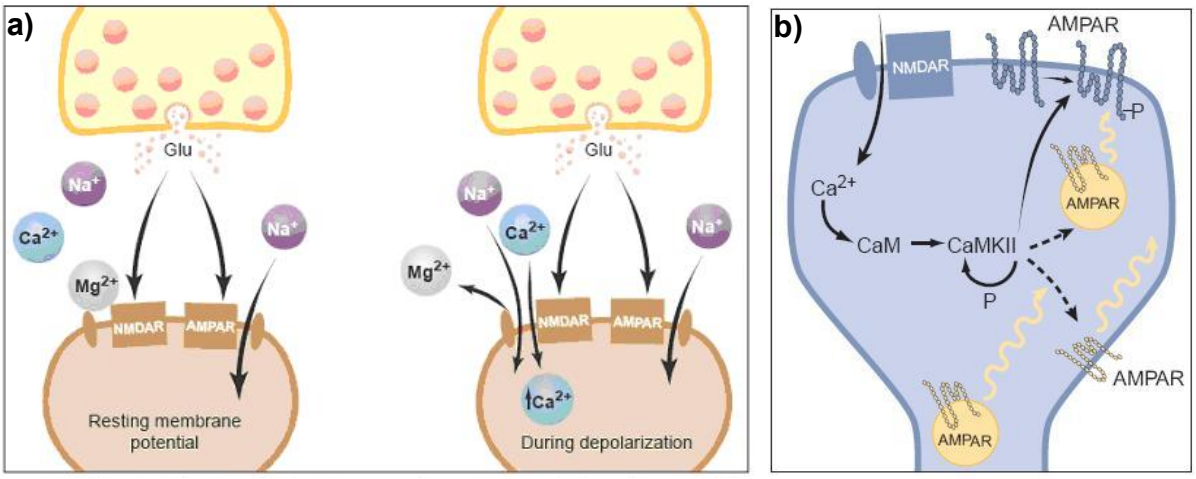


Figure 1-3: **Schematic Illustration of LTP induction**
a) Released glutamate from the presynaptic bouton acts on both AMPA receptors (AMPARs) and NMDA receptors (NMDARs). Under resting membrane potential, NMDARs are blocked by Mg^{2+}, therefore, Na^{+} flows only through the AMPA receptor. Depolarisation of the postsynaptic cell releases the Mg^{2+} block of the NMDA receptor channel, and Na^{+}/Ca^{2+} enters the dendritic spine through the NMDA receptors. This is the critical trigger for LTP. **b)** The postsynaptic increase in Ca^{2+} concentration activates CaMKII, which in turn phosphorylates already inserted AMPA receptors. CaMKII is also believed to influence the subsynaptic localisation of AMPA receptors such that more AMPA receptors are delivered to the synaptic plasma membrane. Adapted from Malenka and Nicoll, (1999).

It has been shown that postsynaptic application of CaMKII inhibitors or the genetic deletion of a critical CaMKII subunit prevents LTP induction (Malenka et al., 1989; Malinow et al., 1989; Bliss and Collingridge, 1993). In contrast, increased expression of constitutively active CaMKII in CA1 cells leads to an enhancement of synaptic transmission and LTP (Pettit et al., 1994; Lledo et al., 1995). Several other protein kinases have also been implicated in LTP: protein kinase C (PKC), cAMP-dependent protein kinase (PKA), the tyrosine kinase (Src), and mitogen-activated protein kinases (MAPK). Whether these kinases are mediators or modulators of LTP remains to be determined (Lynch, 2004).

Is the increase in synaptic strength during LTP primarily generated due postsynaptic modifications or due presynaptic change in transmitter release? It is now widely accepted that the major mechanism underlying the expression of CA1-LTP involves an increase in the number of AMPARs in the plasma membrane at synapses, secondary to activity-dependent changes in AMPAR trafficking (Malenka

and Nicoll, 1999; Malinow and Malenka, 2002; Song and Huganir, 2002; Bredt and Nicoll, 2003). In addition, an increase in the single-channel conductance of AMPARs via direct CaMKII-mediated phosphorylation of the AMPA receptor subunit GluR1 contributes to increased synaptoc strength (Benke et al., 1998; Malenka and Nicoll, 1999; Soderling and Derkach, 2000; Lee et al., 2002).

An important observation is that LTP lasts for hours, days or even weeks. This requires gene transcription and protein synthesis (Abraham and Williams, 2003; Kandel and Pittenger, 2003; Lynch, 2004; Malenka and Bear, 2004) and involves structural remodelling of synapses, including growth of new dendritic spines, enlargement of pre-existing spines and their associated postsynaptic densities and splitting of single postsynaptic densities and spines into two functional synapses (Yuste and Bonhoeffer, 2001; Abraham and Williams, 2003). This structural remodelling of dendritic spines implies modifications of the cell cytoskeleton. *In vivo* studies have demonstrated that LTP is accompanied by a long-lasting increase in F-actin content in spines, thus inhibiting actin depolymerisation (Fukazawa et al., 2003). The notion that actin cytoskeleton reorganisation is strongly involved in maintaining LTP is also supported by studies showing that inhibitors of actin polymerisation impair LTP (Kim and Lisman, 1999; Krucker et al., 2000).

c- Functional significance of LTP

It is commonly accepted that long-lasting changes in the strength of synaptic connections are critical components of the neural mechanisms underlying learning and memory. LTP provided the first experimental analogue of these postulated learning-induced changes in synaptic connectivity in the brain (Bliss and Collingridge, 1993; O'Mara et al., 2000; Morris et al., 2003; Neves et al., 2008).

Since Morris *et al* (1986) discovered that hippocampal NMDA receptor blockade prevents spatial learning but not visual discrimination learning, a variety of experimental studies confirmed that NMDA receptor activation is necessary for a wide range of different forms of learning and memory (Morris et al., 1986). Moser demonstrated that chronic intraventricular infusion of 2-amino-5-phosphonovalerate (AP5), an NMDA receptor antagonist, impaired hippocampal LTP and also spatial learning (Moser et al., 1993; Moser and Moser, 1998b). The findings of this initial pharmacological study are complemented by studies in which the NMDA receptor was genetically modified. Tsien *et al.* (1996) showed, by region-specific gene

targeting that the NMDA receptor channel in the hippocampal CA1 region is essential for LTP and spatial learning (Tsien et al., 1996). By means of analyses of the functional properties of various NMDA receptor subunits, another study showed that disruption of the GluRε1 (NR2A) gene results in the reduction of hippocampal LTP and impairment of Morris water maze learning (Kiyama et al., 1998).

Taken together, the findings described above are in line with the assumption that synaptic plasticity is the cellular correlate of certain forms of learning and memory.

d- Short-term plasticity

Like LTP, short term plasticity has been also observed at many chemical synapses (Zucker, 1989; Wang and Kelly, 1997; Lopez, 2001). One of the most studied forms of short term plasticity is the paired-pulse facilitation (PPF).

PPF is an experimental protocol consisting of giving 2 electrical stimuli on presynaptic terminals with a short interstimulus interval (ranging from 25 to 400 ms) and recording the resulting excitatory postsynaptic potentials (fEPSP). From these recordings, the paired-pulse ratio (PPR) is calculated as: PPR= amplitude fEPSP2/ amplitude fEPSP1. When this PPR is larger 1, we called it PPF. In certain cases, the PPR can be smaller than 1 and we talked about paired-pulse depression (PPD). The PPR is a way to study rapid modifications occurring at synapses and also give an idea about the release probability of neurotransmitter at these synapses.

For example at CA3-CA1 synapses, the application of this protocol leads to a PPF. The underlying mechanism of PPF is associated with a rise in Ca^{2+} levels in the presynaptic bouton in response to the 1^{st} pulse. When the second pulse is given with a short delay (25-100 ms), the residual Ca^{2+} from the 1^{st} pulse cannot be buffered, and the rise in intracellular Ca2+ due to the 2^{nd} pulse leads to the fusion of more vesicles and thus to the release of higher amount of neurotransmitter in the synaptic cleft (Miledi and Parker, 1981; Charlton et al., 1982; Wang and Kelly, 1997). This mechanism is known as the theory of “the residual calcium” (Manabe et al., 1993; Christie and Abraham, 1994; Lopez, 2001).

1.2.1.5 Functional differentiation within the hippocampus

The functional role of a particular brain region depends on the afferent and efferent connectivity with other brain structures. Ramòn y Cajal (1901) and Lorente de Nò (1934) established for the first time a basic cytoarchitectonic scheme of the hippocampus (Cajal, 1901; Lorente De Nó, 1934). In his pioneering work, Cajal described alterations across the longitudinal axis of the hippocampus. He distinguished two perforant pathways coming from the entorhinal cortex (i.e. the "superior" and "inferior"), today referred as to the "dorsal" and "ventral" hippocampus. Lorente de Nò divided the "ammonic system", based on the different afferent inputs across the dorso-ventral axis, into three main segments. He postulated that, even if there is no sharp boundary, each of these segments has specific structural characteristics.

Generally, the hippocampus is defined as a unidirectional transverse loop of excitatory synapses, through the "trisynaptic pathway" and the subiculum. Although this intrinsic connectivity appears to be consistent along the longitudinal axis of the hippocampus, afferent and efferent connectivity changes along the dorso-ventral axis (Moser and Moser, 1998b). The enthorinal cortex can be divided into three parallel band-like zones: the caudolateral, the intermediate, and the rostromedial zone. Each of them acts as a relatively independent functional unit, based on the fact that they receive different inputs and give rise to separate efferent outputs, and that direct connections between them are almost absent (Insausti et al., 1997; Dolorfo and Amaral, 1998; Burwell, 2000). The entorhinal cortex, which innervates all the hippocampus in a topographically ordered pattern, receives also direct projections back from both the CA1 and subiculum following the same topographic organisation along the longitudinal axis (Naber et al., 2001; Kloosterman et al., 2003b; Cenquizca and Swanson, 2007).

a- <u>The dorsal hippocampus</u>

The afferences projecting specifically to the dorsal hippocampus originate from the caudolateral zone of the entorhinal cortex, and mainly carry visuo-spatial information (via the adjacent perirhinal and postrhinal cortex).

The dorsal CA1 pyramidal neurons raise massive sequential, multi-synaptic and presumably feed-forward excitatory projections to the dorsal parts of the

subiculum, as the main output from the dorsal hippocampus (Swanson and Cowan, 1977; Witter and Groenewegen, 1990; Amaral et al., 1991). These efferent projections contact different brain areas. The retrosplenial and anterior cingulate cortices receive the most prominent projections from the dorsal CA1 and subiculum. These two cortical areas are involved in cognitive processing of visuo-spatial information, memory formation and environmental exploration (Han et al., 2003; Frankland et al., 2004; Jones and Wilson, 2005; Lavenex et al., 2006).

Other projections from the dorsal subiculum innervate, through the postcommissural fornix, the medial and lateral mammillary nuclei and the anterior thalamic complex (Kishi et al., 2000; Ishizuka, 2001). This neuronal network represents the most important interface to create a cognitive map for the spatial navigation system, giving the opportunity to orientate in a known environment (Taube et al., 1990; Muller et al., 1996; Jeffery, 2007; Fanselow and Dong, 2010).

Previous studies have shown that the dorsal hippocampus is connected with areas mediating cognitive processes, such as learning, memory, navigation and exploration (Fanselow and Dong, 2010). One famous report is the human case H.M. suffering of severe anterograde and retrograde amnesia after bilateral surgical removal of the medial temporal lobe (Scoville and Milner, 1957). This patient showed a massive impairment of memory functions, including recognition of previously presented words or figures, and the loss of memory for the position of objects (Milner et al., 1968). Patients with locally restricted damage of the hippocampus showed similar impairments, indicating that the hippocampus is involved in many mnemonic operations of the medial temporal lobe (Rempel-Clower et al., 1996).

Selective lesions at different levels of the longitudinal axis of the hippocampus in rats demonstrate that the animal behaviour is differentially affected depending on the dorsal or ventral location of these lesions (Moser et al., 1993). This is confirmed by the observation that rats with lesions within the dorsal hippocampus showed the same behavioural impairment as animals with complete hippocampal disruption, leading to severe impairment in spatial learning (Moser et al., 1993). Numerous other studies revealed similar effects of dorsal cytotoxic lesions on spatial memory (Bannerman et al., 1999; Bannerman et al., 2002; Bannerman et al., 2003). These results were not observed with lesions of the ventral hippocampus (Hock and Bunsey, 1998; Bannerman et al., 1999; Bannerman et al., 2002; Pothuizen et al., 2004).

Pharmacological studies also confirm these findings. Local microinfusions of drugs, disrupting normal neuronal activity in the dorsal hippocampus revealed similar results (Moser and Moser, 1998a; Tuvnes et al., 2003). Electrophysiological single unit recordings (Jung et al., 1994), *c-fos* activation studies (Vann et al., 2000) and structural magnetic resonance imaging (RMI) studies (Maguire et al., 2000) also support a preferential role for the dorsal hippocampus in spatial learning and memory. All these experimental findings are consistent with the anatomical connectivity of the dorsal hippocampus. The major input from primary sensory cortical areas to the dorsal hippocampus, enables this region to serve as a unique regulatory relay station regarding spatial learning and memory.

b- The ventral hippocampus

The ventral hippocampus primarily receives olfactory, visceral and gustatory inputs from the intermediate zone of the entorhinal cortex (Ishizuka et al., 1990; Insausti et al., 1997; Dolorfo and Amaral, 1998; Cenquizca and Swanson, 2007).

The ventral hippocampus is strongly connected with the olfactory bulb and several other primary olfactory cortical areas, including the anterior olfactory nucleus and the endopiriform nucleus (Cenquizca and Swanson, 2007; Roberts et al., 2007). The ventral CA1 pyramidal neurons and ventral subiculum exhibit strong bidirectional connectivity with the major amygdalar nuclei, namely the lateral, basolateral and central nuclei (Kishi et al., 2000; Pitkanen et al., 2000; Petrovich et al., 2001; Cenquizca and Swanson, 2007). Thus, the amygdala does not only receive inputs from the ventral CA1 area and the subiculum, but also projects to this areas.

The ventral CA1 area and the subiculum also share bidirectional connections with the infralimbic, prelimbic, and agranular insular cortices (Chiba, 2000; Jones and Wilson, 2005; Hoover and Vertes, 2007; Roberts et al., 2007). These prefrontal cortical structures establish a network of parallel and segregated descending fibers through the lateral septum and the BNST. This unique network innervates the periventricular and medial zones of the hypothalamus and, therefore, participates in the regulation of three basic classes of motivated behaviours with strong emotional aspects: ingestion, reproduction and defence (Kishi et al., 2000; Dong et al., 2001; Petrovich et al., 2001; Herman et al., 2005; Dong and Swanson, 2006).

The network composed of the ventral CA1 and subiculum as well as the adjacent posterior amygdalar nucleus specifically controls neuroendocrine activating,

via the strong projections to the ventral part of the lateral septum (LSv) and anteromedial nucleus of the BNST (Canteras et al., 1992; Risold et al., 1997; Dong et al., 2001). It is also important to mention that the ventral hippocampus and the medial band of the lateral and medial entorhinal cortices project directly to the caudomedial nucleus accumbens, which is strongly involved in reward processing and motivation as well as feeding behaviour (Kelley et al., 2005a; Kelley et al., 2005b). To summarise, the afferent and efferent connections of the ventral hippocampus suggest that this structure is ideally located to modulate the impact of emotional experience and to regulate affective states, such as anxiety and stress (Fanselow and Dong, 2010).

This idea is supported by previous studies. Lesions of the ventral hippocampus induce effects similar to those induced by benzodiazepine treatment in unconditioned anxiety tasks in rats. The animals show: 1) reduced hyponeophagia, i.e. they eat more readily in potentially anxiogenic and unfamiliar environments (Bannerman et al., 2002; Bannerman et al., 2003), 2) increased social interaction (Bannerman et al., 2002), 3) faster reaction to pass from the black to the white compartment during a two compartment box test (Bannerman et al., 2003), 4) increased proportion of time in a more anxiogenic division of the successive alleys apparatus, a modified version of the elevated plus maze (Bannerman et al., 2002; Kjelstrup et al., 2002). In each of these tests, lesions of the ventral hippocampus induce a strong anxiolytic-like effect. On the contrary, these effects were not observed in rats with dorsal lesions (Bannerman et al., 2002; Kjelstrup et al., 2002; McHugh et al., 2004). One useful parameter to measure anxious behaviour is defecation, which is increased after exposure to an anxiogenic environment. Animals with ventral hippocampal lesions show reduced defecation both in the bright part of the open field test and also during contextual fear conditioning, although there were no differences between sham and lesioned animals in defecation levels in the home cage (Bannerman et al., 2002). Kjelstrup and colleagues reported that complete and ventral hippocampal lesions reduce the defecation score, compared to sham and dorsal hippocampal lesioned animals, following confinement in a bright chamber (Kjelstrup et al., 2002). With respect to the regulatory action of the hippocampus on the stress response, it is important to mention that ventrally lesioned animals show a

smaller increase in plasma corticosterone concentration after stress exposure compared to control animals (Kjelstrup et al., 2002).

In summary, anatomical and lesion studies clearly indicate regional differentiation within the hippocampus. The dorsal part can be seen as the “cold” hippocampus, which stands as the gate of declarative memories, independently of their emotional content. On the other hand, the ventral hippocampus is intimately linked to emotions, regulates stress and anxiety responses and, therefore, can be seen as the “hot” hippocampus. Dysregulation of the “hot” hippocampus is thus often associated with affective disorders, such as diseases and anxiety disorders (Fanselow and Dong, 2010).

1.2.2 The amygdala

The amygdala is a heterogeneous collection of ~13 nuclei located at the medial perimeter of the temporal lobe. A variety of different functions has been attributed to the amygdaloid complex over the last 10-20 years. New imaging data obtained in humans elucidate a role of the amygdala in determining the emotional significance of sensory stimuli, memory consolidation, attention, fear, anxiety and the perception of body movements (Davis, 1992; Adolphs et al., 1994; Pitkanen et al., 1997; Whalen et al., 2001; Adolphs et al., 2002). These functions are important for the successful coping with the daily social environment (Adolphs et al., 1998; Morris et al., 1998). The amygdaloid complex is structurally diverse; its nuclei and cortical areas differ in terms of cytoarchitecture, histochemistry, and connectivity. Additionally the amygdaloid complex is further characterised by extensive internuclear and intranuclear connections.

1.2.2.1 Anatomy

According to the nomenclature established by Price et al (1987), the amygdaloid complex can be separated into various nuclei and cortical areas (JL

Price et al., 1987). Since then, this nomenclature, with some modifications, has been used to investigate the anatomical constitution of the amygdaloid complex (Pitkanen et al., 1997; Jolkkonen and Pitkanen, 1998). Amygdalar nuclei can be divided into three groups (**Figure 1-4**):

1) The deep or basolateral group:	-lateral nucleus
	-basolateral nucleus
	-accessory basal nucleus
2) The superficial or cortical-like group:	-cortical nuclei
	-nucleus of the lateral olfactory tract
3) The centromedial group:	-medial nuclei
	-central nuclei

The intercalated cell masses and the amygdalo-hippocampal area are a separate set of nuclei that do not fall in any of these groups and are listed separately.

As described for the hippocampus, the connectivity between the different nuclei and the afferent and efferent connections between the amygdala and other brain areas are the key to understand how the amygdala responds to biologically significant events and orchestrates appropriate responses. Based on intra-amygdaloid connectivity, there are three separate levels of information processing (Pitkanen et al., 1997):

Internuclear connections: between two nuclei

Intradivisional connections: within one subdivision of a nucleus

Interdivisional connections: between separate subdivisions of a nucleus

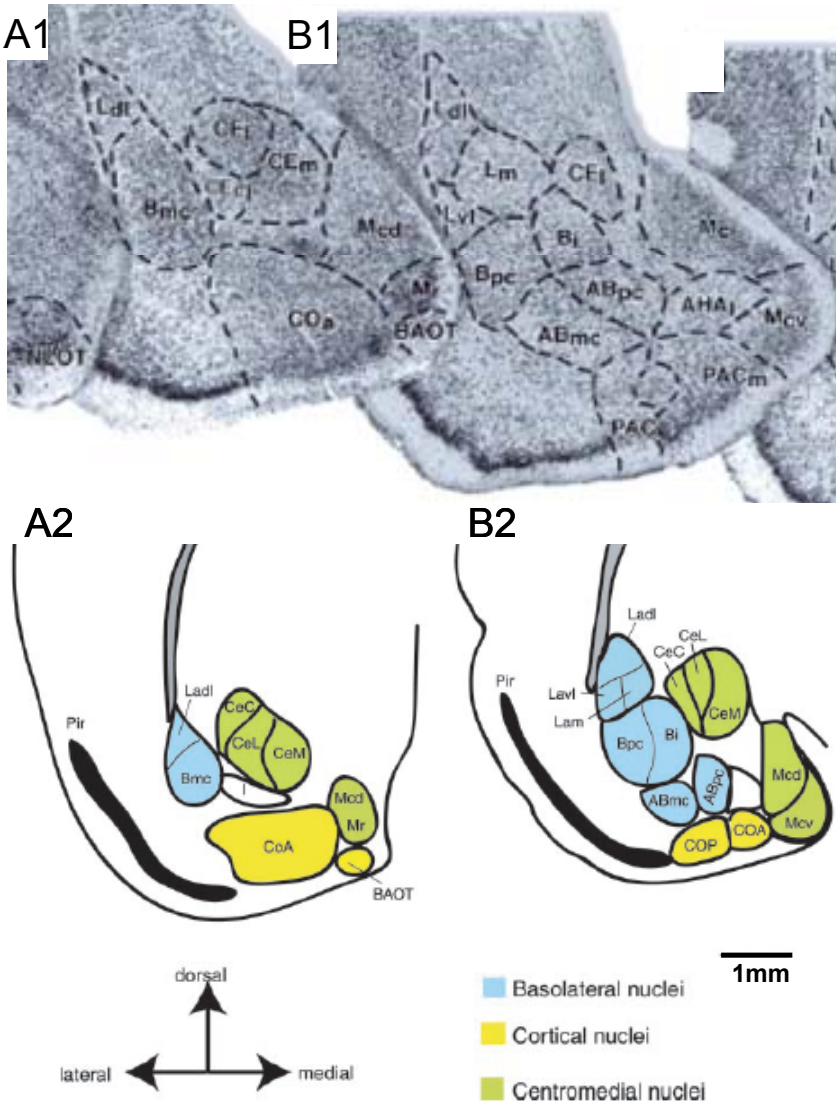


Figure 1-4: **Schematic illustration of the amygdaloid complex of the rat**

The amygdaloid complex in the rat is subdivided into various nuclei and cortical areas. Panel A is more rostral than panel B. Most of the nuclei have subdivisions and compose the anatomical and functional units of the amygdala. The different nuclei are divided into groups as described in the text.

Blue represent the nuclei of the basolateral nuclei, yellow are the cortical group and green for the centromedial group.

ABmc: Accessory basal magnocellular subdivision; **ABpc**: accessory basal parvicellular subdivision; **AHA_l**: amygdalo-hippocampal area, lateral division; **BAOT**: bed nucleus of the accessory olfactory tract; **B_i**: basal nucleus intermediate division; **B_{mc}**: basal nucleus magnocellular division; **B_{pc}**: basal nucleus parvicellular division; **Ce_c**: central nucleus capsular division; **Ce_i**: central nucleus intermediate division; **Ce_l**: central nucleus lateral division; **Ce_m**: central nucleus medial division; **Co_a**: cortical nucleus anterior division; **Co_p**: cortical nucleus posterior division; **L_{dl}**: lateral nucleus dorsolateral division; **L_m**: lateral nucleus medial division; **L_{vl}**: lateral nucleus ventrolateral division; **M_{cd}**: medial amygdala dorsal subdivision; **M_{cv}**: medial amygdala ventral subdivision; **M_r**: medial amygdala rostral subdivision; **PAC**: periamygdaloid cortex; **PAC_m**: periamygdaloid cortex medial division. Adapted from Pitkanen et al. (1997) ; Sah et al. (2003).

1.2.2.2 Divisions and locations

1.2.2.2.1 The basolateral group

The basolateral or deep group of nuclei is composed of the lateral nucleus (LA), the basolateral nucleus (BLA), sometimes also called the basal nucleus, and the accessory basal nucleus, also known as the basomedial nucleus.

The LA is located in the dorsal part of the amygdala, medial to the external capsula and lateral to both the central nucleus (rostrally) and the lateral ventricle (caudally) (Aggleton, 2000). Ventrally, the LA borders the BLA and is separated into three subdivisions: the dorsolateral, the ventrolateral and the medial subdivision (Sah et al., 2003).

The BLA is located rostral to the LA, medial to the external capsula and lateral to the central nucleus. It is composed of three subdivisions: magnocellular, intermediate and parvocellular subdivisions. When the intermediate and parvocellular divisions appear (more caudally), the BLA is located ventral to the LA and dorsal to the accessory basal nucleus. Caudally, the BLA lies lateral to the lateral ventricle.

The accessory basal nucleus borders the BLA dorsally and the periamygdaloid cortex ventrally. At caudal levels, it is located dorsal to the lateral division of the amygdalo-hippocampal area.

1.2.2.2.2 The superficial or cortical-like group

The superficial group consists of nuclei with cortical characteristics since they are located at the surface of the brain and show a layered structure (Sah et al., 2003; Andersen et al., 2007). The nucleus of the lateral olfactory tract, the bed nucleus of the accessory olfactory tract (BAOT), the anterior and posterior cortical nucleus (CoA and CoP) and the periamygdaloid cortex (PAC) form this group. The BAOT lies at the most rostral part of the amygdala and is bordered laterally by the CoA.

The CoA is located lateral to the nucleus of the lateral olfactory tract, rostrally and caudally to the medial nucleus. The CoP is located in the most caudal part of the amygdala where it borders the amygdalo-hippocampal area dorsally and the periamygdaloid cortex laterally (Aggleton, 2000; Sah et al., 2003).

The periamygdaloid cortex is subdivided in: the medial division and the sulcal division, and is located ventral to the BLA.

1.2.2.2.3 The centromedial group

The centromedial group is located in the dorsomedial portion of the amygdaloid complex and consists of the central amygdala (CeA), the medial amygdala, and the amygdaloid part of the BNST (Sah et al., 2003; Andersen et al., 2007).

The CeA is divided in four parts: the capsular, lateral, medial and intermediate subdivision. The CeA is located medial to the LA and the BLA nuclei and lateral to the stria terminalis. Caudally, the CeA ends at the lateral ventricle appears (Aggleton, 2000).

The medial nucleus starts at the edge of the nucleus of the lateral olfactory tract and extends caudally to the beginning of the lateral ventricle. The medial nucleus is also divided in three parts: rostral, central and caudal.

The amygdaloid part of the BNST is based on the innervations of the BNST and the caudodorsal regions of the substantia inominata (ventral pallidum), from the the centromedial amygdala. These two regions have similar efferent connections to the outgoing projections of the amygdala, and are therefore, characterised as part of the amygdaloid complex (Sah et al., 2003).

1.2.2.2.4 Connectivity

Studies, which used anterograde or retrograde tracer injections into various amygdalar, cortical or subcortical regions, provide data about afferent and efferent pathways to and from the amygdaloid complex (Aggleton, 2000). Each amygdaloid nucleus receives inputs from a variety of distinct brain regions. The efferent projections from the amygdala also reach numerous brain regions, including cortical and subcortical areas (McDonald, 1998; Aggleton, 2000).

a- Afferent projections to the amygdala

According to the origin of the afferences, inputs to the amygdala can be separated into two main categories (Aggleton, 2000):

- those coming from the hypothalamus or the brainstem
- those arising from cortical and thalamic structures

Hypothalamic and brainstem inputs arise from regions associated with autonomic responses, whereas cortical and thalamic inputs convey information from sensory areas and structures associated with memory formation.

The cerebral cortex is the major source of sensory information to the amygdala (McDonald, 1998). Most of the projections are ipsilateral and enter the amygdala via the external capsula (Mascagni et al., 1993). The majority of these projections arise from association areas and forward processed information by a series of cortico-cortical loops. The inputs convey modality-specific or polymodal sensory information. The amygdala receives inputs about all modalities: somatosensory, auditory, visual, olfactory, gustatory and visceral.

Cwith respect to somatosensory inputs, most afferences reach the amygdala via the dysgranular parietal insular cortex in the parietal lobe (Shi and Cassell, 1998). These projections target the LA, the BLA, and the CeA (McDonald and Jackson, 1987; Shi and Cassell, 1998, 1999). Somatosensory information also reaches the amygdala via projections from the pontine parabrachial nucleus and thalamic nuclei, the medial portion of the medial geniculate and the posterior internuclear nucleus. All these nuclei have been suggested to be involved in nociception (Ledoux et al., 1987; Bernard et al., 1989; Bordi and LeDoux, 1994; Sah et al., 2003).

Auditory and visual information reach the amygdala from association areas, rather than from the primary cortex (Mascagni et al., 1993; Shi and Cassell, 1997; Sah et al., 2003). Tracing studies have shown that these projections arise from cortical layers II and IV of the auditory cortex (Te3) and reach the LA through the dorsolateral subdivision (LeDoux et al., 1991; Shi and Cassell, 1997). Subcortical acoustic inputs arise from the thalamic medial geniculate nucleus and target the same areas of the LA (LeDoux et al., 1990; LeDoux et al., 1991; Turner and Herkenham, 1991; Sah et al., 2003). Like the auditory inputs, visual cortical projections to the amygdala originate from thalamic and high-order visual areas (Shi and Davis, 2001). Cortical projections from these areas terminate in the dorsal

subdivision of the LA, the CeA, and some in the magnocellular basal nucleus (Sah et al., 2003).

Projections from the main and accessory olfactory bulbs as well as the primary olfactory cortex target the nucleus of the lateral olfactory tract, the anterior cortical nucleus, and the periamygdaloid cortex. The accessory olfactory bulb projects to the bed nucleus of the accessory olfactory tract, the medial nucleus and the posterior cortical amygdala (Scalia and Winans, 1975). The piriform cortex and the anterior olfactory nucleus send projections to the LA, BLA and accessory basal nuclei (Luskin and Price, 1983).

Gustatory and visceral primary areas in the anterior and posterior insular cortices provide strong projections to the dorsal subdivision of the LA, the posterior BLA, and the CeA (Shi and Cassell, 1998). Gustatory and visceral information arise also from subcortical structures, and both cortical and subcortical inputs converge in the amygdaloid complex (McDonald, 1998).

The amygdala also receives major polymodal sensory inputs from the prefrontal and perirhinal cortices and the hippocampus, structures that are strongly involved in the regulation of behaviour and reward circuitries (Rolls, 2000). Projections from the prefrontal cortex reach the BLA, but also the LA, as well as the accessory basal, central, and medial nucleus (McDonald et al., 1996). Areas related to long-term declarative memory (e.g. the perirhinal, entorhinal, parahippocampal cortices, and the hippocampus) have strong reciprocal connections with the amygdala. The perirhinal cortex sends the heaviest projections to the medial division of the LA and also innervates the BLA and the cortical nucleus (Shi and Cassell, 1999). Compared to the perirhinal cortex, the entorhinal cortex seems to project to most of the amygdalar nuclei (McGaugh et al., 1996). The afferent inputs from the hippocampus originate from the subicular region and, although the BLA is the main target structure, other nuclei are also likely to be innervated (Canteras and Swanson, 1992).

In summary, the amygdala receives information from all sensory systems. In combination with afferent inputs from the medial temporal lobe, the amygdala appears to be in a unique position to form associations between current sensory inputs and past experiences.

b- Intra-amygdaloid connectivity and efferences

Tracing studies enable tracking of the intranuclear and internuclear connectivity within the amygdala (Krettek and Price, 1978; Aggleton, 2000). These studies show that sensory information enters the amygdala through the LA where it is processed before a lateral-to-medial progression to the medial and central nuclei, which act as the main output areas of the amygdala (Pitkanen et al., 1997).

Within the LA, strong rostrocaudal as well as interdivisional connections have been described (Pitkanen et al., 1995). The dorsolateral subdivision projects to the medial subdivision and also to the lateral subdivision of the LA. Unimodal sensory inputs enter the LA laterally, while polymodal inputs from the declarative memory system invade the medial subdivision of the LA (Aggleton, 2000). The lateral to medial intranuclear connections within the LA suggests that the medial subdivision acts as a site of integration between sensory information and past experience. Neurons of the LA send heavy projections to the BLA, the accessory basal nucleus and the capsular part of the CeA (Smith and Pare, 1994; Pitkanen et al., 1995). Except for the CeA, these regions also send projections back to the LA, mostly into its medial and ventrolateral subdivision (Savander et al., 1996; Savander et al., 1997).

The BLA and the accessory basal nucleus receive strong cortical inputs and have extensive internuclear and intranuclear connections. The parvocellular subdivision of the BLA projects to the magnocellular and intermediate subdivisions (Savander et al., 1995). The largest projection from the BLA is to the medial subdivision of the CeA (Savander et al., 1995; Savander et al., 1996). Projections from the BLA to the CeA play a key role in controlling the outflow of processed information from the amygdaloid complex (Savander et al., 1995; Savander et al., 1996). The accessory basal nucleus has extensive rostrocaudal connections and sends projections to the LA, the CeA, and the medial nuclei (Savander et al., 1995).

The CeA forms the major source of efferences and receives projections from all other amygdalar nuclei, with only a few reciprocal connections (Pitkanen et al., 1997; Jolkkonen and Pitkanen, 1998). It is important to mention that the lateral subdivision of the CeA is also innervated by cortical and subcortical areas (Aggleton, 2000), suggesting that it acts as a site of integration of input signals.

The internuclear connections to the CeA are mostly restricted to the medial and capsular subdivisions. The CeA also has extensive intradivisional and

interdivisional connections (Jolkkonen and Pitkanen, 1998). The capsular and lateral parts of the CeA send strong projections to the medial, but only few connections to the intermediate subdivision. The medial subdivision projects to the capsular subdivision and generates the main output of the amygdala to the hypothalamus and the brainstem. The projections are responsible for the activation of autonomic and endocrine stress responses (Savander et al., 1996).

1.2.3 Amygdala, stress and anxiety

In the past two decades, the amygdala has increasingly been implicated in the pathophysiology of anxiety disorders, and becomes more and more accepted as a key structure concerning the regulation of fear and anxiety. In contrast to the prefrontal cortex and the hippocampus, the amygdala drives the HPA axis (Herman et al., 2003). Stimulation of the amygdala promotes corticosteroid biosynthesis and secretion from the adrenal cortex (Redgate and Fahringer, 1973; Saito et al., 1989). Studies using localised stimulations and lesions showed that the central, medial and basolateral nuclei control the release of ACTH from the anterior pituitary (Herman et al., 2003).

The BLA has been implicated in emotional arousal and stress-induced CRH-mediated modulation of stress and anxiety states (Roozendaal et al., 2002; Roozendaal et al., 2004). CRHR1 is strongly expressed in BLA magnocellular neurons (Chen et al., 2000), which project to the hippocampus, thereby contributing to the consolidation of emotional memories (Pare, 2003). CRHR1 activation increases the excitability of these projection neurons. Consequently, release of CRH in the BLA has been hypothesised to contribute to the regulation of stress-induced anxiety states (Rainnie et al., 1992; Rainnie et al., 2004). Consistently, repeated administration of low doses of CRH (50 fmol) can lead to increased activity of BLA neurons upon future stress exposure and to the development of sustained anxiety (Sajdyk et al., 1999; Sandi et al., 2008). Stress exposure can also lead to an enhancement in synaptic strength within the BLA, further contributing to the development of chronic enhanced anxiety levels (Rainnie et al., 2004; Shekhar et al., 2005). Repeated local infusions of urocortin (an endogenous CRHR2 agonist) into the BLA increase anxiety levels in rodents, with electrophysiolocical recordings from

BLA neurons showing a reduction in spontaneous as well as evoked GABAergic transmission and, thus, a hyperexcitability of BLA circuits (Rainnie et al., 2004). These findings are consistent with recent evidence for an inverse relationship between GABAergic inhibition in the BLA and anxious behaviour. Interestingly, these effects can be reversed by enriched environmental (E.E.) stimulation (Sztainberg et al., 2010). Rodents exposed to a combination of social, cognitive, sensory, and motor stimulation display reduced levels of emotionality-related measures such as defecation, freezing, and anxiety levels (Fox et al., 2006). Sztainberg *et al.* (2010) showed that one possible molecular mechanism underlying this phenomenon is reduction of CRHR1 expression in the BLA (Sztainberg et al., 2010).

Modulation of electrical activity in the BLA by CRH can lead to long-lasting functional and structural changes. Indeed, acute and chronic stress leads to remodelling of synapses and dendritic branching in the BLA and medial amygdala that correlates with increased anxiety levels (Vyas et al., 2002; Vyas and Chattarji, 2004; Vyas et al., 2004; Vyas et al., 2006). A single stress episode of 2 h in rats is sufficient to increase spine density on principal neurons in the BLA, an effect that also correlates with increased anxiety (Mitra et al., 2005).

Compared to acute stress, chronic stress induces a more widespread increase in spine density, affecting both primary and secondary dendrites of BLA principal neurons (Mitra et al., 2005). This effect is accompanied by a dendritic growth in pyramidal and stellate neurons of the BLA (Vyas et al., 2002; Vyas et al., 2004; Vyas et al., 2006) and enhanced anxiety (Mitra and Sapolsky, 2008). This dendritic hypertrophy probably involves increased corticosterone levels. Mitra and colleagues (2008) showed that chronic, high-dose administration of corticosterone for ten days induces dendritic hypertrophy in the BLA and increases anxiety levels (Mitra and Sapolsky, 2008). Thus, effects of chronic stress on anxiety seem to be mediated at least in part by a remodelling of dendritic arborisation in the BLA (Mitra and Sapolsky, 2008).

Excessive amygdalar activity, with or without presentation of emotional stimuli, has been described in patients suffering from social anxiety disorder (Birbaumer et al., 1998; Stein et al., 2002; Phan et al., 2006), posttraumatic stress disorder (Rauch et al., 2000; Shin et al., 2005), panic disorders, and generalised anxiety disorders (Thomas et al., 2001).

Taken together, clinical and preclinical studies highlight the amygdala as a major relay station for the regulation of anxiety levels, depending on environmental factors. Dysregulation of amygdalar activity leads to the development of anxiety disorders. In particular, the BLA appears to be an important nucleus because of its ability to integrate diverse stressful stimuli. Release of CRH and corticosteroids can alter synaptic strength and dendritic morphology within the BLA, thus leading to an enhanced excitability of pyramidal neurons.

1.3 Animal models of human psychopathology

In the last two decades, new brain imaging techniques have led to major advances in the understanding of psychiatric conditions (Stein et al., 2002; Anand and Shekhar, 2003; Stein et al., 2007). However, research on humans has, beside ethical reasons, been limited by numerous factors. In order to understand the mechanisms underlying such complex disorders, the use of animal models is indispensable.

A variety of animal models have played an important role in the development of many widely used psychopharmacological treatments and strongly contributed to a better understanding of psychopathologies (McKinney, 2001). Nevertheless, many animal models also have certain disadvantages. There is no “perfect” animal model which recapitulates all aspects of a complex human disease such as mood disorders. Ideally, animal models should mimic the specific features experienced by patients with respect to etiology, symptomatology, treatment, and biological background (McKinney, 2001). An animal model must fulfil three main criteria in order to be considered a valid model. Construct validity (the causal conditions) and face validity (diagnosed symptoms) should be similar to those observed in patients, and predictive validity (pharmacological treatment of the animals should produce the same quantifiable effects as those seen in patients).

There are numerous ways to induce psychopathological symptoms in rodents. Exposure of animals to certain environmental conditions, e.g. to a stressful or enriched environment, can modulate the stress response in a way that it mimics human stress-related pathologies (Schmidt et al., 2003; Weaver et al., 2004; Friske and Gammie, 2005; Schmidt et al., 2007; Touma et al., 2008). As an alternative to

transgenic animal models, selective breeding has found to be a strong tool to uncover the genetic background of mental disorders (Phillips et al., 2002; Swallow and Garland, 2005; Touma et al., 2008). In the selective breeding approach, individuals lying at the extremes of the response curve are selectively bred for their contrasting trait phenotypes for multiple generations (Liebsch et al., 1998b; Liebsch et al., 1998a; Kromer et al., 2005; Touma et al., 2008). This selective bidirectional breeding improves the frequency of genetic modifications related to a bidirectional shift in the animal's trait phenotype from the mean of the strain (Kromer et al., 2005; Landgraf et al., 2007). Heritability features of the trait can be analysed and later generations can be evaluated on the basis of neurobiological correlates of the trait. Successfully developed examples of this selective breeding approach comprise mice and rats selected for extremes in anxiety-related behaviour, namely high anxiety-related behaviour (HAB) and low anxiety-related behaviour (LAB) (Kromer et al., 2005; Landgraf et al., 2007)

1.3.1 An animal model of trait anxiety: The HAB/LAB mouse model

One approach to better understand the neurobiological underpinning of anxiety-related behaviour is the development of a psychogenetically selected animal model, selected for trait anxiety. This model originates from one outbred rat or mouse strain selected over generations such that it is reliable, consistent and robust (Landgraf, 2003).

For the high (HAB) and low (LAB) anxiety-related behaviour animal model, Wistar rats were bred and selected based on their anxiety phenotype on the elevated plus maze (EPM) (Landgraf and Wigger, 2002; Landgraf, 2003; Salomé et al., 2004; Landgraf et al., 2007). The EPM represent one of the most widely used behavioural tests to characterise anxiety levels in rodents and the anxiolytic or anxiogenic effects of drugs (Rodgers and Johnson, 1995; Hogg, 1996). This "approach-avoidance" behavioural paradigm relies on the observations that rats and mice show higher levels of exploration in enclosed alleys and avoid the open, non-protected arms of a maze (Pellow et al., 1985; Lister, 1987).

After inbreeding for several generations, HAB and LAB lines exhibit stable bidirectional anxiety phenotypes, with HAB rats showing symptoms of pathological

anxiety (Liebsch et al., 1998b; Liebsch et al., 1998a; Landgraf and Wigger, 2002). These rats fulfill all three criteria of a valid animal model and display high genetic homogeneity. HAB animals allow the investigation of multiple genetic and environmental factors which contribute to trait anxiety. In order to reduce the possible influence of genetic drift and the concomitant risk of false associations between the gene of interest and a given phenotype, different sublines are evaluated at the same time (Kromer et al., 2005; Bunck et al., 2009).

Although HAB/LAB rats represent a powerful tool for the investigation of behavioural and neuroendocrine features of trait anxiety, rats are limited in terms of investigations using molecular genetic approaches (Kromer et al., 2005). Genetic approaches are better realisable in the mouse, whose genome has been fully sequenced making the mouse a powerful model to uncover candidate genes and gene products underlying trait anxiety (Tarantino and Bucan, 2000). To exploit these advantages, a mouse model was developed based on CD1 strain mice, employing the same breeding paradigm as that described for HAB/LAB rats. This bidirectional breeding led to the creation of two inbred lines that, independent of gender, show extreme differences in trait anxiety phenotypes (Kromer et al., 2005). The HAB/LAB mouse model also exhibits differences in other metrics, including depression-like and explorative behaviours. HAB mice are not only more anxious on the EPM, compared to LAB mice, but also show less signs of risk assessment and emit more ultrasonic vocal calls (USV), measures associated with high anxiety levels (Kromer et al., 2005). In the forced swim and the tail-suspension tests, HAB mice display higher scores of immobility than LAB mice, indicating increased depression-like behaviour (Kromer et al., 2005). The latter findings are consistent with the clinical observation of a comorbid appearance of anxiety disorders and depression. Furthermore, cognitive differences are observed between HAB/LAB mice. In the social discrimination test, HAB animals need a longer time to discriminate between a novel and a familiar ovariectomised female as LAB animals. In the Y-maze and the Morris water maze tests, similar cognitive differences can be found between mice of both groups (Bunck, 2008). Thus, the HAB/LAB mouse model serves as a good tool to investigate neurophysiological, genetic, and environmental factors.

2 Aim of the study

Although it is obvious that the physiological architecture of neuronal networks and its modifications are essential for the brain to process incoming information and to control highly organised behaviour, the mechanisms which control anxiety are still poorly understood. The aim of this thesis was to uncover changes in the characteristics of neuronal networks that may contribute to abnormal anxiety states.

This work addressed the following questions:

a. Do HAB and LAB mice differ in terms of basal neurotransmission and/or plasticity at CA3-CA1 synapses in the ventral hippocampus?

b. Is there a regional dissociation within the hippocampus (dorsal *vs*. ventral) in basal neurotransmission and/or plasticity at CA3-CA1 synapses between HAB/LAB mice?

c. Is the efficacy of evoked neuronal activity propagation through the amygdalar network different in HAB and LAB mice?

d. Are environmental factors sufficiently strong to modulate the anxiety phenotype and the efficacy of neuronal activity propagation through the amygdala network in HAB/LAB animals?

3 Material & Methods

3.1 Animals

All animal lines, except for the HAB enriched environment mice (HAB E.E.), were housed in same sex groups ranging from two to five mice. The transparent polycarbonate cages (type 2 – macrolone, 25.5x19.5x13.8 cm) furnished with wood chip bedding and wood shaving nesting material (Product codes: LTE E-001 and NBS E-001, ABEDD-LAB and VET Service GmbH, Vienna, Austria) were kept at the animal facility of the Max Planck Institute of Psychiatry in Munich and maintained under standard laboratory conditions (light-dark cycle: 12 h, light on at 7:00 AM; temperature: 22 ± 1°C; relative humidity 55 ± 10%). Food and tap water were available *ad libitum*.

The HAB E.E. animals, which were exposed to an environmental enrichment, were housed under conditions allowing increased stimuli interactions. The E.E. cages, constructed of a 85 x 75 x 20 cm transparent polycarbonate, contained the following enrichment items: polyvinyl chloride box; increased nesting material; the Mouse House a red transparent Perspex nest box; the Refuge, a plastic tubes tunnel system; and wooden toys. Nesting material was replaced weekly, whereas some of the enrichment items, such as the running wheels, were never removed.

The data presented here were obtained from 8- to 12- week old male mice. Behavioural paradigms and electrophysiological experiments were separated in order to allow at least one week of intermittent recovery.

3.2 Electrophysiology

3.2.1 Preparation of brain slices

The following slice preparation procedure was carried out between 6:00 and 9:00 AM. The animals were anesthetised using isoflurane (Abbott Deutschland, Wiesbaden, Germany) and decapitated using an animal guillotine. All of the following steps were carried out in ice-cold artificial cerebrospinal fluid (ACSF) saturated with

carbogen (95% O_2/5% CO_2) The ACSF (pH 7.4) contained (in mM): NaCl, 125; KCl, 2.5; $NaHCO_3$, 25; NaH_2PO_4, 1.25; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 25. The brain was rapidly removed from the cranial cavity and the frontal cortex and the cerebellum were dissected. The brain was then glued on the slicing stage with Histoacryl®. The slicing stage was immediately placed in the cutting dish of the vibratome (HM650V, Thermo Scientific). Depending on the kind of experiment, various types of slices were prepared:

1) 400 µm coronal slices containing the amygdala (Rammes et al., 2000).
2) 350 µm parasagittal slices containing the dorsal hippocampus (Eder et al., 2003).
3) 350 µm horizontal slices containing the ventral hippocampus.

To clearly distinguish between the dorsal and the ventral part of the hippocampus, the brain was glued on the dorsal surface of the cortex and slices were obtained by cutting from the ventral to the dorsal axis. Brain slices, containing the structure were transferred into a storage glass vial filled with carbogenated ACSF. Slices were incubated for 30 min at 34°C and afterwards stored at room temperature (22-24°C) for at least 90 min. For patch-clamp recordings, slices were only incubated for 30 min at 34°C before the beginning of the experiment.

3.2.2 Field excitatory postsynaptic potential (fEPSP) recording

3.2.2.1 Equipment

All electrophysiological recordings were performed on a steel plate platform mounted on a vibration-cushioned table (TMC, USA, Peabody; MA). In order to minimize extraneous electrical noise, a Faraday cage (TMC, USA, Peabody; MA) was placed around this steel plate platform. In the recording chamber, brain slices were permanently superfused with carbogenated ACSF via a peristaltic pump (Ismatec, Glattburg, Germany) with a constant flow of 3.5 ml/minute. A platinum frame covered with two nylon threads (“grid”) fixes the slice on the bottom of the

recording chamber. The stimulation and recording electrodes were positionned in the slice by means of micromanipulators.

For stimulation, a custom-made bipolar tungsten electrode (insulated to the tips 50 µm diameter) was placed within the CA1 *Stratum radiatum* to stimulate the Schaffer collateral-commissural pathway (**Figure 3-1**). Recording electrodes were pulled from 1.5 mm diameter fibre-filled borosilicate glass pipettes (Harvard Apparatus, Edenbridge, Kent, UK) on a horizontal puller (DMZ Puller; Zeitz, Martinsried, Germany). The open tip resistance of these electrodes varied between 0.7 and 1.1 MΩ. The electrodes were backfilled with ACSF.

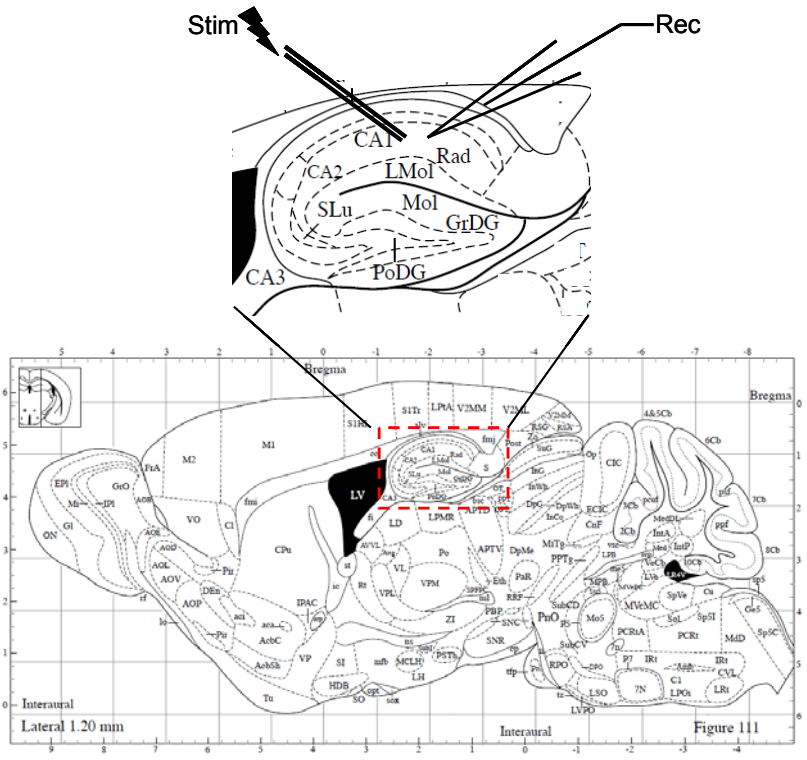


Figure 3-1: **Section of the mouse brain adapted from "The mouse brain" (G. Paxinos, 2008)**
Parasagital section of the mouse brain adapted from "The mouse brain" (G. Paxinos, 2008). For field potential recordings, stimulus and recording electrodes were placed in the *Stratum radiatum* of the dorsal CA1 hippocampus subfield. **CA1-3***: Cornu ammonis*; **Rad**: *Stratum radiatum*; **SLu**: *Stratum lucidum*; **LMol**: *Stratum lacunosum moleculare*; **Mol**: molecular layer of the *Dentate gyrus*; **GrDG**: granular layer of the *Dentate gyrus*; **PoDG**: Polymorphic layer of the *Dentate gyrus*.

3.2.2.2 Data recording

An external stimulus generator (Npi electronic; Tamm, Germany) was triggered every 15 s by the Pulse software 8.0 (HEKA Elektronik; Lambrecht/ Pfals, Germany), through a digital/analog converter (HEKA Elektronik; Lambrecht/ Pfals, Germany). Square pulse electrical stimuli (50 µs pulse width, voltage stimulation) were applied to the neuronal tissue. The recorded field potentials were low-pass filtered at 1 kHz and digitised at 5 kHz (**Figure 3-2**).

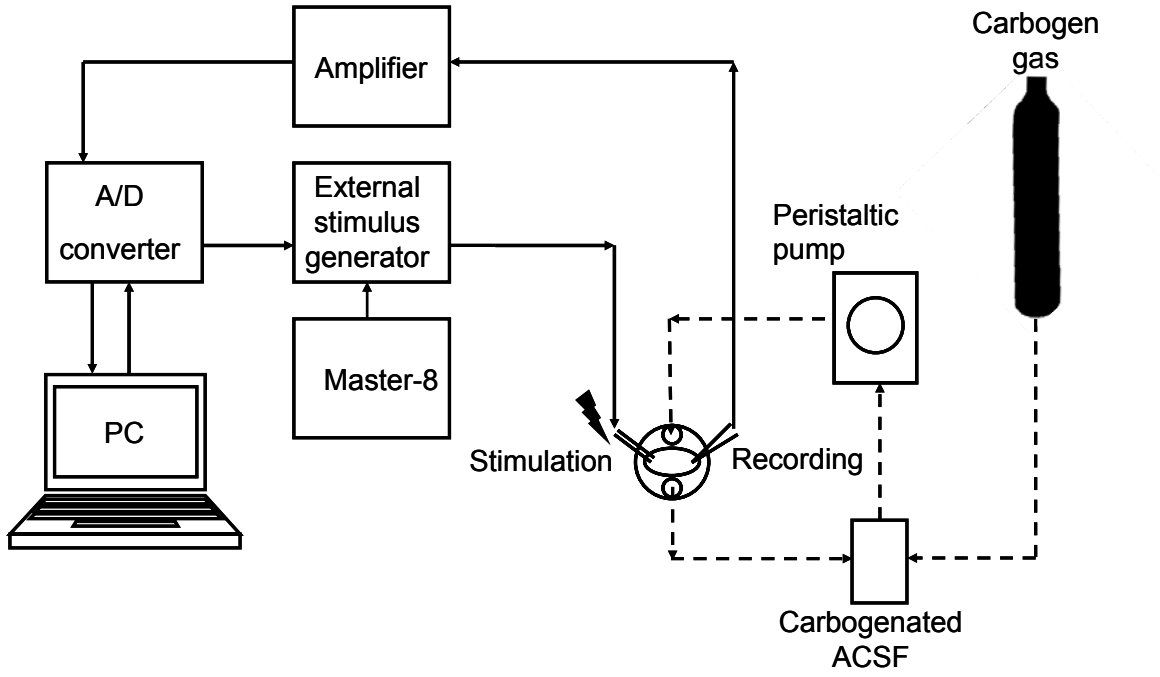


Figure 3-2: Schematic illustration of the extracellular recording set-up

3.2.2.3 Experimental protocols

For each experiment in the hippocampus, the recordings were conducted in three steps:

1) Basal neuro transmission at CA3-CA1 synapses was evaluated by means of input-output relationships. The input is defined as the amplitude of the fibre volley (**Figure 3-3**), representing the strength of action potential activity in the Schaffer collateral-commissural pathway. The stimulation intensity was adjusted in a manner to produce fibre volley amplitudes of approximately 20, 40, 80, 120 and

200µV. The amplitudes of the resultant fEPSPs, are defined as the output.

2) Paired-pulse facilitation (PPF) was analysed. PPF was assessed using interstimulus intervals of 25, 50, 100, 200 and 400 ms.

3) Long-term synaptic plasticity was investigated by evoking LTP by application of high-frequency stimulation (HFS, 100 Hz/1 s). For this purpose triggering device (Master-8; Uziel St., Jerusalem, Israel), connected to the external stimulator, was manually activated for the generation of the HFS. The amplitudes of the fEPSPs were normalised to the mean amplitude of the fEPSPs acquired of the last 10 min of baseline recording.

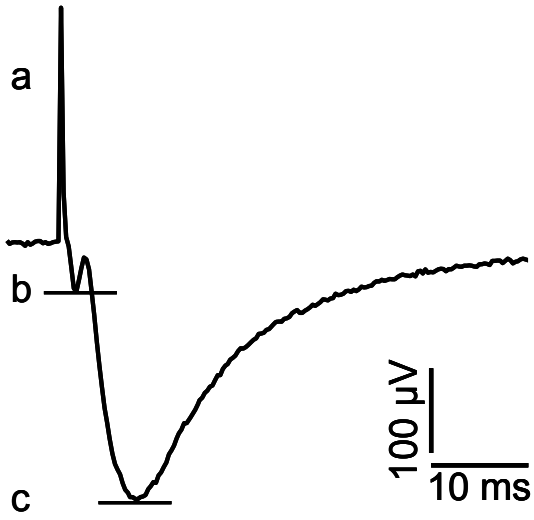


Figure 3-3: **Representative recording trace acquired during fEPSP recording in area CA1 of the hippocampus**
a) Stimulus artefact; **b)** Amplitude of the fibre volley; **c)** Amplitude of the fEPSP

3.2.2.4 Data storage and analysis

Recorded signals were stored on the hard disk of a power Macintosh 7100 computer. Amplitudes of fEPSPs were analysed using the Pulse Software and macros written in Igor Pro (version 6.12a; WaveMetrics, Oregon, USA). SigmaStat 3.5 (Statcon, Witzenhausen, Germany) was used for statistical analysis.

3.2.3 Patch clamp recordings

Slices were placed in a submerged recording chamber and fixed with a grid. Slices were permanently superfused with carbogenated ACSF via a peristaltic pump (Ismatec, Glattburg, Germany) at a flow rate of 3.5 ml/ minute.

Patch-clamp recordings were made from principal neurons of the BLA that were visually identified using an infrared videomicroscope (Zeiss Axioskop; Carl Zeiss, Munich, Germany). The enhanced visibility of neuronal structures reached in brain slices imaged with infrared light comes from the reduced dispersion of infrared light compared to visible light (Dodt and Zieglgansberger, 1990; Dodt, 1993). Neurons were selected depending on their appearance. Cells with a high contrast, a rough and angular appearance, and a visible nucleus were excluded. Only cells with smooth and soft-looking membranes, that could easily dimpled by the tip of the patch pipette, were selected for recordings.

The recording chamber, the microscope, and the recording pipette were independently moved by means of three micromanipulators (Luigs and Neumann; Ratingen, Germany).

3.2.3.1 Recording electrodes & data acquisition

The recording electrodes were made in a two-step process. First, patch pipettes with an open tip resistance of 6 to 8 MΩ were pulled and subsequently heat-polished. Both steps were carried out using a horizontal puller (DMZ Puller; Zeitz, Martinsried, Germany). The electrodes were backfilled with intracellular solution and placed in a pipette holder. An Ag/AgCl wire is required to provide electrical connection between the intracellular solution and a head-stage (SEC 10L NPI Electronic GmbH, Tamm, Germany). The head-stage serves as a first amplifying interface between the main amplifier and the recorded cell. The patch clamp amplifier contains the measuring and clamping circuitry, and in turn process the experimental commands, such as holding potential and voltage steps (Molleman, 2003). The amplifier, according to the principle of discontinuous single-electrode voltage clamp, periodically alternated between measuring the voltage and injection current. To provide commands and acquire data, electrical signals were low-pass filtered at 1

KHz and converted from analog to digital format, and vice versa. An analog/ digital digital/ analog interface (ITC 16; HEKA Elektronik Dr. Schulze GmbH, Lambrecht/Pfalz, Germany) digitalised the data with a sampling rate of 10 KHz. Data were recorded and stored with Pulse-software (version 8.5, HEKA Elektronik Dr Schulze GmbH) on a MacIntosh computer.

3.2.3.2 Whole-cell recording

One major factor to obtain a successful patch clamp recording is to reach the cell membrane without damage or contamination of the pipette by debris in the bath. To avoid such a contamination, a positive pressure was applied to the pipette through a syringe connected to a three-way-stopcock. The electrode was placed in the recording chamber by careful navigation with the micromanipulators. Once in the bath, the offset of the pipette was eliminated and secondly the resistance of the electrode was compensated.

After choosing a cell in the BLA, the pipette tip was positioned close to the plasma membrane by visual control. The contact between the pipette's tip and the cell should not traumatise the membrane of the neuron. In order to have the most optimal experimental conditions, the final approach was monitored on a high magnification camera. After removing the positive pressure, the plasma membrane and the tip of the pipette started to seal together. After reaching a resistance of 100 MΩ, the amplifier was switched to the voltage clamp mode (holding potential of -70 mV) to reach a resistance of at least 1 GΩ. This step is confirmed by the virtual absence of leak current between the tip of the electrode and the membrane fragment inside the pipette, as the result of the high resistance of the gigaseal.

To achieve the whole-cell configuration, the patch of membrane under the pipette`s tip is broken by applying a small negative pressure. The intracellular solution of the pipette is then in direct contact with the cytoplasm, and dialysis of both solutions can take place. Before starting the recording, a resting period of 5 min was observed to allow the complete dialysis between the intracellular solution and the cytoplasm of the recorded cell.

3.2.3.3 Recording of $GABA_A$ receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs)

The pipette solution for $GABA_A$ receptor-mediated mIPSCs recordings was composed of (in mM): Cesiummethanesulfonat, 100; CsCl, 60; Lidocain N-Ethyl-Chlorid, 5; Hepes, 10; EGTA, 0.2; $MgCl_2$, 1; Mg-ATP, 1; Na_3GTP, 0.3; pH 7.3.

To isolate $GABA_A$ receptor-mediated mIPSCs, different blockers were added to the extracellular bath solution: D-AP5 (50 µM) to block NMDA receptors, NBQX (5 µM) to block AMPA and kainate receptors, and TTX (1 µM) to block the voltage-gated sodium channels. Control experiments showed that the remaining postsynaptic currents are completely abolished after addition of the $GABA_A$ receptor antagonist bicuculline (10 µM), confirming that the recorded mIPSCs were mediated by $GABA_A$ receptors.

3.2.3.4 Data analysis & statistics

Only neurons with a resting membrane potential (RMP) between -60 and -75 mV were analysed. Recordings showing changes in the access resistance by more than 15% were collected. $GABA_A$ receptor mediated mIPSCs were recorded for 15 min and analysed using the Mini Analysis software (version 6.0.9; Synaptosoft Inc., Fort Lee, NJ, USA). The signal was separated from the noise by definition of a threshold: this threshold was set to five times of the mean square root of noise value.

SigmaStat (version 3.5.; Systat Software, Inc., Point Richmond, CA, USA) was used for statistical analysis.

3.3 Voltage-sensitive dye imaging (VSDI)

3.3.1 Di-4-aminonaphtylethenylpyridinium (Di-4-ANEPPS)

A stock solution (20.8 mM) of Di-4-ANEPPS (Sigma-Aldrich GmbH; Munich, Germany) was prepared in 100% DMSO and stored at -20ºC in 7.6 µl batches. The

ANEPP dyes were developed by Leslie Loew and colleagues (Fluhler et al., 1985). The maximum fluorescence excitation/emission wavelengths of di-4-ANEPPS are ~475/617 nm.

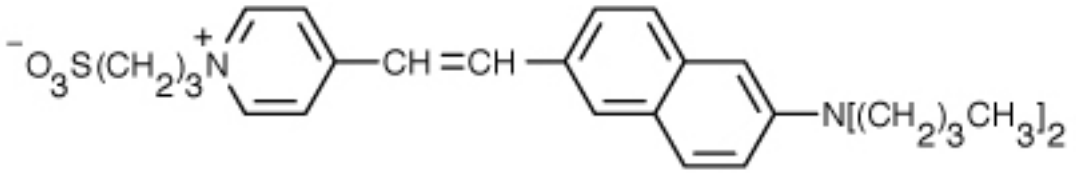


Figure 3-4: **Chemical formula of Di-4-ANEPPS**
Di-4-ANEPPS was used for the VSDI recordings. **4**: the number of carbons in each of the alkyl chains; **ANE**: aminonaphthethenyl; **PS**: propylsulfonate

3.3.2 Preparation and staining of brain slices for VSDI recordings

Coronal amygdalar slices (400 µm) were prepared as described in the paragraph 3.2.1. After 30 min of incubation at 34°C, the slices were stained with Di-4-ANEPPS. Slices were submerged in carbogenated ACSF containing 7.5 µg/ml of the dye (< 0.1% DMSO) and incubated at room temperature (22-24°C) for 15 minutes. Following the staining procedure, slices were set aside in pure carbogenated ACSF for at least 30 min (von Wolff et al., 2011).

3.3.3 VSDI equipment & data acquisition

The MiCAM02 hard- and software package (BrainVision, Tokyo, Japan) was used for the VSDI recordings. The software triggered the MiCAM02 processing unit, which in turn was connected to a halogen lamp (Moritex, MHAB-150W), the shutter and the charge-coupled device (CCD) camera (MiCAM02-HR). For excitation of the dye, light was passed through green light filter (wavelength of ~475 nm), while emitted fluorescence was collected in the range of red light (wavelength of ~617 nm). The tandem-lens fluorescence microscope was equipped with the MiCAM02-HR camera and the 2X and 1X lens at the objective and condensing side, respectively.

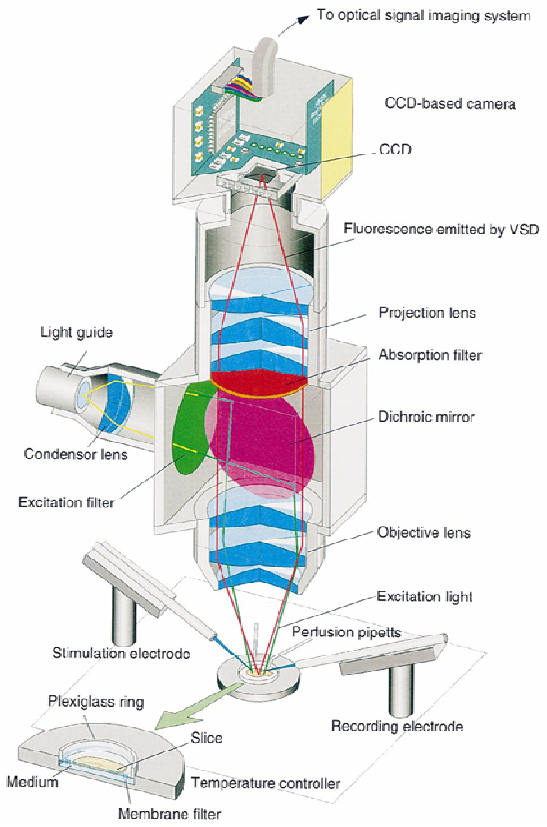


Figure 3-5: **Schematic illustration of the VSDI set-up adapted from Tominaga et al., (2000)**

Collected images were transmitted via the processing unit to the BrainVision software on the computer. Acquisition settings were as followed: 88x60 pixels frame size, 36.4x40.0 µm pixel size, and 2.2 ms sampling time. For each acquisition, 8 images were recorded at intervals of 10 sec and averaged. To minimize noise during optical recordings, slices were superfused with carbogenated ACSF using a combination of gravity system (for influx) and a peristaltic pump (for outflow). That way, vibrations at the surface of the submerged slices were avoided. A bipolar platinum/ iridium cluster electrode (25 µm pole diameter; FHC, Bowdoinham, ME, USA) was used to evoke neuronal activity. The stimulus electrode was placed in the lateral amygdala, closed to the *capsula externa* (**Figure 3-6**). Square pulse electrical stimuli (200 µs pulse width, voltage stimulation) were applied to the neuronal tissue of three different intensities (10, 17.5, 25 Volt).

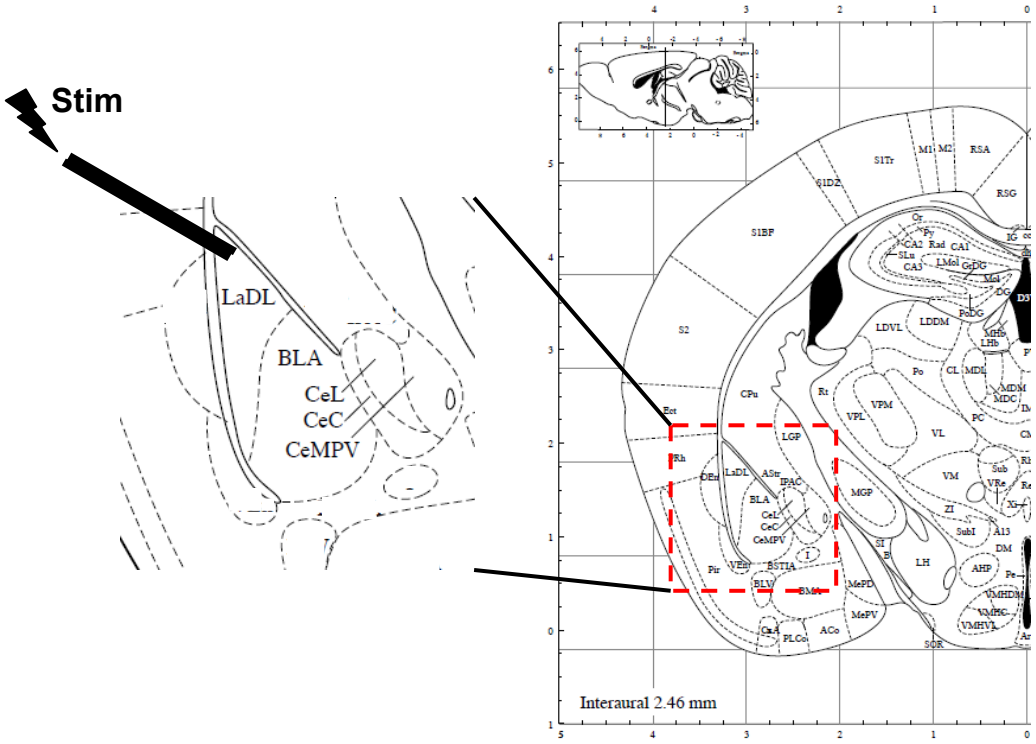


Figure 3-6: **Coronal section of the mouse brain adapted from "The mouse brain" G. Paxinos, (2008)**
For VSDI recordings, the stimulus electrode was placed in the dorso-lateral part of the lateral amygdala. **LaDL**: lateral amygdaloid nucleus dorsolateral; **BLA**: basolateral amygdaloid nucleus anterior; **CeL**: central amygdaloid nucleus lateral division; **CeC**: central amygdaloid nucleus capsular division; **CeMPV**: central amygdaloid nucleus medial posteroventral division.

3.3.4 Data processing & analysis

The fractional change in fluorescence (ΔF/F) was calculated. For all recordings, ΔF/F values were temporally and spatially smoothed by a 3x3x3 average filter. For quantification of neuronal population activity propagation in amygdalar nuclei, three circular regions of interest (ROIs) were manually set according to anatomical landmarks. The first ROI (4 pixels radius) was placed in the LA between the stimulus electrode and the BLA. A second ROI (6 pixels radius) was placed in the BLA, ventral to the dorsolateral part of the LA and close to the medial division of the *capsula externa*. A third ROI (5 pixels radius) covered the CeA. (**Figure 3-7,** left panel; representative recording are depicted in the right panel).

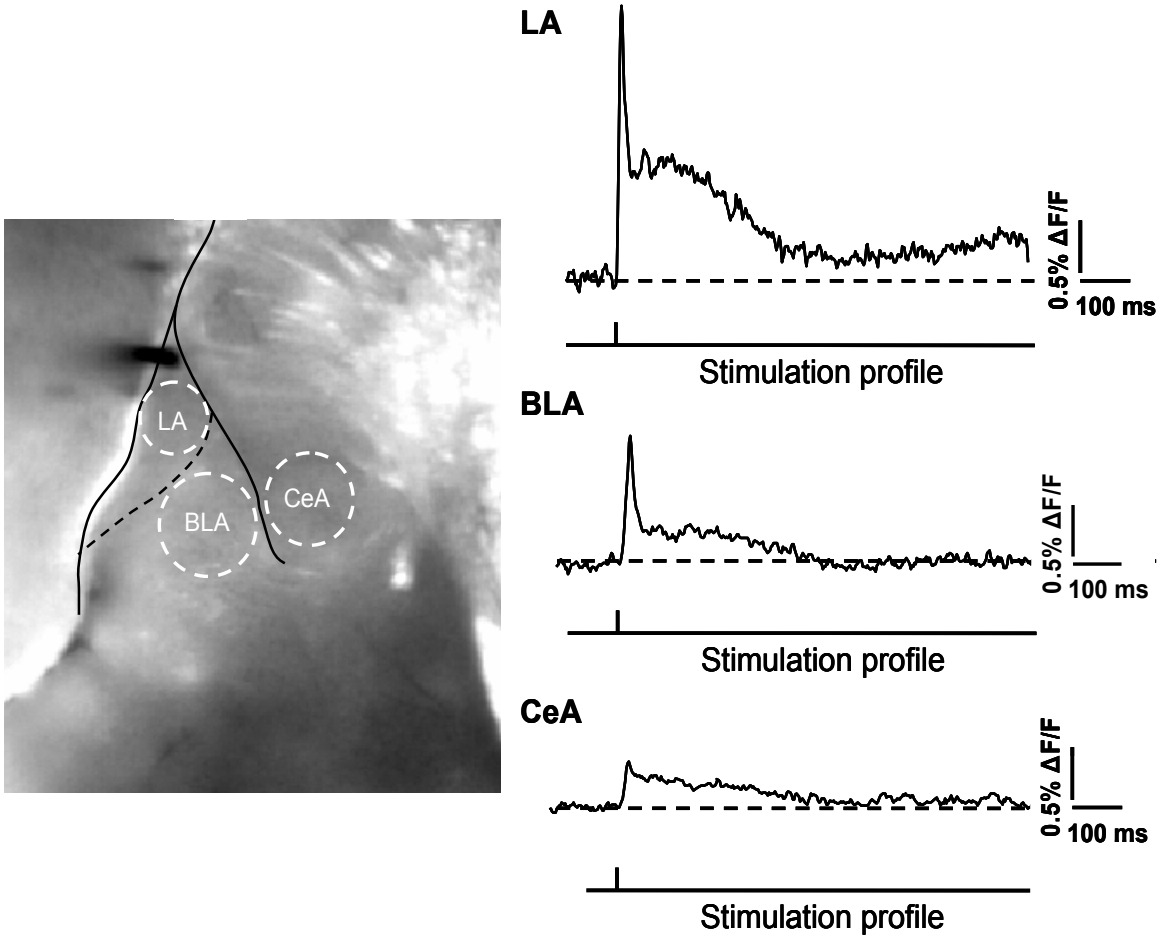


Figure 3-7: **Regions of interest (ROIs) in the three amygdalar nuclei under investigation**

The average of smoothed ΔF/F values within a particular ROI served as final measure of neuronal population activity. For further analysis, the peak amplitude of the fast, depolarisation-mediated VSDI signal (FDS) was calculated (**Figure 3-8**).

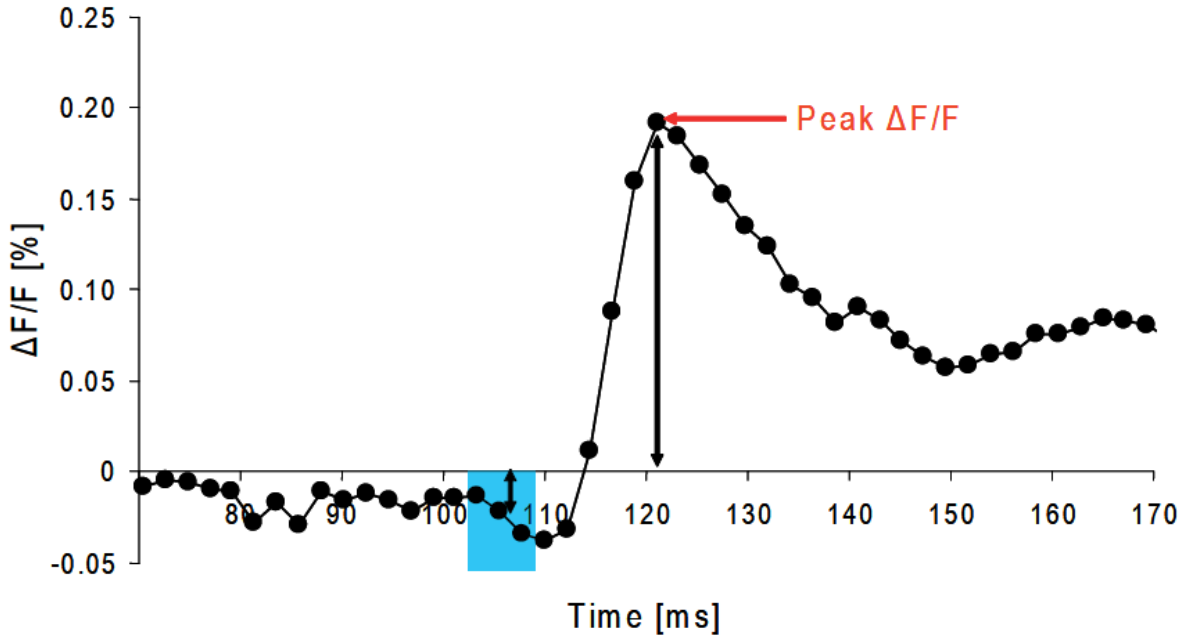


Figure 3-8: **Quantification of the fast, depolarisation-mediated VSDI signal**
To quantify the fast, depolarisation-mediated VSDI signal (FDS), the mean value of the background ΔF/F between 103.4-107.8 ms (blue box) was subtracted from the value of the peak ΔF/F (red arrow)

3.3.5 Statistics

The unpaired student t-test run in SigmaStat (version 3.5.; Systat Software, Inc., Point Richmond, CA, USA), was used for the statistical analysis.

4 Results

4.1 HAB/LAB animal model

4.1.1 Behavioural characterisation of the HAB/LAB mice

All animals used in this study were tested on the elevated plus-maze (EPM) at the age of 7 weeks. Based on the results of this behavioural characterisation, animals were separated in different groups:

-HAB
-LAB
-Control CD1
-HAB E.E.
-HAB E.E. not responding to the enriched environment (HAB E.E. n.r.)
-LAB exposed to chronic unpredictable stress (LAB Str.)

The behavioural phenotyping was performed by members of the RG Landgraf.

The main criterion for the separation in the different anxiety phenotypes was the percentage of time spent on the open arms of the EPM. Animals spending less than 10% of the time on the open arms were defined as HAB mice (**Figure 4-1**). On the contrary, animals spending more than 70% of the time on the open arms were defined as LAB mice (**Figure 4-1**). CD1 mice were chosen as the control group (same genetic background) exhibiting intermediate percentage of time spent on the open arms (30-50%, **Figure 4-1**).

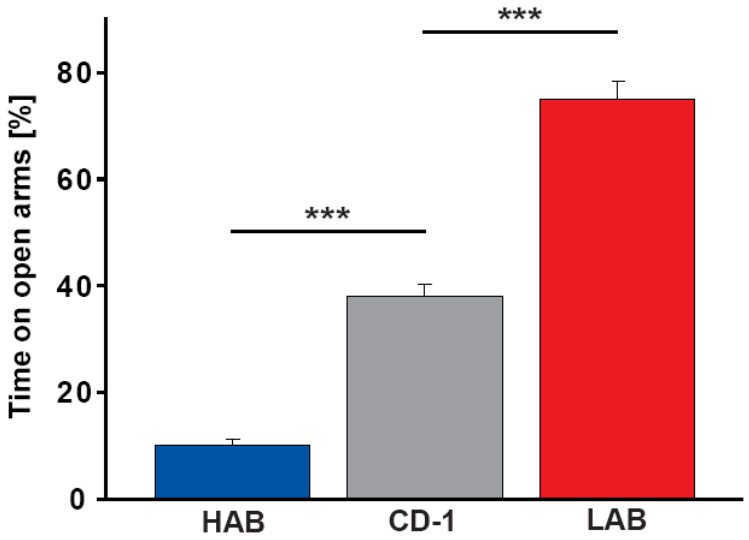


Figure 4-1: **Anxiety-related behaviour on the elevated plus-maze (EPM)**
The graph shows the percentage of time spent on the open arms of the EPM. HAB animals spent significantly less time on the open arms (9.7 ± 1.1%) than control CD1 (37.8 ± 2.5%) and LAB (75.7 ± 3.2%) mice indicating the most anxious behaviour of all groups. Data are shown as mean ± SEM. n_{HAB} = 7, n_{CD1} = 8, n_{LAB} = 9; *** $p < 0.001$.

4.1.2 Synaptic transmission and plasticity in the hippocampus of the HAB/LAB animals

4.1.2.1 Ventral hippocampus

There is increasing evidence that the hippocampus is not only involved in the formation of memories, but also plays a major role in sensory information processing and control of emotional-behaviour. The nature of the anatomical connections to and from the different parts along the septo-temporal axis, together with recent studies using selective lesions of the dorsal and ventral part of the hippocampus, point to the ventral part as an important area for the regulation of anxiety behaviour.

Based on this evidence, the ventral hippocampus was chosen as the first target of investigation in this study. In order to uncover potential differences in glutamatergic synaptic transmission and plasticity between HAB and LAB animals, fEPSP recordings were performed in the ventral hippocampus. HAB animals showed a decrease in basal neuronal transmission at CA3-CA1 synapses compared to LAB animals (**Figure 4-2a**). On the contrary, the paired-pulse ratio (PPR) was not significantly different between the two groups, indicating that CA3-CA1 synapses in

HAB and LAB animals exhibit an identical probability of glutamate release (**Figure 4-2b**). However, compared to the CD1 control mice, both HAB and LAB showed increased PPR, but no significant difference in the input-output relationship.

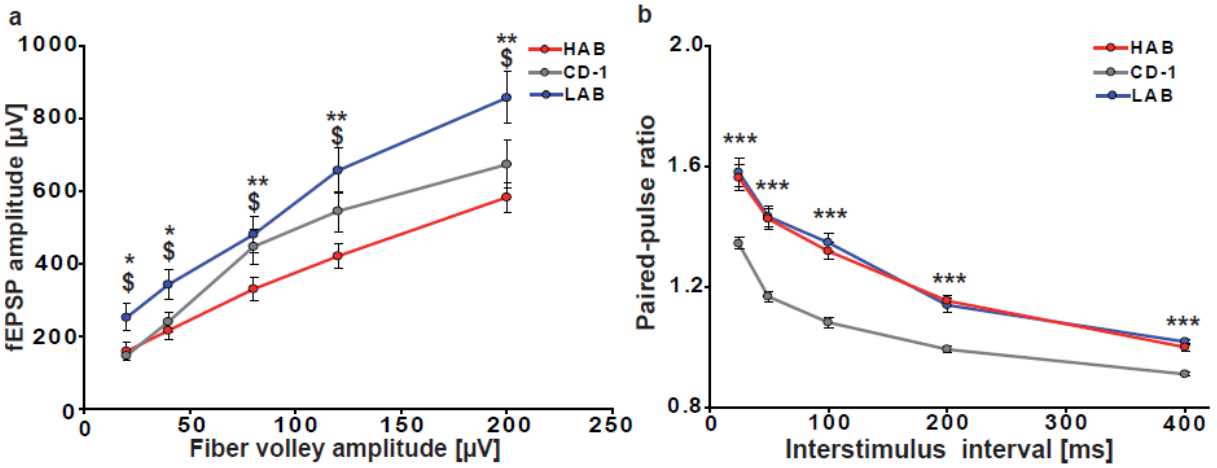


Figure 4-2: **Basal synaptic transmission and short-term plasticity at CA3-CA1 synapses in the ventral hippocampus**
(a) Input-output relationship: fEPSP amplitude is plotted as a function of the fiber volley amplitude. Basal synaptic transmission at CA3-CA1 synapses is decreased in HAB (red dots) compared to LAB (blue dots) animals. This relationship is not significantly different between HAB and LAB animals compared to the CD1 control (grey dots) counterparts.
(b) Paired-pulse ratio (PPR): The PPR is significantly increased in LAB (blue dots) and HAB mice (red dots) compared to the CD1 control group (grey dots) at interstimulus intervals of 25, 50, 100, 200 and 400 ms.
$: comparison of LAB and HAB animals. Data are all shown as mean ± SEM. n_{LAB} = 17, n_{CD1} = 18, n_{HAB} = 21 ; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

To test for potential differences in long-term synaptic plasticity, LTP was induced by high-frequency stimulation (HFS, 100 Hz/1 s) after 20 min of stable baseline recording. The results showed a significantly increased LTP in HAB compared to LAB and CD1 animals (LTP1: min 50-55, 49.11 ± 3.9 % in HAB *vs.* 32.53 ± 3.7 % in LAB *vs.* 25.14 ± 1.8 % in CD1; LTP 2: min 85-90, 70.26 ± 6.3 % in HAB *vs.* 50.02 ± 6 % in LAB *vs.* 39.33 ± 3.3 % in CD1; **Figure 4-3**).

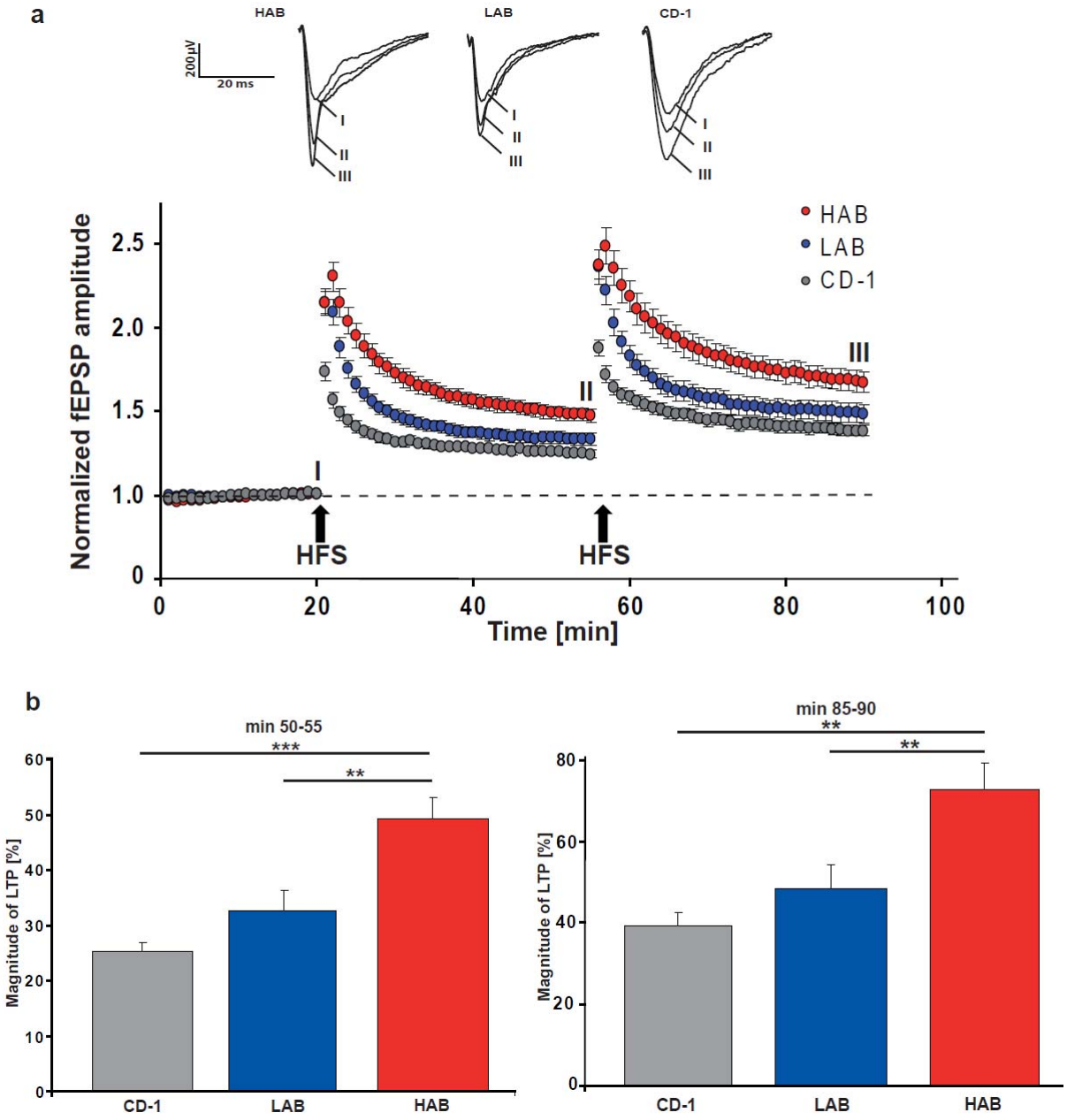


Figure 4-3: **Long-term potentiation (LTP) at CA3-CA1 synapses in the ventral hippocampus of HAB, LAB, and CD1 animals**

(a) LTP at CA3-CA1 synapses is increased in HAB (red dots) compared to LAB (blue dots) and CD1 control animals (grey dots). After 20 min of baseline recording, high-frequency stimulation (HFS, black arrow) was applied to the Schaffer collateral-commissural pathway. Changes in synaptic strength were then monitored for 35 min before applying a second HFS. Data are normalised to the mean fEPSP amplitude of the last 10 min of baseline recording.

(b) Statistical evaluation of the LTP experiment.

Data are shown as mean ± SEM. n_{LAB}= 17, n_{CD1} = 17, n_{HAB} = 18; ** $p < 0.01$, *** $p < 0.001$.

4.1.2.2 Dorsal hippocampus

Our previous findings for the ventral hippocampus raised the question whether similar differences between HAB and LAB mice can also be observed in the dorsal hippocampus. On that account we performed the same electrophysiological experiments in the dorsal CA1 subfield.

HAB and LAB mice showed a weaker basal synaptic efficacy than CD1 mice. Contrary to the ventral hippocampus, no significant difference was found between HAB and LAB animals (**Figure 4-4a**). The PPR at the interstimulus intervals of 25, 50 and 100 ms significantly differed between LAB and CD1 control animals. At an interstimulus interval of 400 ms, we also detected a difference between HAB and CD1 mice (**Figure 4-4b**).

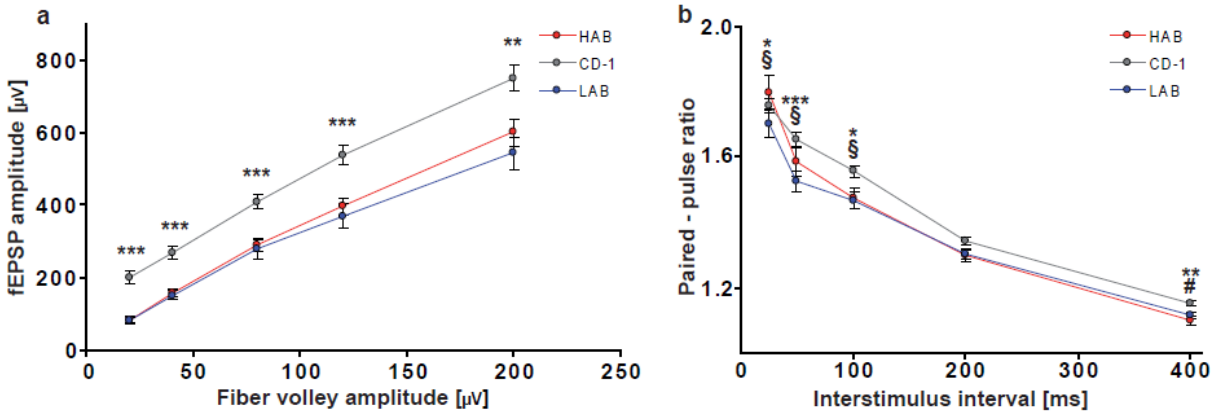


Figure 4-4: **Basal synaptic transmission and short-term plasticity in the dorsal hippocampus**
(a) Input-Output relationship: fEPSP amplitude is plotted as a function of the fiber volley amplitude. The basal synaptic transmission at CA3-CA1 synapses is decreased in HAB (red dots) and LAB (blue dots) animals compared to the CD1 control (grey dots) counterparts. This relationship is not significantly different between HAB and LAB animals.
(b) Paired-pulse ratio (PPR): The PPR is significantly reduced in LAB (blue dots) compared to the CD1 control group (grey dots) for interstimulus intervals 25, 50, 100, and 400 ms, indicating an increased release probability of glutamate at CA3-CA1 synapses
§: comparison between LAB and CD1 animals; * p<0.05, ** p< 0.01, *** p<0.001. For interstimulus interval 400 ms, PPR is also decreased in HAB compared to CD1 mice (# comparison between HAB and CD1 animals; ** p< 0.01).
Data are shown as mean ± SEM. n_{LAB} = 17, n_{CD1} = 18, n_{HAB} = 21

In the LTP experiments, we obtained opposite results as in the ventral hippocampus. In slices from HAB animals the magnitude of LTP was significantly lower than in slices from LAB and CD1 control animals (LTP1: min 50-55, 22.50 ± 2.2

% in HAB *vs.* 34.41 ± 3.3 % in LAB *vs.* 38.76 ± 3.1 % in CD1; LTP2: min 85-90 in HAB *vs.* 55.35 ± 5.6 % in LAB *vs.* 53.76 ± 4.7% in CD1; **Figure 4-5**).

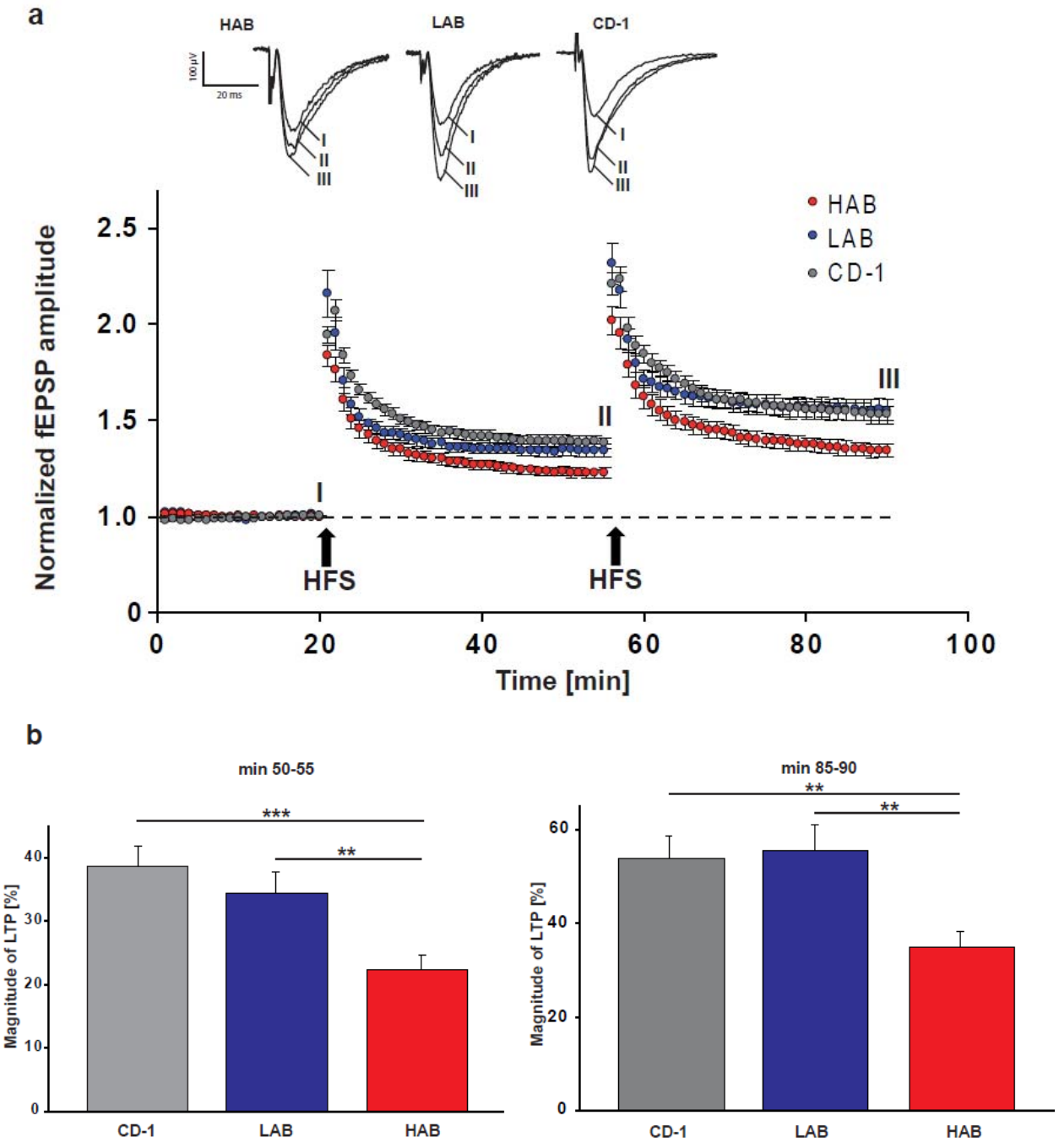


Figure 4-5: **Long-term potentiation (LTP) at CA3-CA1 synapses in the dorsal hippocampus of HAB, LAB, and CD1 animals**

(a) LTP at CA3-CA1 synapses is reduced in HAB (red dots) compared to LAB (blue dots) and CD1 control animals (grey dots). After 20 min of baseline recording, high-frequency stimulation (HFS, black arrow) was applied to the Schaffer collateral-commissural pathway. Changes in synaptic strength were then monitored for 35 min. Data are normalised to minutes the mean fEPSP amplitude of the last 10 min of baseline recordigs.

(b) Statistical evaluation of the LTP experiment.

Data are shown as mean ± SEM. n_{LAB} = 17, n_{CD1} = 17, n_{HAB} = 18; ** $p < 0.01$, *** $p < 0.001$

4.1.3 Investigation of amygdalar network features

4.1.3.1 Neuronal network activation and VSDI recordings

Based on the observation that patients with generalised anxiety disorder exhibit abnormal neuronal activity in the amygdala and due to the fact that this limbic structure plays a crucial role in the regulation of anxiety states, we considered that differences in neuronal activity propagation through the amygdalar network might be causally related to different anxiety phenotypes.

We first established an *in vitro* assay allowing a quantitative imaging of evoked neuronal activity propagation through important amygdalar subfields. Electrical stimuli (10, 17.5 and 25 V) were applied to the dorsal part of the LA to elicit a fast, depolarisation-mediated VSDI signal (FDS) propagating through the LA, the BLA and the CeA. The FDSs in these amygdalar subregions were strong enough for a clear discrimination from the background noise and could be enhanced by increasing the stimulus intensity indicating that they were not saturated (**Figure 4-6**).

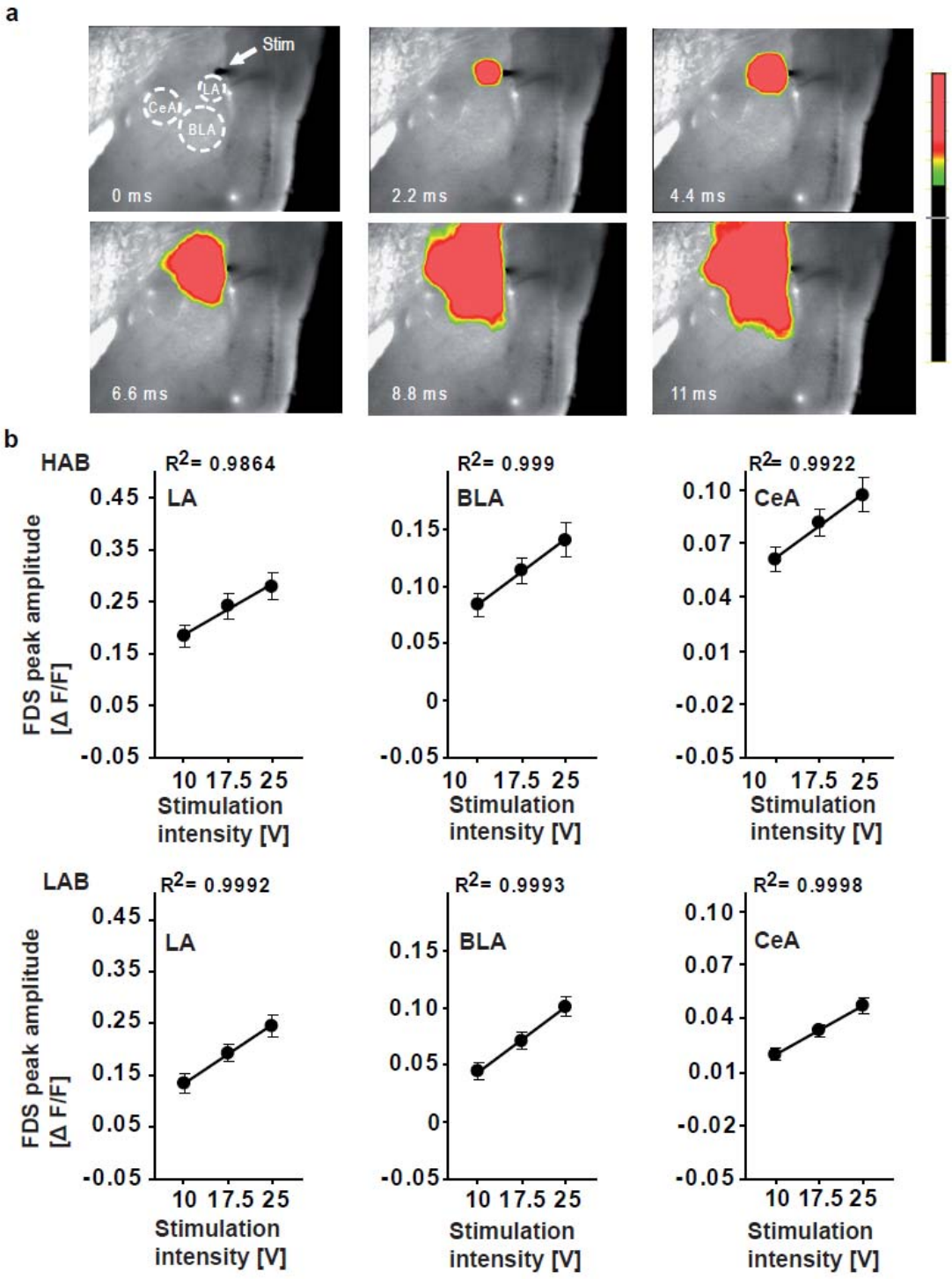


Figure 4-6: **Voltage-sensitive dye imaging (VSDI) of neuronal activity propagation through the amygdalar network**

(a) Representative filmstrip showing the propagation of an evoked **f**ast, **d**epolarisation-mediated VSDI **s**ignal (FDS) after electrical stimulation (25 V). The white circles illustrate the regions of interest (ROIs) used for the calculation of neuronal activity within the three amygdalar subnuclei. The position of the stimulus electrode is given by the white arrow. Changes in neuronal activity are reflected by the fractional change in fluorescence (ΔF/F). Warmer colours indicate stronger neuronal activity.

(b) Relationship between stimulation intensity and FDS peak amplitudes in the LA, BLA, and CeA of slices obtained from HAB and LAB mice. Note the linear relationship between the stimulation intensity and FDS peak amplitudes in both groups.

In order to further characterise the FDS, a series of pharmacological experiments was performed. The FDSs, which reflect action potentials as well as excitatory postsynaptic potentials (von Wolff et al., 2011), were strongly diminished by the glutamate receptor antagonist AP5 [50 µM) and NBQX (5 µM) and completely abolished by the voltage-gated sodium channel blocker TTX (1µM) (**Figure 4-7**).

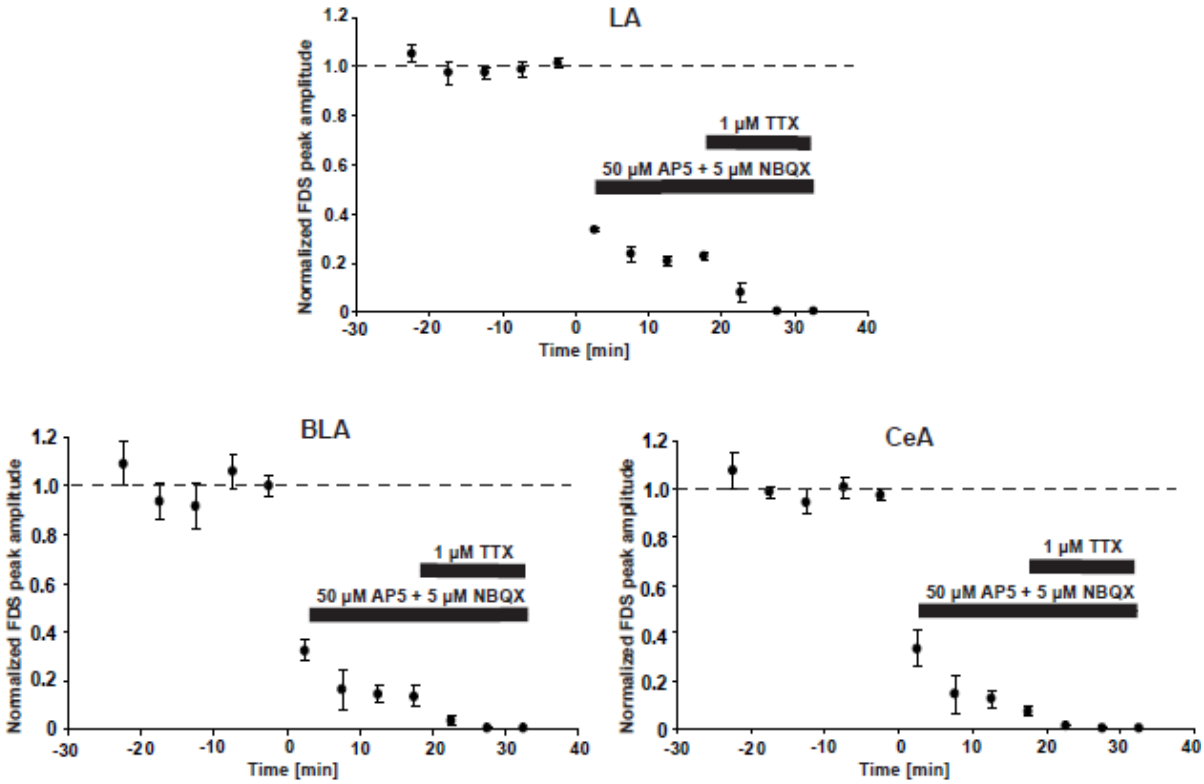


Figure 4-7: **Pharmacological characterisation of fast, depolarisation-mediated imaging signals (FDSs) in the amygdala**
Acquisitions were made at intervals of 5 min. Data are normalised to the mean value of the fractional change in fluorescence (ΔF/F) of the VSDI recordings during baseline conditions. Time courses of experiments depicted for the LA, BLA and CeA. Bath application of the ionotropic glutamate receptor antagonists NBQX and AP5 at maximal effective concentrations strongly reduced the FDSs in the LA, BLA, and CeA. Coapplication of the voltage-gated sodium channel blocker TTX at a maximal effective concentration fully abolished the caused FDSs in all three subnuclei of the amygdala.

An antagonism of $GABA_A$ receptors with bicuculline (Bic.; 10µM) induced an increase in the FDS peak amplitude, indicating that the FDS signal also comprises a GABAergic component. The FDS peak amplitude of the BLA nucleus shows a stronger increase after bicucullin application compared to the enhancement of the FDS peak amplitude in LA and CeA, indicating a higher density of interneurons within the BLA compared to the LA and CeA (**Figure 4-8**).

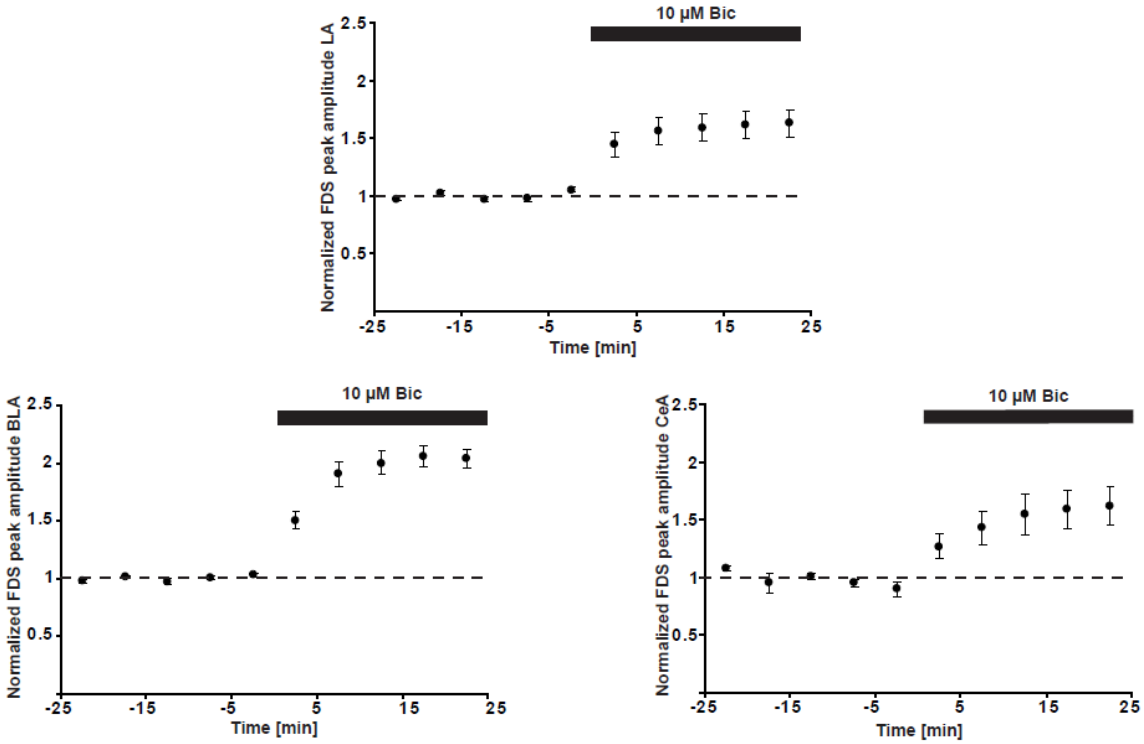


Figure 4-8: **Pharmacological characterisation of fast, depolarisation-mediated imaging signals (FDSs) in the amygdala**
Bath application of the $GABA_A$ receptor antagonist bicuculline (Bic) increased the FDS peak amplitude in the LA, BLA, and CeA (LA: 56.8 ± 5.6%, BLA: 90.2 ± 9.5%, CeA 49.2 ± 6.3%)

For a quantitative analysis of neuronal activity flow through the amygdalar network, ratios of FDS peak amplitudes, serving as a kind of Input-output relationship, were calculated (BLA/LA and CeA/LA). These ratios did not significantly differ for the three stimulation intensities. Therefore, we calculated average ratios over the three stimulation intensities (**Figure 4-9**).

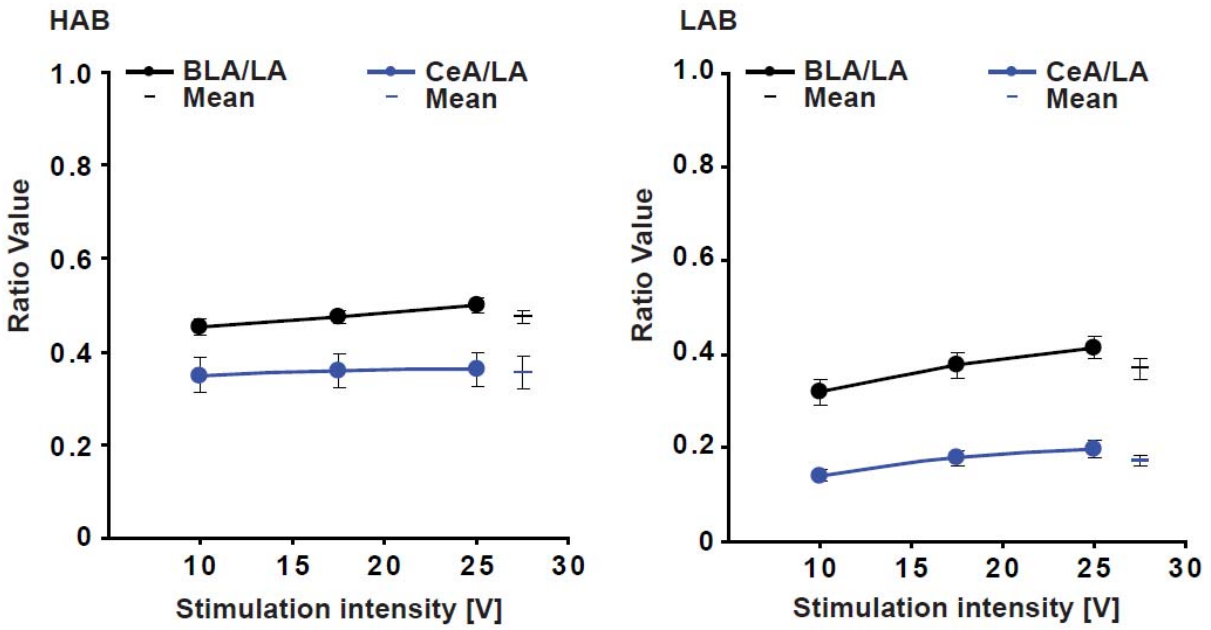


Figure 4-9: **Ratios of FDS peak amplitudes (BLA/LA and CeA/LA) for the different stimulus intensities**

HAB animals showed significantly higher BLA/LA and CeA/LA ratios of FDS peak amplitudes than LAB and CD1 control mice (HAB; BLA/LA: 0.47 ± 0.01 & CeA/LA: 0.31 ± 0.03 *vs*. LAB; BLA/LA: 0.36 ± 0.02 & CeA/LA: 0.17 ± 0.01 *vs.* CD1; BLA/LA 0.35 ± 0.02 & CeA/LA: 0.22 ± 0.01, **Figure 4-10**). LAB animals did not show a significant difference in the BLA/LA ratio but a lower CeA/LA ratio compared to the CD1 mice.

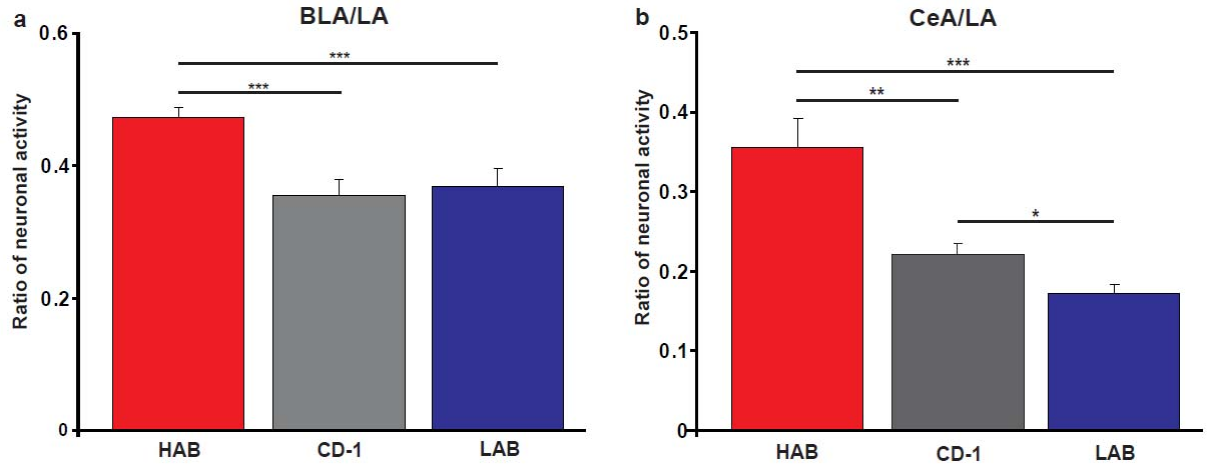


Figure 4-10: **Ratio of neuronal activity in the amygdala**
VSDI reveals differences in evoked neuronal activity propagation through the amygdalar network between HAB, CD1, and LAB mice.
Data are shown as mean ± SEM. n_{HAB} = 13, n_{CD1} = 16, n_{LAB} = 12; * p<0.05, ** p < 0.01, and *** p < 0.001.

Numerous studies provide strong evidence for a link between anxiety levels, stress, and amygdalar network activity. Stress hormones increase anxiety concomitantly with an enhancement in neuronal network activity in the amygdala, whereas the exposure to an enriched environment reduces anxiety, indicating an anxiolytic effect of the environmental enrichment. It thus appears of high importance to investigate potential relationships between environment, neuronal activity in the amygdala and anxiety levels. On that account, HAB mice were housed in an enriched environment (HAB E.E.) and LAB mice exposed to chronic unpredictable stressful conditions (LAB Str.). These environmental changes indeed affected anxiety-like behaviour in both groups (**Figure 4-11**).

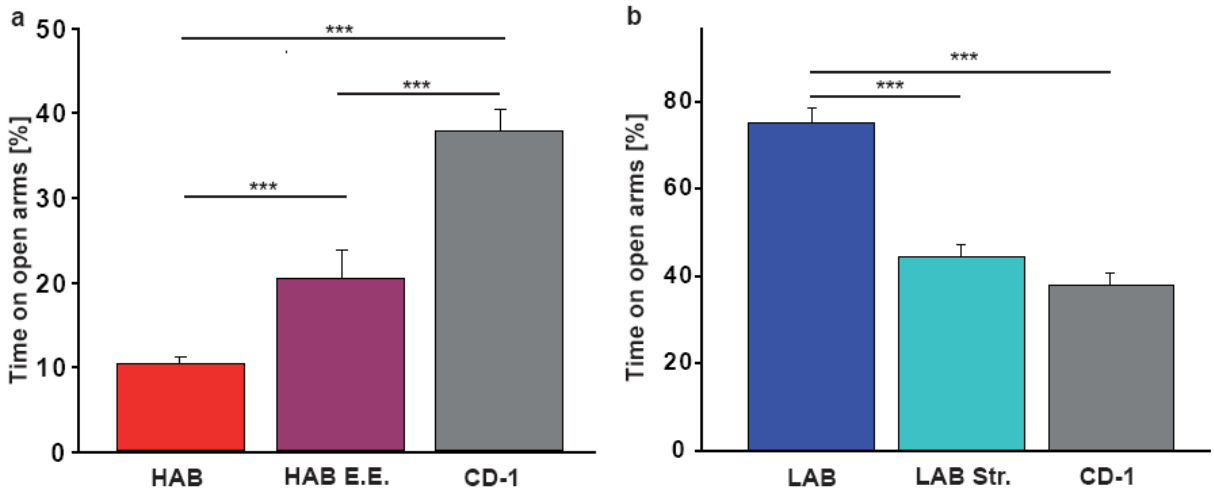


Figure 4-11: **Effects of environmental alterations on anxiety levels on the EPM**
(a) Effect of enriched environment on anxiety in HAB mice. Enrichment of the environment induced an anxiolytic effect in HAB mice. Note the still significant difference existing between HAB E.E and CD1 mice.
(b) Effect of chronic unpredictable stress on anxiety in LAB mice. The stress caused an anxiogenic effect in LAB mice.
Data are shown as mean ± SEM. n_{HAB} = 7, $n_{HAB\ E.E.}$ = 14, n_{CD1} = 8, n_{LAB} = 9, $n_{LAB\ Str.}$ = 7; *** $p < 0.001$.

Next, we investigated whether observed effects of stress and enrichment on anxiety-related behaviour are reflected in changes in the BLA/LA and CeA/LA ratios. In fact, in slices obtained from HAB E.E. mice, we observed significantly lower BLA/LA and CeA/LA ratios than in slices prepared from HAB animals. As for the behavioural measurements, HAB E.E. mid displayed a phenotype between the HAB and CD1 group with respect to the BLA/LA ratio. This was, however, not the cause

for the CeA/LA ratio (HAB; BLA/LA: 0.47 ± 0.01 & CeA/LA: 0.31 ± 0.03 *vs*. HAB E.E; BLA/LA: 0.39 ± 0.02 & CeA/LA: 0.18 ± 0.01 *vs.* CD1; BLA/LA: 0.35 ± 0.02 & CeA/LA: 0.22 ± 0.01, **Figure 4-12**).

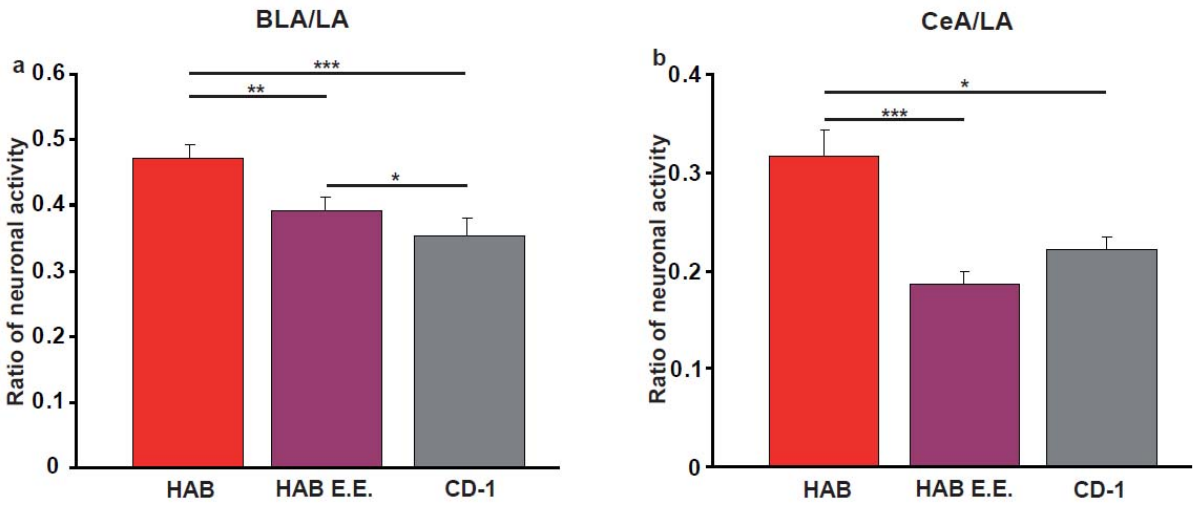


Figure 4-12: **Effects of environmental enrichment on amygdalar network activity in HAB animals**
Data are shown as mean ± SEM. n_{HAB} = 13, $n_{HAB\ E.E.}$ = 25, n_{CD1} = 16; *** $p < 0.001$.

However, the anxiolytic effect of environmental enrichment can not be observed in all animals which are exposed to enriched conditions. Therefore, a new group was created, consisting of animals which were not responding to the environmental enrichment (HAB E.E.n.r.). Animals of the HAB E.E.n.r. group show no decrease in anxiety levels indicated by the time that these animals spent in the open arms of the EPM (HAB: 9.68 ± 1.2%, HAB E.E.: 19.23 ± 2.5%.; HAB E.E.n.r.: 9.22 ± 2.8% **Figure 4-13**). In these animals, the BLA/LA ratio was not significantly different to the animals of the HAB and HAB E.E. group. This was also the case for the CeA/LA ratio (HAB; BLA/LA: 0.47 ± 0.01 & CeA/LA: 0.31 ± 0.03 *vs*. HAB E.E; BLA/LA: 0.39 ± 0.02 & CeA/LA: 0.18 ± 0.01 *vs.* HAB E.E.n.r.; BLA/LA 0.45 ± 0.03 & CeA/LA: 0.27 ± 0.03, **Figure 4-13**).

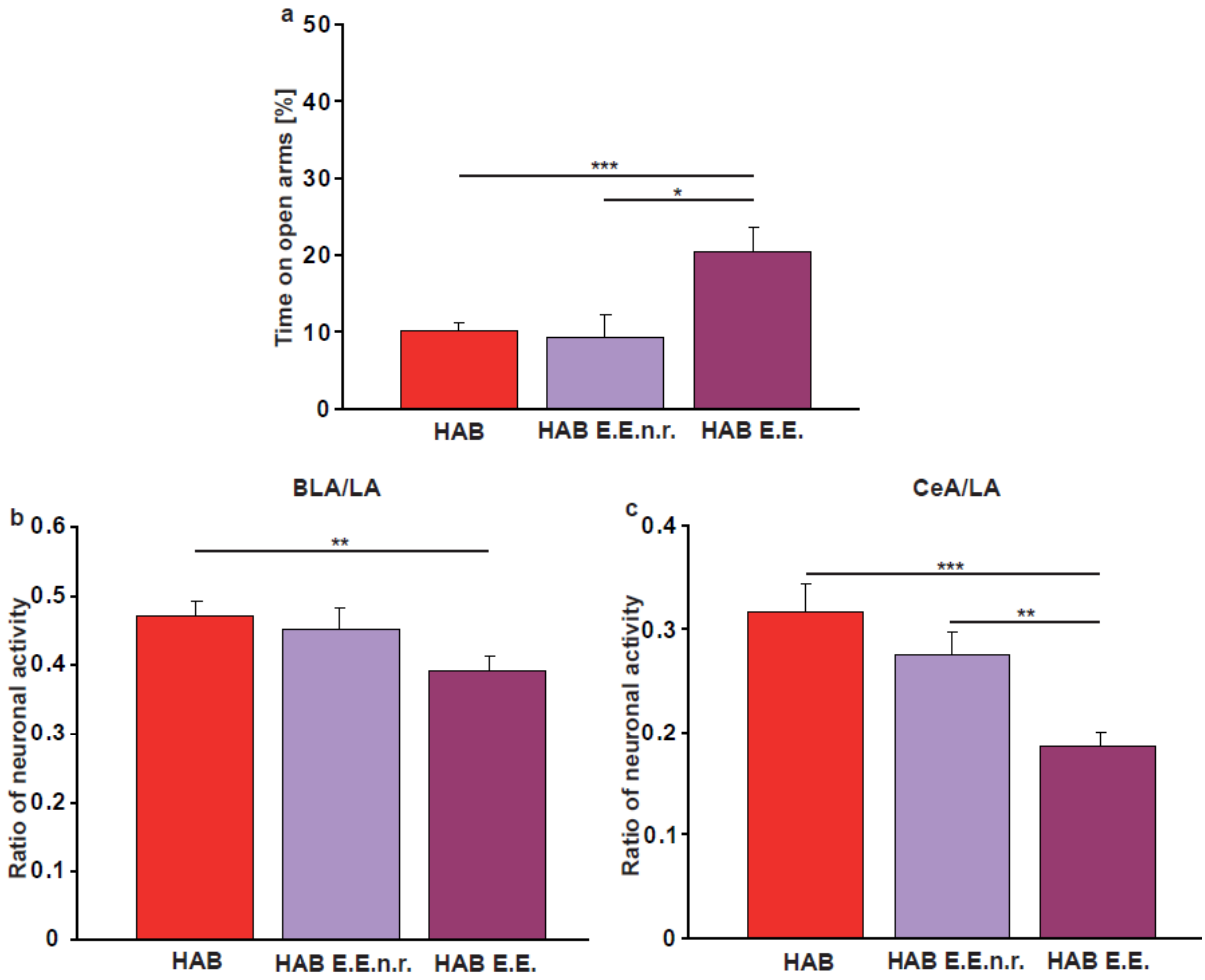


Figure 4-13: **Characterisation of anxiety levels and amygdalar network activity in animals insensitive to enriched environment (HAB E.E.n.r)**
(a) Anxiety-related behaviour on the EPM.
(b) Neuronal activity propagation in the amygdala
Data are shown as mean ± SEM. n_{HAB} = 7, $n_{HAB\ E.E.n.r.}$ = 6, $n_{HAB\ E.E.}$ = 14;* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

An opposite environmental modulation of anxiety-related behaviour could be observed in LAB mice exposed to chronic unpredictable stress. The time that LAB Str. animals spent on the open arms of the EPM was significantly reduced compared to LAB mice (**Figure 4-14**), indicating an anxiogenic effect of the stress. The BLA/LA ratio was significantly higher than measured in the animals of the LAB and CD1 group. This was, however, not the case for the CeA/LA ratio (LAB; BLA/LA: 0.36 ± 0.02 & CeA/LA: 0.17 ± 0.01 *vs*. LAB Str.; BLA/LA: 0.51 ± 0.06 & CeA/LA: 0.22 ± 0.02 *vs.* CD1 BLA/LA: 0.35 ± 0.02 & Ce/LA: 0.22 ± 0.01, **Figure 4-14**).

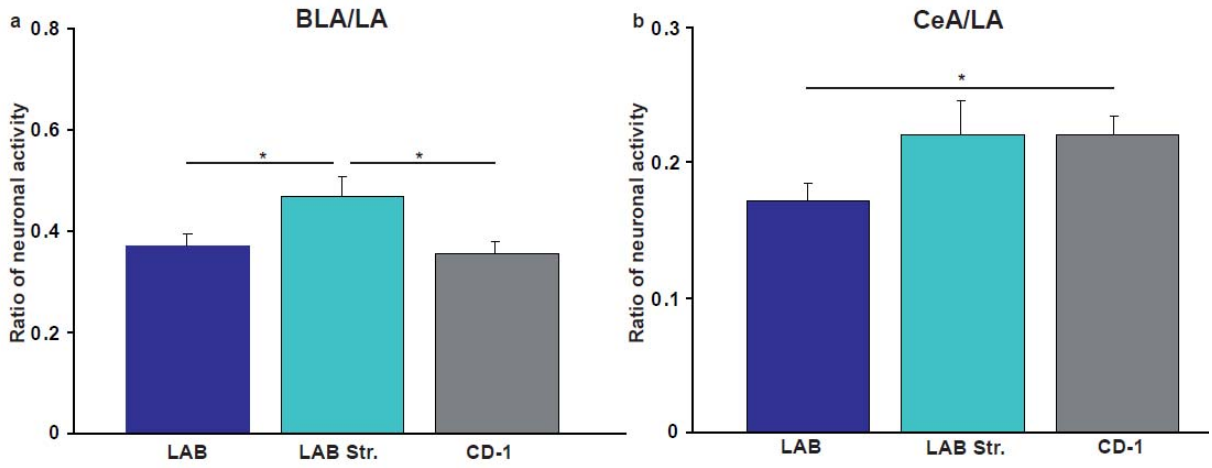


Figure 4-14: **Effects of chronic unpredictable stress on amygdalar network activity in LAB animals**
Data are shown as mean ± SEM. n_{LAB} = 9, $n_{LAB\ Str.}$ = 7, n_{CD1} = 16; * $p < 0.05$.

Up to here, I show that there is a clear correlation between the strength of anxiety-related behaviour on the EPM and the magnitude of evoked neuronal activity propagation from the LA to the CeA. As a final step in data analysis, I therefore plotted the values of time on open arms as measured in all mice in the study, against the respective CeA/LA ratio. This preceding confirmed a relationship between anxiety levels and the neuronal network dynamics investigated in the amygdala (**Figure 4-15**).

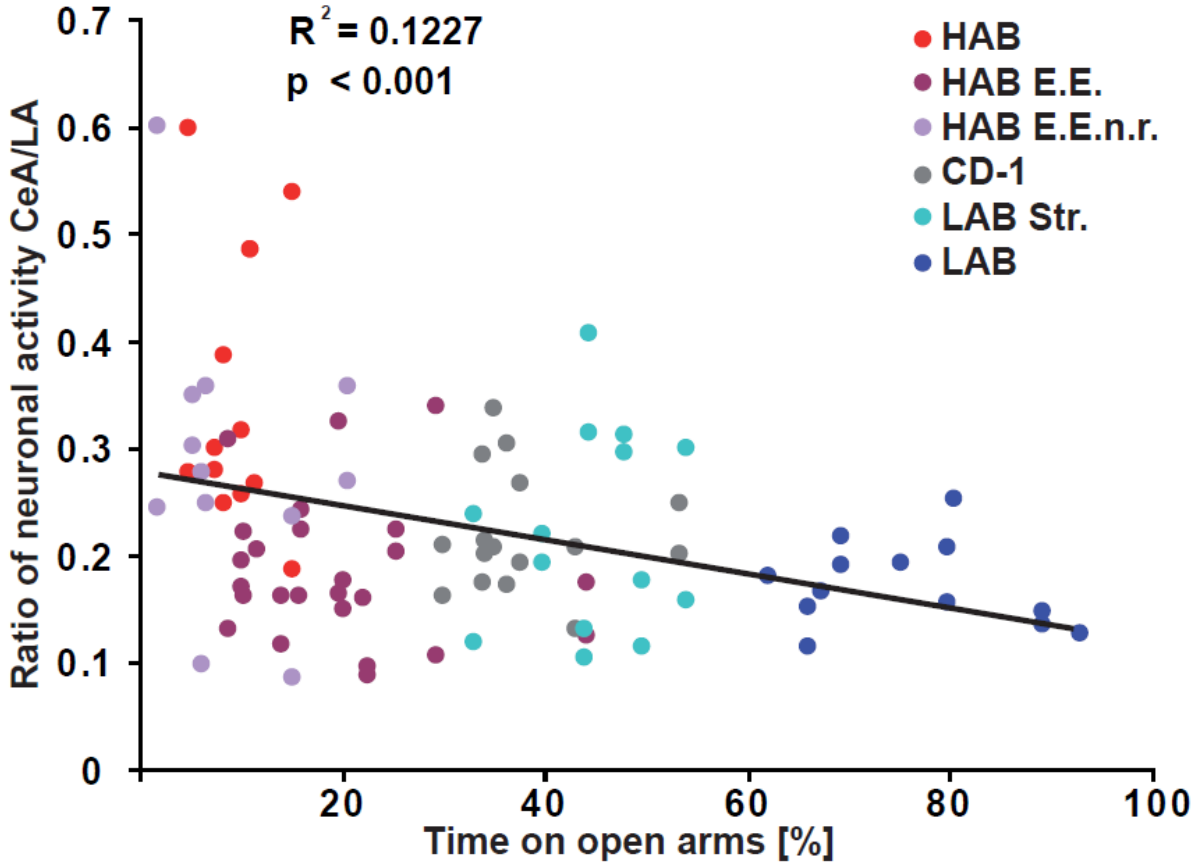


Figure 4-15: **VSDI revealed a statistically significant correlation between anxiety phenotypes in mice and neuronal activity propagation in the amygdalar network**

4.1.3.2 Investigation of GABAergic neurotransmission in BLA neurons of HAB and LAB mice

There is evidence for an altered GABAergic inhibitory control of amygdalar activity in anxiety disorders (Millan, 2003). Benzodiazepines, the most frequently clinically used anxiolytic drugs, exert their effects through the modulation of $GABA_A$ receptors (Nemeroff, 2003). In addition, changes in $GABA_A$ receptor expression were observed in the limbic system of patients suffering from panic disorder by [^{14}C] flumazenil positron emission tomography (Hasler et al., 2008). Alterations in GABAergic neurotransmission in the amygdala do not only modulate fear and anxiety under non-pathological conditions, but also predispose individuals to pathological trait anxiety (Shen et al., 2010). Therefore, experiments were performed to test for potential differences in GABAergic inhibition in the BLA of HAB and LAB animals. For this purpose, recordings of $GABA_A$ receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) were conducted in principal BLA neurons. These

recordings did not reveal differences in the frequency or amplitude of mIPSCs between HAB and LAB animals (HAB mean frequency: 1.56 ± 0.2 Hz *vs.* LAB mean frequency: 1.76 ± 0.2 Hz; HAB mean amplitude: 13.54 ± 0.7 pA *vs.* LAB mean amplitude: 12.92 ± 0.5 pA, **Figure 4-16**).

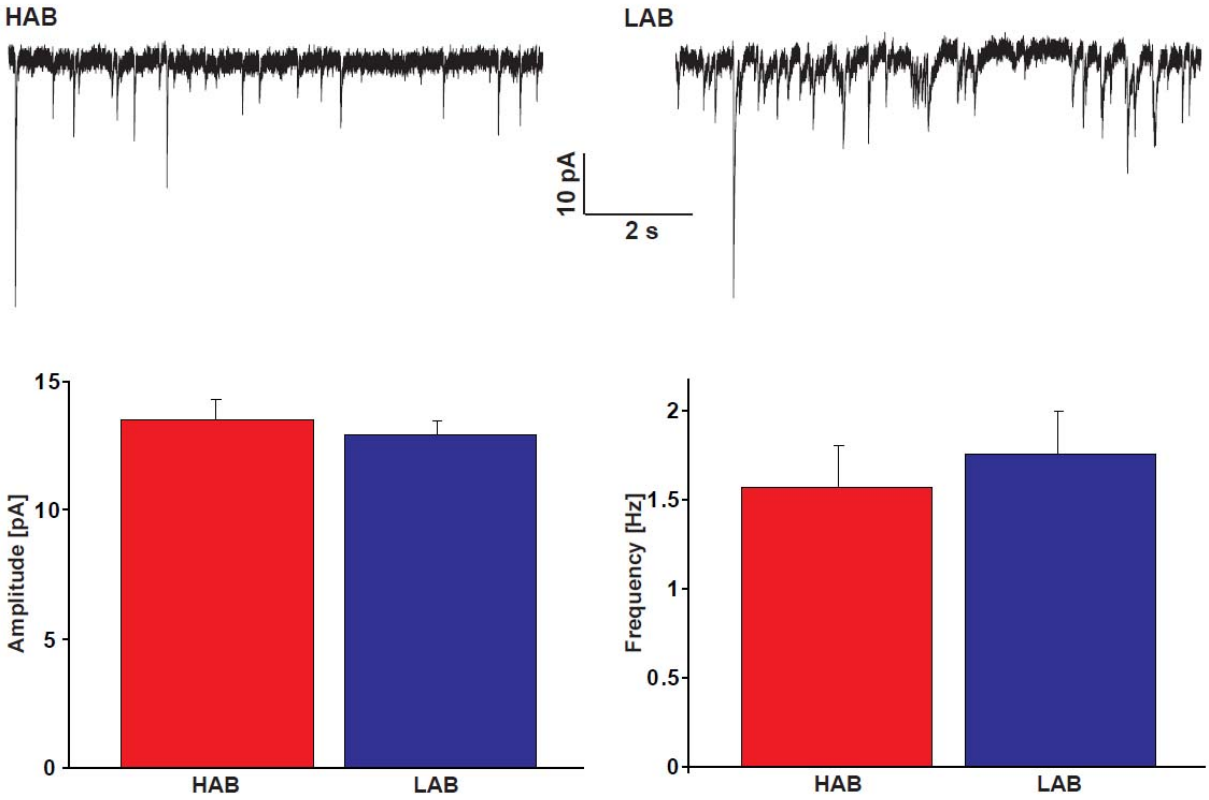


Figure 4-16: **Comparison of the amplitude and frequency of mIPSCs in BLA principal neurons between HAB and LAB mice**
Representative recordings traces.
Amplitude and frequency analysis of mIPSCs in HAB and LAB mice.
Data are shown as mean ± SEM. n_{LAB} = 26, n_{HAB} = 38

5 Discussion

5.1 Striking variations in the expression of long-term potentiation along the septotemporal axis of the hippocampus between animals of the HAB/LAB mouse model

Using acute brain slices, I investigated basal neurotransmission and plasticity at CA3-CA1 synapses along the septotemporal axis of the hippocampus in HAB, LAB and CD1 mice. Anxiety disorders, as well as other stress related brain disorders, frequently result from a dysregulation of the amygdala and HPA axis. Thus, it is of particular important to understand how the hippocampus regulates the activity of these two neuronal systems. The present study revealed that basal neuro-transmission at CA3-CA1 synapses in the dorsal hippocampus (DH) is weaker in HAB and LAB animals compared to CD1 mice, without a significant difference between HAB and LAB animals. Also paired-pulse facilitation was not different between HAB and LAB mice. In the ventral hippocampus (VH), LAB animals showed a stronger synaptic transmission compared to HAB animals. The paired-pulse ratio, however did not point to difference in the probability of glutamate release between the two groups.

It has been shown that stress can affect several brain functions, including synaptic transmission and plasticity (such as LTP) (Kim and Yoon, 1998; Garcia, 2002; Kim et al., 2006). For example, CA1 LTP induction is impaired in rats exposed to stress, such as an inescapable foot-shock (Foy et al., 1987; Shors et al., 1989). This effect of stress on hippocampal plasticity has been postulated to be mediated by the release of high doses of corticosterone. Indeed, administration of corticosterone either *in vivo,* controlled by implanting corticosterone pellets in adrenalectomised rats (Diamond et al., 1992), or *in vitro* (Pavlides et al., 1996; Alfarez et al., 2002) produced the same impairment of LTP. However, recent studies indicate that the corticosterone effect on LTP is more complex than originally believed. These studies show that animals exposed to stressful conditions display a decrease in the magnitude of LTP at the CA3-CA1 synapses in the DH (Yang et al., 2004). In contrast, LTP in the VH of stressed animals is markedly enhanced (Maggio and Segal, 2007a, b, 2010, 2011).

The latter results obtained after acute stress exposure are similar to the here presented findings in the HAB/LAB mouse model, supporting the idea of a functional differentiation between the DH and the VH. This idea was first described after lesion studies. Lesions of the DH impair cognitive performances whereas lesions of the VH affect emotional processes (Kjelstrup et al., 2002). The importance of a regulation of amygdala and HPA axis activity by the VH has been well established over the last decade (Degroot and Treit, 2004). Thus, the increased anxiety levels in HAB mice could result, at least in part, from the enhancement of LTP in the VH, providing stronger excitatory input to the amygdala, leading to an “overactivation” of this brain structure.

Possible explanation for the decreased LTP in the DH and increased LTP in the VH of HAB mice is an asymmetrical expression of corticosteroid receptors along the septotemporal axis. Corticosterone mediates its effects through the activation of two types of receptors, the mineralocorticoid (MR) and the glucocorticoid (GR) receptors (de Kloet et al., 2005). The original view was that both types of receptors act as nuclear transcription factors, modifying protein synthesis and, therefore, producing slow, long-lasting changes in the functioning of neurons (de Kloet et al., 1993; Joels, 1999, 2001). However, recent findings point to a localisation of MR and GR also in the plasma membrane (Maggio and Segal, 2010; Groeneweg et al., 2011). These membrane-bound MRs and GRs induce fast, non-genomic effects, such as a modulation of ionic conductances. This can lead to changes in the excitability of neurons (Karst et al., 2005; de Kloet et al., 2008).

In a recent study, Maggio and Segal described a differential effect of corticosterone on IPSCs in the DH and VH inherited from differential expression of corticosteroid receptors in these hippocampal regions. In the DH, corticoterone increased the amplitude of IPSCs in CA1 pyramidal cells by approximately 60% (Maggio and Segal, 2009). This effect is mediated by GR since it is completely abolished by specific GR antagonist RU38486 (mifepristone) (Avital et al., 2006). The increase in GABAergic inhibition should hyperpolarise CA1 pyramidal neurons, thus possible impairing induction of CA1-LTP.

On the contrary, corticosterone decreases the frequency of mIPSCs in CA1 pyramidal neurons of the VH, suggesting a reduction in inhibitory inputs to these cells. This decrease in inhibition could facilitate CA1 LTP induction. Another study shows similar results. In this work, physiological concentrations of corticosteron

enhance LTP in the VH through the activation of MRs. This effect could be blocked by the MR antagonist spironolactone (Avital et al., 2006; Maggio and Segal, 2010). These results are supported by a recent publications demonstrating that GRs and MRs are differentially expressed along the septotemporal axis of the hippocampus. The VH is enriched in MRs, whereas the DH contains more GRs (Robertson et al., 2005).

Although all of the above mentioned effects of corticosterone were observed after acute stress, they may also underlie the electrophysiological alterations here observed in HAB mice. It would be necessary to investigate whether a selective GR antagonist is able to block the impairment of LTP in the DH of HAB animals. The enhancement of LTP in the VH, however, should be reversed by application of a selective MR antagonist. In this context, it appears also important to study the expression of GRs and MRs in the hippocampus of HAB and CD1 control animals. This could be done by Western blot analysis and/ or *in situ* hybridization. Since the HAB/LAB mouse model is based on inborn trait anxiety, it also would be interesting to examine effects of a chronic treatment with a MR antagonist on anxiety levels in HAB mice. That way, one could get important hints for an involvement of the VH in high anxious behaviour.

Our data clearly show an enhancement of LTP in the VH and an impairment of LTP in the DH in HAB mice compared to control CD1 animals. Reduced DH LTP and simultaneously enhanced VH LTP could at the same time suppress the cognitive route of the hippocampus to cortical structures (e.g. cingulated and prefrontal cortex), and enhance the emotional route of the VH to the amygdala. This may explain why patients suffering from mood/stress-disorders often show an exaggeration of acquired fear responses as well as enhanced activity levels in the amygdala that is accompanied by a suppression of prefrontal cortical functions (Rauch et al., 2006; Milad et al., 2009).

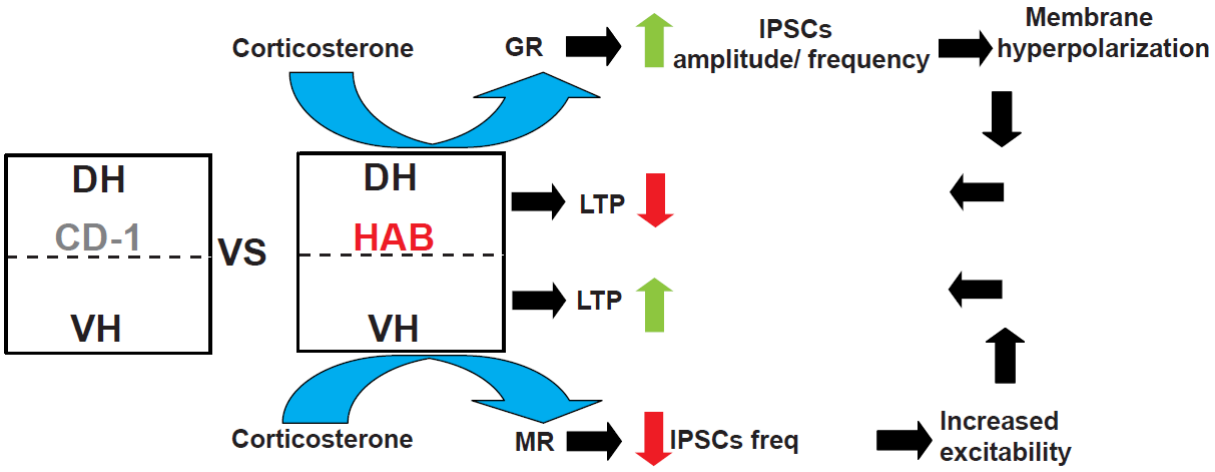


Figure 5-1: **Schematic diagram of proposed mechanism by which corticosterone could differently modulate LTP in the dorsal (DH) and ventral hippocampus (VH)**
In the DH, activation of membrane-located GRs increases IPSCs amplitude and frequency. This leads to a hyperpolarisation of the cell membrane impairing induction of LTP. In the VH, activation of membrane-located MRs reduces IPSCs frequency, leading to an increased excitability of neurons which could favour LTP induction. Adapted from Maggio and Segal (2010).

5.2 Correlations between neuronal activity propagation through the amygdala network and anxiety phenotypes in the HAB/LAB mouse model

Understanding the contribution of neuronal network activity to the development of certain behavioural patterns could improve the ability to design more effective therapeutic approaches to treat affective and stress-related disorders. The present study demonstrates a correlation between neuronal activity flow through the amygdala and anxious behaviour, which appears not to be mediated by differences in GABAergic synaptic transmission. Another important finding is that anxiety levels can be modulated by alteration in environmental conditions, leading to a down- or up-regulation of the amygdala network activity studied. Exposure of animals to an environmental enrichment exerts an anxiolytic effect, which is associated with decreased activity propagation through the amygdalar network. On the contrary, chronic unpredictable stress induces an anxiogenic effect, correlated with stronger neuronal activity propagation. Based on the existing literature, it is likely that changes in the CRH system represent the mechanism underlying the environmentally induced alterations in neuronal network activity.

The unique anatomical circuitry of the amygdala has repeatedly been shown, in both animal and human studies, to be crucial for the regulation of anxiety responses (Pesold and Treit, 1995; LeDoux, 2000; Rauch et al., 2000; Stein et al., 2002; Anand and Shekhar, 2003; Phan et al., 2006; Siegle et al., 2007; Stein et al., 2007). Different lines of evidence corroborate that glutamatergic excitation and GABAergic feed-forward inhibition are in a sensitive equilibrium in order to appropriately regulate anxiety states. It is widely accepted that an enhancement in GABAergic feed-forward inhibition in the different amygdalar nuclei leads to an anxiolytic effect. Application of glutamate receptor antagonists into the BLA reduces anxious behaviour (Kim et al., 1993; Davis et al., 1994; Sajdyk and Shekhar, 1997). The BLA is under tonic GABAergic inhibition and a blockade of this inhibition causes increased anxiety levels, conditioned avoidance, and sympathetically mediated cardiovascular activation (Davis et al., 1994; Shekhar et al., 2003). Excitation of BLA neurons via stress-mediated CRH release enhances anxiety (Shekhar et al., 2003). All these studies point to the BLA as a regulatory subnucleus, which significantly contributes to the modulation of anxiety, autonomic responses, and the development of anxiety disorders.

In order to address the question if there are differences in amygdalar network activity between HAB, LAB, and control CD1 mice, I employed the VSDI technique for the experiments. The high temporal and spatial resolution of this method allows the propagation of evoked neuronal activity through the whole amygdalar network to be studied. We calculated ratios of neuronal activity between the main input area (LA) and two important output regions, namely the CeA and the BLA. These ratios gave us the ability to form a kind of input-output relationship of the amygdala. The main emphasis was to characterise circuit dynamics at the whole network level. In order to analyse “pathological modifications”, it is particularly important to understand how the activation of a complete brain “module” can lead to an altered behavioural outcome. Therefore, it is essential to not only analyse the characteristics of single neurons, but also the “whole aggregate response”, which is the result of complex cellular interactions within the neuronal network (**Figure 5-2**).

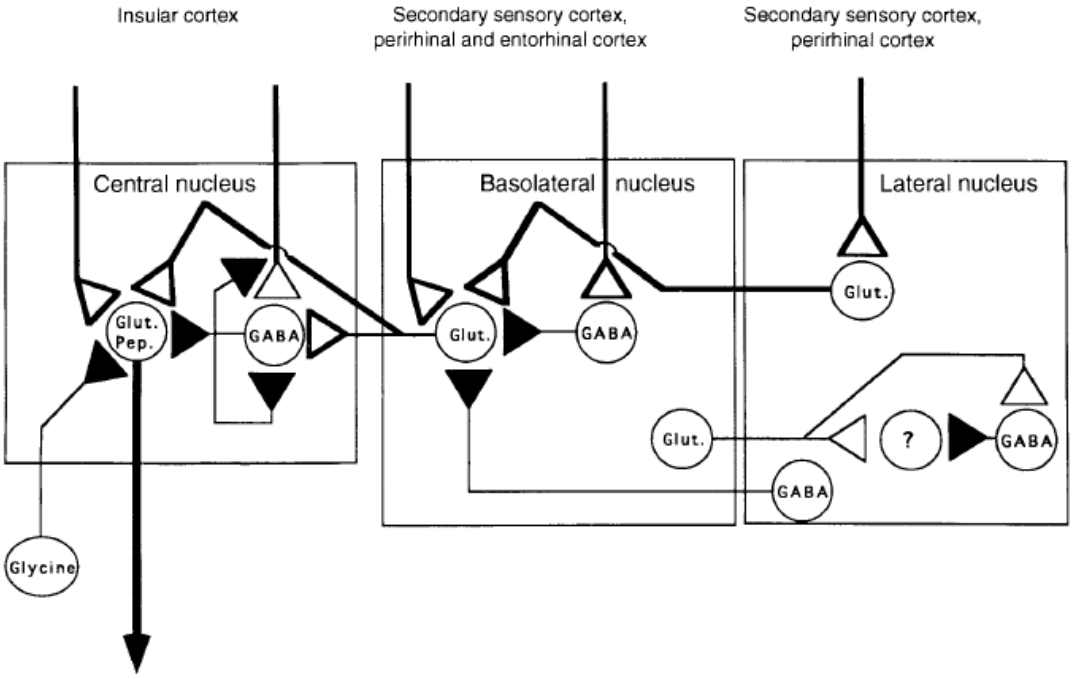


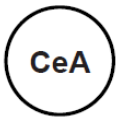
Figure 5-2: **Schematic diagram of principal connections within the three main areas of the amygdaloid complex based on *in vitro* electrophysiological recording and anatomical data**
Many other connections, mostly subcortical projections, are not depicted to simplify the illustration. It is important to characterise the whole network response, in order to understand the contribution of a particular brain area to the formation of complex behavioural patterns. Therefore, the focus of this study was not only directed to single synapses or a certain neuronal population, but also to the whole amygdala. Adapted from Davis et al. (1994).

The data presented here indicate that different anxiety levels can correlate with differences in the effectiveness of neuronal activity propagation through the amygdalar network. HAB animals which spent the shortest time on the open arms of the EPM, showed the highest BLA/LA and CeA/LA ratios. In contrast, LAB mice which spent the longest time on the open arms of the EPM, displayed no significant differences in the BLA/LA ratio compared to CD1 animals, but a reduction in the CeA/LA ratio. According to these results, it is clear that there are differences in intrinsic amygdalar network features between HAB, LAB and CD1 mice.

To investigate whether the differences in amygdalar network characteristics in the HAB/LAB mouse model might come from changes in GABAergic neurotransmission, I performed mIPSCs recordings in principal BLA neurons. The analysis of the amplitude and the frequency of the mIPSCs, however did not provide evidences for such alterations.

The enhanced activity propagation within the amygdala of HAB animals could contribute to the development of the high anxious behavioural phenotype. Considering the anatomical connections of the CeA with brain structures such as the

hypothalamus or the locus coeruleus (Davis et al., 1994), increased amygdala innervation of these structures may lead to the physiological concomitant effects of anxiety (sympathetic activation, increased respiration, activation of the HPA axis; **Figure 5-3**).



Anatomical target	Effect of amygdala Stimulation	Behavioural sign of anxiety
Lateral hypothalamus	Sympathetic activation	Tachycardia, galvanic skin response, paleness, pupil dilation, blood pressure elevation
Dorsal motor n of vagus Nucleus ambiguus	Parasympathetic activation	Ulcers, urination, defecation
Parabrachial nucleus	Increased respiration	Panting, respiratory distress
Ventral tegmental area Locus coeruleus Dorsal lateral tegmental n	Activation of dopamine, noradrenaline and ACh neurons	Behavioural and EEG arousal, increased vigilance
Pontine reticular formation	Increased reflexes	Increased startle
Paraventricular n (hypothal)	ACTH release	Stress response, CRH release

Figure 5-3: **Description of selected efferences of the central nucleus of the amygdala to hypothalamic and brainstem regions**
Possible contribution of these connections to specific behavioural characteristics of anxiety. Adapted from Davis et al. (1994).

A key question of this study was whether manipulations of environmental conditions can modify innate anxiety and amygdalar network activity. Answering this question is of particular interest, since a huge body of evidence indicates that the interaction between environmental parameters and genetic factors plays a critical role in the pathogenesis of stress-related mental disorders (Sztainberg et al., 2010). It has been shown that physiological and psychological health can benefit from environmental stimulation (Fox et al., 2006). Animals which maintained under enriched environment (E.E.), exhibit reduced levels of emotionality-related responses such as defecation, freezing, and anxiety (Chamove, 1989; Benaroya-Milshtein et al., 2004).

An anxiolytic effect of E.E. was tested on the EPM. HAB E.E. mice spent significantly more time on the open arms corroborating the anxiolytic effect of environmental stimulation. This anxiolytic effect was accompanied by a reduction of

the BLA/LA and CeA/LA activity ratios. Not all HAB E.E. animals responded to the environmental enrichment. These non-responders also did not show changes in BLA/LA and CeA/LA activity ratios. This suggests that the decrease in the activity propagation within the amygdala is causally linked to the anxiolytic effect of the E.E..

The molecular mechanisms underlying the effects of E.E. have been the focus of many studies investigating memory formation and neurological disorders (Nithianantharajah and Hannan, 2006). Concerning the anxiolytic effect of E.E., it has been hypothesised that E.E. causes an increase in the expression of glucocorticoid receptors in the hippocampus (Mohammed et al., 1993; Olsson et al., 1994), which enhances glucocorticoid sensitivity and, thus, negative feedback from the hippocampus to the HPA axis (Fox et al., 2006; Sztainberg et al., 2010). Furthermore, the anxiolytic effect of E.E. has been reported to be associated with a reduction in CRHR1 mRNA expression in the BLA (Sztainberg et al., 2010). These findings are in line with an other study showing that chronic administration of the benzodiazepine alprazolam decreases CRHR1 mRNA expression in the BLA (Skelton et al., 2000). Moreover, the anxiogenic action produced by CRH in rats is most likely mediated via activation of CRHR1 (Heinrichs et al., 1997; Sajdyk et al., 1999; Rainnie et al., 2004; Shekhar et al., 2005).

Prolonged exposure to stressful conditions leads to behavioural abnormalities inducing cognitive impairments as well as affective disorders. Early studies on the effects of stress demonstrated that changes in the amygdala are more likely to be responsible for the affective aspects of stress-related disorders (Vyas et al., 2004). The amygdala is crucially involved in emotional learning and represents a critical part of the neural circuitry which regulates stress response and anxiety (Vyas et al., 2004). In the present study, LAB animals exposed to chronic unpredictable stress (LAB Str.) confirm the anxiogenic effect of some stressful conditions. LAB Str. animals exhibited a stronger anxious behaviour then LAB animals, as revealed by behavioural testing on the EPM. These animals showed a significant increase in the BLA/LA activity ratio. This finding is in line with results from other studies demonstrating that chronic stress exposure can cause dendritic hypertrophy in the BLA, leading to an enhanced anxiety-like behaviour (Vyas et al., 2003). Two hours of immobilisation stress per day repeated for 10 consecutive days increase anxiety levels and dendritic growth in the BLA. The latter effect is accompanied by a robust increase in spine density across primary and secondary dendrites of BLA spiny

neurons (Mitra et al., 2005). Hence, the higher BLA/LA ratio in LAB Str. animals compared to the LAB counterparts could be caused by a stress-induced neuronal remodelling in the amygdala involving an increased spinogenesis (Mitra et al., 2005).

Although various neurotransmitter and peptide systems have been implicated in the regulation of the stress response, the CRH system seems to play an outstanding role in the control of biological cascades during stressful conditions (Vale et al., 1981; Rivier and Vale, 1983; de Kloet et al., 2005). Dysregulation of CRH-mediated stress responses can lead to the development of physiological and psychological effects and many studies have linked chronic hyperactivation of the CRH-system to stress-related emotional diseases such as depression and anxiety disorders (Holsboer, 1999a; Zorrilla and Koob, 2004; de Kloet et al., 2005; McEwen, 2005; Holsboer and Ising, 2008). Data from studies using animal models indicate anxiogenic-like behavioural effects after CRH administration and an anxiolytic-like impact of selective CRHR1 antagonists. These findings, point to an important role of CRH in the pathophysiology of anxiety disorders (Holsboer, 1999a; Zorrilla and Koob, 2004; de Kloet et al., 2005; McEwen, 2005; Holsboer and Ising, 2008). Several anatomical and behavioural studies could show that the BLA is strongly involved in CRH-induced anxiety-related physiological and behavioural responses. Administration of CRH into the BLA induces a dose-dependent and long-lasting anxiety-like response, mediated through the CRHR1 (Gehlert et al., 2005). Contrary knockdown of CRHR1 in the BLA provided an anxiolytic-like phenotype (Sztainberg et al., 2010).

Environmental factors could exert their anxiolytic or anxiogenic effect through a modulation of the CRH system. Innate differences in the CRH system in HAB and LAB animals might thus contribute to the different anxiety phenotypes. In a previous study performed in brain slices (Avrabos, 2008), CRH (125 nM) caused a stronger increase in the excitability of BLA neurons in HAB mice compared to LAB animals. This difference in CRH sensitivity between HAB and LAB mice might result from a difference in the CRHR1 density, with HAB animals having a higher expression rate of CRHR1 in BLA neurons. Chronic hyperactivation of the CRH system in HAB animals could result in an increased synaptic density and hypertrophy of BLA neurons. This may explaining the stronger neuronal activity propagation thought the amygdalar network of these mice. Activation of CRHR1 and remodelling of BLA neurons could also be the reason for the increase in anxiety-like behaviour and

activity propagation observed in LAB Str. compared to LAB mice. The anxiolytic effect of E.E. may be attributed to a downregulation of the CRHR1 mRNA in the BLA, as it is described by Sztainberg and colleagues (Sztainberg et al., 2010). This could explain the weaker neuronal activity propagation in the amygdala of HAB EE compared to HAB and HAB EE non responders.

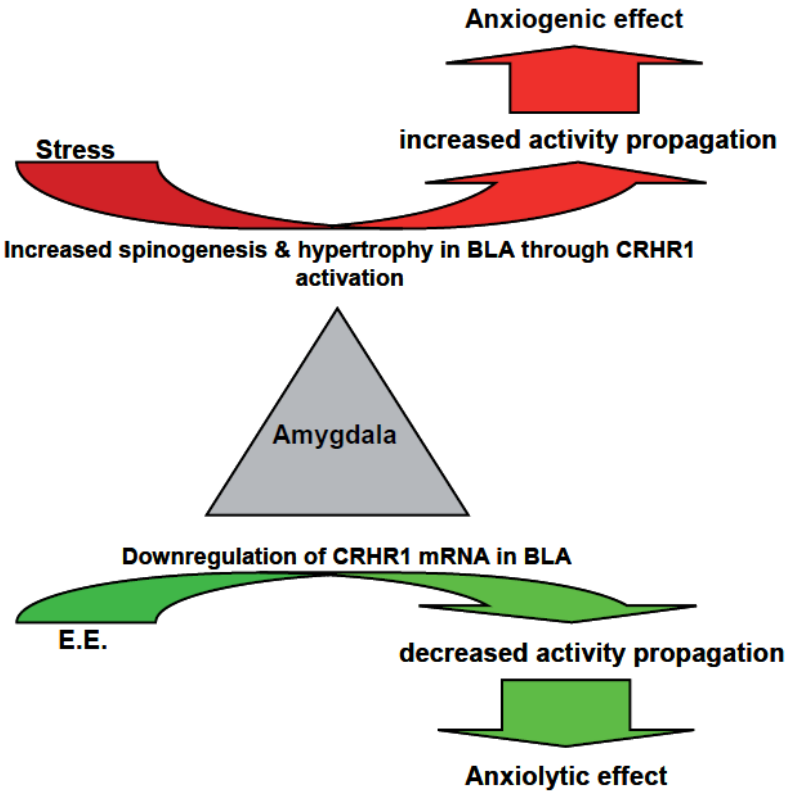


Figure 5-4: **Schematic diagram of possible mechanisms involved in the regulation of anxiety-like behaviour**
The CRH system could act as an interface between environmental factors and regulation of neuronal activity. The amygdala, and especially the BLA nucleus, shows a high expression of CRHR1. According to the results of other studies, stress seems to mediate the anxiogenic effect at least in part by activation of the CRHR1 in the BLA. Exposure to chronic stressful conditions leads to remodulation of dendritic spines within the amygdala and to a hypertrophy of principal BLA neurons. These observations are in line with findings of the present study. Exposure to stressful conditions induced a clear anxiogenic effect and an increase in activity propagation through the amygdala of stressed animals. On the contrary, animals maintained in an enriched environment showed reduced anxiety levels and a decrease in the activity propagation. Previous studies support the view that E.E. exerts his anxiolytic effect via a downregulation of CRHR1 in the BLA.

Despite the need of further investigations, the present study revealed differences in the efficiency of neuronal activity propagation through the amygdala network, which strongly correlates with different levels of anxiety. The data also provide evidence that environmental factors are able to counterbalance behavioural phenotypes through the modulation of neuronal network activity in the amygdala.

According to recent studies, a modulation of the CRH system within the amygdala could significantly contribute to the appearance of altered anxiety phenotypes.

6 List of abbreviations

ACSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone/corticotrophin
AP5	2-amino-5-phosphonovalerate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AVP	arginin-vasopressin
BAOT	bed nucleus of the accessory olfactory tract
Bic	bicuculline
BLA	basolateral amygdalar
BNST	bed nucleus of the stria terminalis
CA1	cornu ammonis 1
CA3	cornu ammonis 3
CaMKII	α-calcium-calmodulin-dependent protein kinase II
CCD	charge-coupled device camera
CeA	central amygdala
CRH	corticotropin-releasing hormone
CRH_{R1}/CRH_{R2}	corticotropin-releasing hormone receptor 1/2
CoA/CoP	anterior and posterior cortical nucleus
DG	dentate gyrus
DH	dorsal hippocampus
Di-4-ANEPPS	di-4-aminonaphtylethenylpyridinium
E.E.	enriched environment
EPM	elevated plus maze
FDS	fast depolarization-mediated VSDI signal
fEPSP	field excitatory postsynaptic potential
ΔF/F	fractional change in fluorescence
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GR	glucocorticoid receptor
HAB	high anxiety-related behaviour
HAB E.E.	HAB enriched environment mice
HAB E.E. n.r.	HAB E.E. not responding to the enriched environment
HFS	high-frequency stimulation
HPA	hypothalamic-pituitary-adrenocortical axis
LA	lateral nucleus
LAB	low anxiety-related behaviour
LAB Str.	LAB stress mice
LTP	long-term potentiation
LSv	ventral part of the lateral septum

MAPK	mitogen-activated protein kinases
mEPSCs	miniature excitatory postsynaptic currents
mIPSCs	miniature inhibitory postsynaptic currents
mPFC	medial prefrontal cortex
mpPVN	medial parvocellular subdivision of the paraventricular nucleus
MPOA	medial preoptic area
MR	mineralocorticoid receptor
NMDA	*N*-methyl-D-aspartate receptors
PAC	periamygdaloid cortex
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PPD	paired pulse depression
PPF	paired-pulse facilitation
PPR	paired-pulse ratio
PVN	paraventricular nucleus of the hypothalamus
RMI	structural magnetic resonance imaging
RMP	resting membrane potential
ROIs	regions of interest
SAS	sympatho-adrenergic system
USV	ultrasonic vocal calls
VH	ventral hippocampus
VSDI	voltage-sensitive dye imaging

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8 List of figures

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10 Publications

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12 Declaration / Erklärung

Hiermit versichere ich, Dass ich die vorliegende Dissertation selbständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt habe. Alle Ausführungen, die wörtlich oder sinngemäß übernommen wurden, sind als solche gekennzeichnet.
Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die vorliegende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

München, den 07.05.2012

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