Extinction Learning in Mice

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Ort, Datum Unterschrift Wolfgang Plendl

“Da es sehr förderlich für die Gesundheit ist,
habe ich beschlossen, glücklich zu sein.”
François Marie Arouet

Für das seltenste Tier der Welt,
für meine Eltern und meinen Bruder

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1. Abstract

Anxiety disorders are characterized by an exaggerated fear response towards a non-harmful stimulus or situation. Exposure therapy is an effective treatment and consists of repeated non-reinforced exposures to the stimulus. Currently the index of beneficial learning is the decreased level of fear at the end of an exposure session. For patients this therapy is emotionally very demanding and dropout rates are high. In addition, many patients are prone to relapse in a context and time dependent manner.

In rodents, the explicit model for exposure therapy is extinction learning. After initial pairing of a previous neutral stimulus (tone) with a negative outcome (shock), animals will show a directed fear reaction (conditioned response) during re-exposure. Multiple re-exposures of the animal to the stimulus without further reinforcement lead to a gradual decay of fear (extinction training). This fear decay is shown to be based on relearning processes. Over time the original memory trace of the tone predicting the shock is opposed and inhibited by the new association that the tone does not predict the shock any longer.

The focus of this work was to dissect extinction learning at the behavioural and the molecular level in a mouse model of conditioned fear. We want to critically revise nomenclature and to provide mechanistic insights and possible transfers to clinical application and to increase the persistence of fear extinction.

In the first set of experiments, we showed that fear deacy during exposure (within-session) to a permanent stimulus is mediated by the endocannabionid system, involving glutamatergic neurons but not stress hormone signalling.

In the second set of experiments, we challenged the view that the fear level at the end of an exposure session (within-session extinction) is the indicator for the strenght of between-session extinction. By comparing different exposure approaches, we were able to show that the decline of fear during exposures did not predict formation of between-session extinction, nor was it a prerequisite. Multiple short and unpredictable exposures to the stimuli were most effective in producing between-session extinction, compared to a permanent exposure. Starting from our results obtained in the first set of experiments we further demonstrated that the endocannabinoid system is essential for within-session extinction but not for between-session extinction. The dissociation of within-session and between-session extinction allowed us to pin down the basolateral amygdala, the cingulate cortex and the dentate gyrus as possible centers of relearning by measuring c-Fos immunoreactivity in various brain areas.

Even after successful completion of extinction training subjects are prone to relapse. Reconsolidation, elicited by re-exposure, was recently identified as a phase where the original fear memory becomes vulnerable again to selective modifications. Therefore in the third set of experiments, we tried to interfere with reconsolidation in order to achieve a long lasting decrease in conditioned fear. By inhibition of intracerebral activity of PKC, within- and between-session extinction were facilitated and moreover renewal and spontanous recovery, as a control for relapse, were abolished. We showed for the first time that reconsolidation can be manipulated without having any negative influences on memory consolidation during extinction training. By means of c-Fos immunohistochemistry we were able to identifiy the cin-

gulate cortex as a candidate region to mediate reconsolidation processes.

In summary this study demonstrates for the first time that endocannabinoid signalling at glutamatergic neurons in the forebrain is essential for within-session extinction but also independent of CRH signalling and corticosterone secretion. Second, within-session and between-session extinction can be dissociated at the behavioural, anatomical and molecular level. Third, by blocking reconsolidation, the conditioned fear memory trace can be degraded during extinction, thus resulting in a lasting decrease of fear. These findings challenge a number of facts, which have been taken for granted in current psychotherapy practice. We hope that this thesis will contribute to develop new therapeutic approaches for the treatment of phobias or trauma-related anxiety disorders.

2. Synopsis

2.1. Introduction

2.1.1. Phobias and trauma related anxiety disorders and exposure therapy in humans

Anxiety disorders were descibed as early as the fourth century B.C. in the "corpus hippocraticum", but their impact on society was not fully appreciated until some decades ago. The first specialists in psychiatry were mainly concerned with the description and classification of psychotic disorders. As a result the development of the field of anxiety disorders was left to specialists in internal medicine and neurology, like S. Freud (Pichot, 1994). Since then psychologists and psychiatrists have tried with pharmacological and phsychological treatments to change undesired emotional memories.

Excessive fear is a hallmark of a variety of disabling anxiety disorders . However, most treatments only eliminate fearful responses, leaving the original fear memory intact. Once aquired, emotional memory is very long lasting. From an evolutionary point of view, it is extremely important to never forget the most significant events in life. In order to survive, individuals require mechanisms that avert threat. To this end, defensive responses, including fear, evovled. Fear is an emotional state which can appear without previous experience of threat and whose shape can be altered by learning to produce new patterns of defensive behaviour. This, however, can also be harmful and maladaptive, such as in some trauma victims who suffer from dreadful memories.

The development of reliable diagnostic criteria set the stage for a critical appraisal of the magnitude of the problem of anxiety disorders. According to statistical evaluations over 80 million women and men of all ages in the EU are estimated to suffer from this mental disorder. Considering ICD-10 and DSM-IV criterion there is clear evidence that more than one third of the adult European population is or has been affected, or 50% of the population, if lifetime risk is considered (Olesen and Leonardi, 2003; Andlin-Sobocki et al, 2005). The enourmous suffering that can be associated with theses disorders is self evident. Typically school, academic career, social environment, somatic health and also social life are heavily impaired. Panic disorders, or phobias can emerge already in childhood and adolescence. They are also a major cause for premature mortality, either caused by suicide or by the effects of complications arising from associated risk factors.

The most effective strategies for phobias and trauma related anxiety disorders include exposure based interventions and pharmacotherapy (Norton and Price, 2007). It has been generally assumed that combining two effective interventions should result in greater gains than only one. Surprisingly some clinical trails showed that patients who received a combination of cognitive behavioural therapy and anxiolytics showed a loss of efficacy once the medication was discontinued (Otto et al., 1995; Gelder et al., 1998; Barlow et al., 2000) A specific reason for this not yet known. Discontinuation of medication may alter the internal state which may in turn interfere with learning that took place during a different internal state.

Or it is possible that the fear reduction is attributed to the fear expression, based on pharmacological disinhibition, rather than on a learning effect. It is also possible that the anxiolytic effect of pharmacotherapy inhibits the full activation of the fear structure, leading to a suppression of emotion processing of the feared stimuli. Thus the combined treatment may not outperform cognitive behavioural therapy alone, but may even place patients at relatively greater risk of relapse. However, it has been shown that pharmacotherapy aimed not at treating symptoms of anxiety but instead at improving the relearning that takes place in exposure therapy might acutally improve the treatment effectiveness. When D-Cycloserine, a partial NMDA agonist, is combined with exposure the beneficial effect, of exposure, is significantly enhanced. This has already been shown for a variety of psychiatric diseases like phobia and other anxiety disorders (Ressler et al, 2004; Hofmann et al, 2006; Kushner et al, 2007).

The effect of D-Cycloserine was not first reported in humans, but rather in a rodent model of exinction of conditioned fear (Walker et al, 2002). Recently it was also shown in rodents that fear can be reduced for a long period of time not only by pharmacological means but also by modification of behavioural protocols (Monfils et al, 2009). The strategies of this model were readily transferred to human subjects and the findings proved consistent (Schiller et al, 2010). This examples show that results obtained in rodent models for conditioned fear and extinction prove applicable also in human populations.

2.1.2. Extinction as explicit model of exposure therapy in rodents

I.P. Pavlov (1849-1936) was the first to show the learned association between a unconditioned stimulus (US) and a conditioned stimulus (CS) that leads to a reproducable conditioned response (CR). Therefore this procedure is called "Pavlovian Conditioning", or classical conditioning (Pavlov, 1927). In classical conditioning paradigms a subject has no control over presentations of the US by its own behaviour and the stimulus-stimulus association (CS-US) is explicit and fixed. After repeated exposure to the CS without US the CR diminishes over time. This decrease is consistent across paradigms and species and is referred to as extinction. It was shown that extinction is not due to forgetting of the original US-CS association.Extinction rather is an active learning process that requires repeated training to develop. Sometimes extinction is also described as an unlearning process dependent on violation of the CS-US expectancy (Rescorla, 1988).

However this unlearning view has been challenged repeatedly during the last years. It was shown that extinction is not simply forgetting the original association, because, first the CR does not dissapear over time without reexposure to the non-reinforced CS. Second, the expression of the extinguished fear vanishes over time and the CR reappears (spontanous recovery) after a appropriate time without reexposure (Robbins, 1990). The fact that extinction is context dependent (renewal) is a third argument against unlearning . If an association is aquired in context A and extinction is performed in context B animals will only show reduced fear responses in context B but not in context A when retested(Bouton and Bolles, 1979). Both, renewal and spontaneous recovery demonstrate that the original CS-US association stays intact during extinction procedures. Because of these findings a new theory was developed that extinction is a form of new learning that opposes the expression of the CR

(Bouton, 1993). During extinction a second, inhibitory, association of CS- no US is formed and strengthened, which directly acts in parallel to the conditioned association which predicts the US after the CS onset. This inhibitory learning theory is able to explain most of the basic behavioural features of extinction to this day (Ehrlich et al., 2009).

However some authors claim that not only associative processes are involved in extinction (Kamprath and Wotjak, 2006). Extinction of conditioned fear seems to include habituation (Harris, 1941; McSweeney and Swindell, 2002). When a stimulus of the same characteristics is presented in a uniform way for a long time, responsivness of a subject to the stimulus will be decreased. Also the strength of the US during acquisition influences the intensitiy of fear displayed during later exposures. Therefore extinction obviously involves many different factors which could influence the short-term and the long-term development of behaviour during repeated exposures towards the conditioned stimuli.

In rodents fear is defined as a cessation of all bodily movements except for respiraton (freezing) increase in the amplitude of acoustic starte responses, changes in blood pressure, ultrasonic distress calls, place avoidance, or other behavioural changes in presence of the CS. In most studies during conditioning, a tone is paired with a mild foot shock. For reexposures animals are transferred in a defined context and, for practical reasons, freezing to the CS is quantified.

Defensive behaviours consist basically of three categories relevant for the entire animal kingdom, including humans: freezing, fleeing and fighting (Eilam, 2006). These defense reactions reflect the integrated final output of different brain systems. In order to trace processes involved in the control of defensive responses, it is necessary to standardize the fear response and to control its releasing stimuli. For this, a simple response elicited by a single stimulus, such as a certain odour or noise, is best fitting. Recently it was suggested that defensive behaviour is two dimensional. Defensive reactions can vary dependend on the distance to the threat (defensive distance) and the avoidability (defensive direction) (McNaughton and Corr, 2004). In people suffering from phobias or trauma related anxiety disorders there seem to be a shift in balance between defensive reactions. They tend to show inadequate reactions in realtion to the defensive distance or defensive reaction. For example, people already show panic-like reactions while the fear eliciting stimulus is still far off. During exposure therapy the defensive distance is deliberately minimized to evoke a maximum emotional reaction. Therefore, by regulation of the defensive distance certain behavioural defensive responses can be evoked, for example, freezing in rodents. Since inhibitory interactions between brain structures mediating defensive behaviour can rapidly switch as the threatening situation varies (Gonzalez et al., 2003) a fixed, controlled environment has to be provided throughout extinction experiments. This allows reproducability of a distinct defense behaviour and comparable involvement of certain brain regions.

2.1.3. The endocannabionid system and fear expression

In recent years the endocannabionid system (ECS) has emerged as a intrinsic neuromodulatory system involved in a magnitude of physiological processes, including the regulation of fear responses. The system consists of two receptors, endocannabionid receptor type 1 (CB1) and type 2 (CB2), (Matsuda et al., 1990; Munro et al., 1993), endocannabi-

noids, namely N-arachidonoylethanolamide (AEA) and 2-Arachidonylglycerol (2-AG) (Devane et al., 1992; Mechoulam et al., 1995) and associated enzymatic machinery for synthesis (enzymes of the lipid metabolism) and degradation (FAAH: fatty acid amino hydrolase, MGL:monoacylglycerol lipase) of endocannabionids (McKinney and Cravatt, 2005; Dinh et al., 2002). Both, CB1 and CB2 receptors, were found following the identification of the main psychoactive compound of marijuana, delta9-tetrahydrocannabinol (THC) (Mechoulam and Gaoni, 1965), which bind the receptors directly. The CB2 receptor is almost exclusively expressed on cells of the immune system and only in restricted areas in the central nervous system (Skaper et al, 1996; Van Sickle et al, 2005). In contrast, the CB1 receptor is among the most abudant expressed G protein-coupled receptors in the central nervous system (Herkenham et al., 1990; Howlett et al.,1999; Freund et al., 2003; Piomelli, 2003). At subcellular levels most CB1-receptors are found on axon terminals of GABAergic and glutamatergic syn-

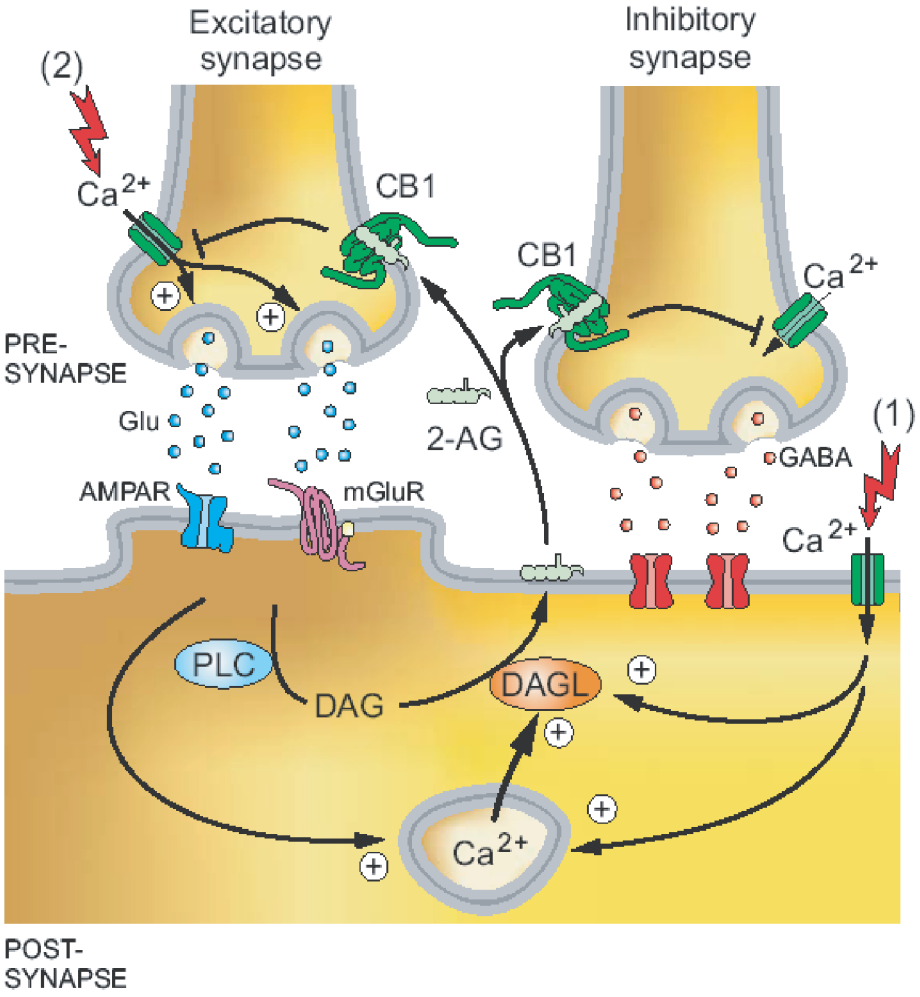

Figure 1: Endocannabionid mediated short-term plasticity
Endocannabinoid production can be incuded by two processes: (1) post synaptic depolarization induces Ca2+ via voltage-gated calcium channels (VGCC); (2) brief excessive stimulation or mGluR activation by endocannabinoids (e.g. 2-AG) will activate presynaptic CB1 receptors, leading to a short term depression of neurotransmitter release. (Adapted from Lutz and Marsicano, 2006)

apses (Monory et al., 2006) and their activation leads to presynaptic inhibition of transmitter release (Schlicker and Kathmann, 2001) by hyperpolarisation of the neuron. Endocannabinoids are synthesised „on demand" in particular conditions, such as during anxiogenic or fearful situations (Wotjak, 2005; Marsicano and Lutz, 2006) and excert their effect on transmitter release in a retrograde fashion (Figure 1).

Clearance of endocannabinoids from the extracellular matrix is rapidly accomplished via a high affinity, selective and temperature dependent process, which suggests a carrier mediated transport by which diffusion is faciliated. Also considering the fast kinetics of endocannabionid synthesis, release and degradation, there seems to be a closely drawn time and location dependency of effects (Piomelli, 2003). This could explain why exogenous cannabionid application could not mirror endocannabinoid effects in some cases, because in this case spatial and temporal connections are violated (Marsicano and Lutz, 2006).

It has been shown that the endocannabionid system, and more specifically the CB1 receptor is not only involved in neuroendocrine responses (Steiner et al.., 2008), but also in processing of acquired fear, including fear conditioning and extinction. In 2002 Marsicano, Wotjak et al. showed, in a rodent model, that the exinction of conditioned fear is controlled by the endocannabionid system. In this study, mice lacking the CB1 receptor were able to show normal acquisition but failed to show reduction of fear during several reexposure sessions. This was also reproducable by blocking the function of the receptor pharmacologically. Later on it was shown that this effect depends mainly on non-associative factors (habituation) in extinction (Kamprath et al., 2006). These findings have some striking similarities when linking them to pathological fear and anxiety in humans (LeDoux, 2000). As mentioned before, fear regulation during exposure to a feared stimulus is essential for adequate behaviour and is often disturbed in humans with anxiety disorders. Several experiments showed that, in rodents, fear adapation could be accelerated by additional CB1 stimulation (Chhatwal et al., 2005.; Pamplona et al., 2006; Varvel et al., 2007). Also CB1 activation seems to have differential effects on distinct phases of memory consolidation, dependent on the pharmacons used for CB1 activation (Lin et al., 2006).

Marijuana and its derviates have been used in humans for medicinal purpose for quite a long time. The shown results, obtained in rodents, suggest that they may have also therapeutic potential in anxiety related disorders. The endocannabinoid system could represent a target for diseases with inappropriate fear adaptaion (Haller et al., 2004a + 2004b). A promising compound could be cannabidiol, a non psychotropic constituent of cannabis. It has already been shown to reduce anxiety in humans (Zuardi et al., 1982; Williamson and Evans, 2000). However, cannabidol signalling does not involve CB1-receptors (Moreira et al., 2006) but it seems to mediate its effects by opioid receptors (Kathmann et al., 2006).

The endocannabionid system has shown to be a promising target for new therapeutics augumenting treatment for anxiety disorders but still many things are unknown.

2.1.4. Fear expression and Stress

The CB1 receptor is expressed in various regions of the brain (Herkenham et al., 1990) and plays a important role not only in fear expression, but also in locomotion, pain perception, feeding, and more (Porter and Felder, 2001). In addition, the endogeneous cannabinoids affect the production of various hormones, including growth hormones, thyroid hormones and glucocorticoids (Brown and Dobs, 2002) and also play a role in the control of the hypothalamo-pituitary-adrenocortical (HPA) axis (Weidenfeld et al., 1994; Pagotto et al., 2001, Di et al., 2003; Barna et al, 2004; Steiner et al, 2008). At the level of the HPA axis endocannabionids seem to interact with the corticotropin-releasing hormone (CRH) system (Wenger et al., 1997) and a functional interaction between these two systems could be shown in multiple studies (Corchero et al., 1999; Cota et al., 2003; Haller et al., 2004; Hermann and Lutz, 2005). Given the key role of CRH and the HPA axis in the regulation of stress and anxiety related responses (DeKloet, 2003; Muller et al., 2003), there may be a functional relationship between the effects of cannabinoids on anxiety and the HPA axis.

CRH is a potent mediator of endocrine, autonomic and immune responses to stress (Holsboer and Barden, 1996) and has been implicated in the modulation of a wide range of different types of behaviour, including arousal and anxiety-related behaviour (De Souza, 1995). CRH activates two distinct types of G protein-coupled receptors, CRHR1 and CRHR2, which are present with different expression pattern in the brain. CRHR1 is expressed at different levels in neocortical areas, the hippocampus, basolateral amygdala, ventral tegmental area, pontine gray, lateral dorsal tegmentum, pedunculopontine tegmental nucleus and anterior pituitary, which is not a part of the brain (Potter et al., 1994; Sanchez et al., 1999; Van Pett et al., 2000). In some areas it is differentially coexpressed with the CB1 receptor, in particular, in olfactory regions, some cortical and limbic areas and some hypothalamic and thalamic nuclei (Hermann and Lutz, 2004). CRHR2 is found in the lateral septum, the bed nucleus of the stria terminalis, medial and cortical nuclei of the amygdala, dorsal raphe and in the nucleus of the solitary tract. CRH is a high affinity ligand for CRHR1m but binds CRHR2 with a much lower affinity, where the primary ligand is urocortin. CRH receptors are linkked to different intracellular singalling pathways, which differ between brain regions, thus resulting in a neuroanatomically signaling specifity of CRH receptors (Artz and Holsboer, 2006).

Stress involves a large number of neuronal circuits, including the prefrontal cortex, the hippocampus, the amygdala, the septum and the hypothalamus. Activation of these circuits by stress results among other things, in the release of corticotropin-releasing hormone. CRH is released from parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN) (Swanson and Simmons, 1989) into portal vessels and activates the HPA axis by triggering the immediate release of adrenocorticotropic hormon (ACTH) from the anterior pituitary (DeSouza, 1995). This in turn stimulates the cortex of the adrenal gland to synthesize and release glucocorticoids.

Studies in rodents have shown anxiogenic-like effects of CRH administration (Dunn and Berridge, 1990). Moreover, brain structures with high expression of CRHR1, such as the amygdala, the BNST, prefrontal cortex and the periaqueductal grey have been strongly implicated in mediating emotional processes such as fear and panic (Coplan and Lydiard, 1998). It was shown that intracerebroventriculary (ICV) administration of CRH increased condi-

tioned fear to a conditioned stimulus (Radulovic et al., 1999). In addition, another study showed that injection of a CRH receptor agonist directly into the basolateral amygdala produced a very potent anxiogenic-like effect (Sajdyk et al., 1999). This suggests that CRH may induce fear, or anxiety, related behaviour in rodents by activation of CRH1.

Across species THC and CB1 receptor agonists and antagonists stimulate corticostrone secretion, irrespective of sex or route of administration. It has been proposed that the HPA axis stimulating actions of CB1 receptor agonists and antagonists are centrally mediated and result in the activation of neurons in the paraventricular nucleus (PVN) of the hypothalamus. Observations imply a model for glucocorticoid-mediated fast feedback-regulation in these neurons, which is based on endocannabionid signalling (Di et al., 2003). Increased corticosterone levels feek back onto membrane bound and G-protein-coupled glucocorticoid receptors in PVN neurons. Thereby local synthesis of anandamide and 2-AG is triggered, which travel retrogradely and bind to presynaptic CB1 receptors on glutamateric afferences of the PVN and result in a reduction of glutamate release.. This results in a reduced activation of the CRH-containing PVN neurons and, in consequence, in a re-setting of the HPA axis following stressor exposure (for review see Steiner and Wotjak, 2008).

These studies lead to the assumption, that, because of the close relationship to the endocannabionid system, CRH mediated effects could play a role in fear adaption during exposure to a conditioned stimulus. By investigating this relationship potential new targets for phamacological augumentation of exposure therapy could come apparent.

2.1.5. Second messengers in cell to cell signalling

Overall there are two types of receptors in the cell membrane neurotransmitters can bind to. The first group are the ionotropic receptors. The receptor is an integral part of the same macromolecule that forms the ion-channel it regulates. Upon binding the neurotransmitter the receptor undergoes a conformational change that results in the opening of the channel. These types of receptors have a very short reaction time and therefore serve as the first instance of cell signaling. The second group of receptors are the metabotropic receptors, where the receptor and the effector functions are carried out by different proteins. This receptor type consists of different subgroups, including the G-protein coupled receptors and the tyrosin-kinase receptors. The G-protein coupled receptors are coupled to a guanine nucleotide binding protein (G-protein) (Figure2). The intracellular tyrosin kinase domaine autophosphorylates itself after extracelluar ligand binding, allowing it to bind and also phosphorylate other intracellular proteins, including other kinases. The response kinetics of metabotropic receptors are much slower and due to their indirect action pose the second instance of cell signaling, because of the indirect action.

The best known targets for G-proteins are the cyclic adenosine monophosphate (cAMP) and the phosphoinositol (IP) second messenger pathways. In the cAMP pathway a stimulatory G-protein (G_s) phosphorylates the adenylyl cyclase which in turn catalyses the conversion of ATP to cAMP. cAMP in turn activates the cAMP-dependent protein kinase (PKA). In the inositol pathway a receptor associated G_q protein activates the phospho lipase C (PLC), which cleaves phosphatidylinositol-1,4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and

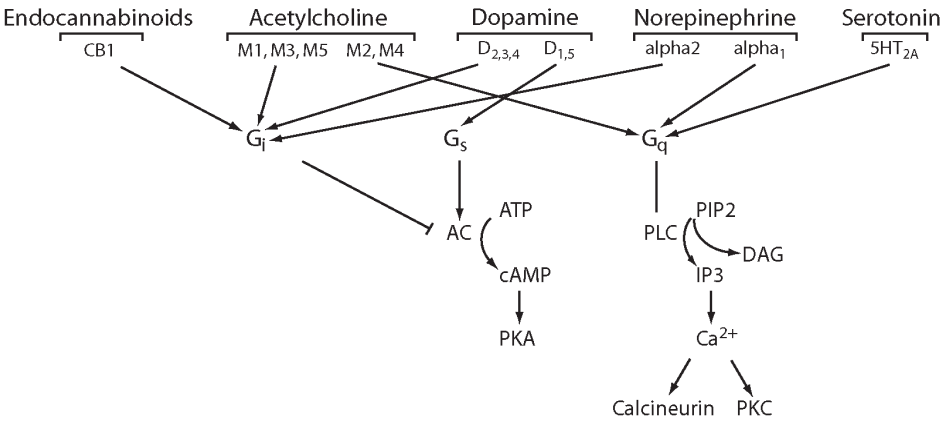

Figure 2: Selected modulatory neurotransmitters and their receptors that couple to either G_i, G_s, or G_q

Receptors that are necessary for working memory initiate second messenger pathways. G-Proteins have distinct effects on intracellular signalling. G_s enhances cAMP concentrations via the adenylylcyclase (AC) resulting in protein kinase A (PKA) activation. G_i reduces cAMP levels. G_q proteins couple to phospho-lipase C (PLC), causing increases in intracellular diacylglycerol (DAG) and inositol triphosphate (IP_3). (Adapted from Dash et al., 2007)

inositol-1,4,5-triphosphate (IP_3). The DAG activates the protein kinase C (PKC), whereas IP3 mobilized intracellular Ca^{2+} stores by docking to a receptor in the endoplasmatic reticulum. PKC is synergisticly activated by DAG and Ca^{2+}. Both of these second messengers control downstream processes which leads to long term changes of synaptic excitability, like transcription and translation. Moreover they can influence signal input to neurons by translocation, internalisation or enhanced recruitment of receptors to the synaptic cleft. So it is not surprising that both messenger systems are also critically involved in learning and memory processes (for detailed information see Cammorota et al., 2005; Dash et al., 2007). Taken together, the second messenger systems integrate a vast amount of receptor signals and translate them straightforward into long and short term adaptive neuronal changes.

The above mentioned CB 1 belongs to the metabotropic group. Interestingly, not a stimulatory, but an inhibitory G-protein (G_i) (Howlett et al., 1999) is coupled to the receptor and, by that, it seems to downregulate synaptic transmitter release. Activation of the receptor leads to the inhibition of adenylyl caclase, activation of extracellular signal regulated kinases 1 and 2, increased activation of inwardly rectifying K^+ channels (GIRK) and inhibition of voltage-gated calcium channels (Howlett et al., 2002). Some details about interactions between the endocannabinoid system and the PKC are also known. Data shows that protein kinase C is activated by cannabinoids, like THC, cannabinol and cannabidiol, in vitro (Hillard and Auchampach, 1993). Also arachidonic acid, which is the precursor of Anandamide and 2-AG, interacts with PKC and seems to increase its activity (Nishizaki et al., 1999), which leads to facilitation of hippocampal synaptic transmission. Moreover PKC seems to disrupt endocannabionid signaling by direct phosphorylation of the CB1 receptor in the third intracellular loop, which is important for G-coupled signalling (Garcia et al., 1998).

Combined findings suggest that PKC activity could be involved in a regulatory feedback mechanism for the endocannabionid system. It is possible that influencing this regulatory mechanism and not the the endocannabionid system directly could lead to new insights in extinction, or ,respective, exposure therapy.

2.1.6. Consolidation and Reconsolidation

Memory consolidation is the process by which the long-term memory of an item stabilizes and becomes resistant to certain sources of interference, such as distracting stimuli, electric shocks or metabolic inhibitors (McGaugh, 2000; Dudai). The question of whether is any item in long term memory consolidation occurs just once, or wether memories reconsolidate each time anew after retrieval, (Dudai, 2004), has potential practical implications. Evidence indicates that after activation, items in long term memory may regain transient sensitivity to consolidation blockers (Nader, 2000; Taubenfeld et al, 2001; Eisenberg et al., 2003). The stability of a memory in general, has high relevance in the attempt to understand and develop treaments for certain pathologies that involve persistent activation of undesired memories.

A well-established type of consolidation blocker used in memory consolidation experiments are the protein synthesis inhibitors (Davis and Squire, 1984). But none of these agents is completely specific (Kyriakis et al., 1994). In recent years, a lot of attempts have been made to identify molecular mechanisms of reconsolidation by inhibitors of cellular targets ranging from receptors and channels to intracellular signal transduction cascades, transcription factors and immediate early genes (for review see Dudai and Eisenberg, 2004). There are also studies which indentify differences between consolidation and reconsolidation, for example, there is a dissociation between the roles of BDNF and Zif268 (Lee et al., 2004). It is not yet known whether such differences are fundamental or emerge only in specific memory types or protocols. Although the pharmacological and molecular data suggests that consolidation and reconsolidation share many mechanistic components, there are, for sure, also differences. By finding targets which are selectively involved in reconsolidation processes but not consolidation, a specific fear memory knock-down should be possible.

2.1.7. Aims

The aim of this thesis was to identify new approaches to exposure therapy.

Our working hypotheses have been as follows:

(1) Endocannabinoids excert their acute effect on fear extinction via glutamatergic transmission

(2) Corticosterone secrection plays an important, though indirect, role in fear extinction by stimulating endocannabinoid signalling

(3) A permanent stimulus exposition during training is not efficient in building up between session extinction

(4) Acute fear relief is not the prerequisite for relearning of the tone-shock associationt

(5) Inhibition of PKC activity promotes fear extinction by inhibition of reconsolidation

2.2 Discussion

In the first part of the thesis we were able to show, that endocannabionids mediate acute fear adapation independently of the CRH system (manuscript 1).We sensitized mice deficient for the endocannabinoid 1 receptor (CB1 $^{-/-}$) and their wildtype littermates (CB1$^{+/+}$) to various footshock intensities and were able to show that only with high intensities the CB1$^{-/-}$ mice showed a sustained fear response to the tone. Moreover, when testing conditional mutants with a deletion of the CB1 receptor either in principal neurons of the forebrain (CaMK-CB1$^{-/-}$) or in cortical glutamateric neurons (Glu-CB1$^{-/-}$), all display a similar phenotype compared to CB1$^{-/-}$ animals. This indicates, that glutamateric transmission, under endocannabionid control, is essential for acute fear adaptation after traumatic experiences. By these findings we were able to verify our working hypothesis 1.

To find out, wether this inability for fear adaptation, after high intensity shocks, is related to the experienced stress we blocked the CB1 receptor pharmacologically by rimonabant (SR141716) in mice deficient for the corticotropin releasing hormone receptor type 1 (CRHR1 $^{-/-}$) and type 2 (CRHR2 $^{-/-}$) and their wildtype littermates. All genotypes showed a sustained fear response. These results suggests that the fear alleviating effect of the endocannabionid system is not connected to the CRH system. Moreover ,because CRHR1-/- mutants are known to be severely impaired in stress induced corticosterone secretion, our observations imply that corticosterone is dispensable for CB1-mediated acute fear adaptation. This clearly asks for rejection of our second working hypothesis.

In the second part (manuscript 2) we wanted to investigate the involvement of acute fear adapation in formation of long-term extinction memory. Therefore, we conditioned mice to a tone and afterwards assigned them to three different exposure paradigms. The paradigms were identical in total exposure duration but differed in the duration of a single stimulus duration and the time between stimuli intervals. All groups showed the same acute fear adapation on the first day after conditioning. On the following days, the groups exposed to the ten single tones with constant and variable inter-stimulus intervals showed memory buildup. Furthermore, the group with variable intervals proved most effective, compared to the group exposed to a single permanent tone, which did not show any long term extinction memory over all days. Additionally, the experiment showed that acute fear reduction during exposure (within session extinction) does not predict extinction memory buildup (between session extinction), neither is it a prerequisit, as we showed in extinction of remote fear memory. These findings proofed our third and fourth working hypothesis.

To further strenghten the point we exposed CB1$^{-/-}$ and their wildtype littermates to the permanent and variable interval protocols. In a parallel experiment we injected wildtype animals with a CB1 receptor antagonist, or vehicle. The groups with impaired endocannabionid signalling showed impaired acute fear adapation (within-session extinction) but developed between session extinction memory. We also implemented further exposures on day 10 and day 40 to test animals for the consitency of the extinction memory. The groups with memory buildup on d3 showed a similar level of fear on d10 and spontaneous recovery, which is a reapperance of the fear reaction towards the tone, on day 40. C-Fos immunoreactivity was

upregulated in several brain areas after rimonabant injection and exposure, but only in the basolateral amygdala, the cingulate cortex and the dentate gyrus the activation showed to be consistent, irrespective of pharmacological treatment, after exposure to the variable interval protocol. These areas are likely to mediate between session extinction. Taken together the results show that within and between session extinction are dissociable processes. As a secondary finding, we showed that endocannabinoids are mostly dispensable for long term memory buildup and seem to only play a role in acute fear adapation (within-session extinction).

As our fifth working hypothesis we stated that PKC activity is essential for reconsolidation. In manuscript 3 we inhibited protein kinase C activity during exposure to asses possible effects on extinction learning. On d1 and d2, we saw, after injection, increased fear adapation during exposure to the permanent tone, but more interestingly we also saw increased between-session extinction, compared to vehicle group. The phenotype is consistent up to d10 and spontanous recovery on d40 was abolished. To ensure extinction specifity we performed several control experiments. Inhibition of PKC activity alone on d1 and d2 was not sufficient to decrease initial freezing or fear adapation during reexposure on d3. Also, shortened exposure combined with pharmacon treatment did not show any effects on freezing levels. Inhibiting PKC activity during conditioning or during preexposure to the tone was not able to produce any differences, compared to vehicle treated animals. This showed that the initially observed phenotye was specific for extinction and was also dependent on exposure length.

Extinction learning consists of two distinct phases, which describe sensitive time windows, in which learning is prone to external manipulations, namely consolidation and reconsolidation. We injected animals again on d1 and d2 but exposed them to the variable interval protocol, which we showed produces between session extionction. From d1 to d10 all groups showed similar within and between session extinction. This shows that memory consolidation during extinction learning is not disturbed by PKC inhibition. However on d40 spontaneous recovery was abloished in the inhibitor injected group. Inhibition of PKC activity impaired reconsolidation processes, but did not influence consolidation. Thus, our fifth working hypothesis proofed valid. Therefore, as a secondary finding we can state that consolidation and reconsolidation are independent processes. By c-Fos immunohistochemistry we were able to identify the cingulate cortex as a potential player in reconsolidation processes.

Since all results are already discussed in detail in the attached manuscripts, a more general discussion will give insights into the scientific impact of the obtained results in the field of extinction and exposure therapy in a translational perspective.

2.2.1. Terminology for extinction progression

A lot of different research groups work with models of extinction in order to reveal basic learning mechanism. This asks for a common standard of nomenclature. However, this is not the case and similar processes are described by different terms (Myers and Davis, 2007). We encountered several problems in terms of description of our findings with the given nomenclature. The following description bases on real behavioural data (Plendl and Wotjak, 2010). In principle, we ask for a dissociation of within- (1) and between-session extinction (2) (Figure 3A). Within-session extinction describes the decrease of freezing over the course of one session and is identical to the term fear relief, which is often used in the human field.

Between-session extinction describes the fear decrease between two sessions and is the comparison of the initial fear levels of two different sessions (Figure 3B). It expresses the active learning component in extinction and, therefore, can also be referred to as the actual relearning event. The relapse of fear after a long period of non-exposure is called spontaneous recovery (3) and can also be described by the comparison of initial freezing levels between two distant sessions, for expample d10 and d40. It is wrong to compare the end of a session with the beginning of a new session as a measurement of spontaneous recovery, because of the shown dissociation between within- and between-session.

A good quantification can be achieved, if fear responses are evaluated in time bins. In our case we split the total 200 seconds duration in ten 20 second bins, defined by the length of the CS during conditioning. But in other experiments, with longer exposure durations, single bin length could be adjusted accordingly. Also, between-session extinction and spontaneous recovery can be presented as changes in initial fear levels (Figure 3 B). We believe that this detailed mode of analysis helps to better visualize extinction progess. A common way to visualize freezing data is to show freezing scores averaged over the whole session duration, but this way of analysis could produce false positives. To demonstrate this we condensed the behavioural data of Figure 3 A into a single data point per day, which is the average of freezing over the whole sesion (Figure 3 C). By looking at the figure, a decline in freezing in the permanent tone group seems obvious, however by looking at the detailed analysis in Figure 1 A, or the initial freezing levels in Figure 3 B, one could see that actual there is no between session extinction in the pt group. Thus, this decline in overall freezing must be dependent on other factors. When plotting within session extinction of d1 and d10 of the pt group together (Figure 3 D), it becomes clear that the decline of freezing in C is caused by an accelerated within-session extinction. Because of the uniformity of the stimulus, this is most likely based on non-associative processes (Kamprath and Wotjak, 2004) and, therefore, we term this fear decline between-session habituation (4) (Figure 3C).

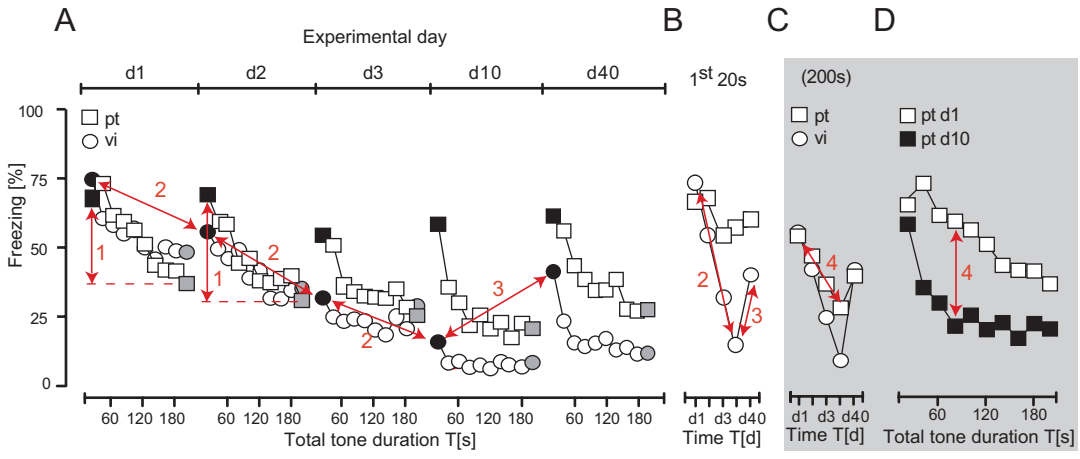

Figure 3: Extinction Terminology

The graphs introduce a new terminology of fear extinction on basis of the behavioural performance of wild-type controls exposed to a permanent tone (pt; A-D) or to repeated tones at variable intervals (vi; A-C) during extinction training. Development of freezing is either described in 20s bins (A,B,D) or averaged over the whole exposure time of 200s (C). (1) Within-session extinction or acute fear relief (A); (2) between-session extinction or relearning (A,B); (3) spontaneous recovery or relapse (A,B); (4) between-session habiutation (C,D) (Adapted from Plendl and Wotjak, 2010)

With this proposed terminology the progress of extinction data could be expressed in detail and comparability between different data sets should be increased. Moreover, reanalysing already existing data might give new insights into extinction and could possibly reveal new exciting results out of experiments already finished some time ago. Additionally it has to be mentioned that this nomenclature is meant for behavioural paraidgms, which are designed to elicit freezing as the main behavioural response. This is especially important if the freezing level during the first 20 seconds of tone exposure is used as a measurement of between-session extinction.

For example, initially CB1 -/- mice were described as being impaired in long-term extinction (Marsicano et al., 2002). Later it was shown that this impairment is dependent on the lack of acute fear adapation (Kamprath et al., 2006). Our study now showed that mice deficient for the endocannabionid system are able to develope between-session extinction, despite the lack of within-session extinction. This now clearly defines the role of the endocannabinoid system in acute fear adaptation and shows that more detailed analysis can lead to more and better conclusions.

2.2.2. Implications for exposure therapy

A major aim of our work was to provide hints to improve behavioural therapy. In the second manuscript we concentrated on exposure pardigms and therefore created three different protocols, which relied on already reported findings. We decided to confront a massed versus a spaced approach and also to introduce variablity into the spaced protocol. This led to the permanent (pt) paradigm, as a massed approach, the constant interval paradigm (ci), as a spaced, and the variable interval, as a spaced approach with a temporal unpredictability factor (vi). For all paradigms we used a fixed context, which was different in many features from the conditioning context. Our results showed that most efficient in producing relearning was the variable interval protocol (vi). This means that not the total amout of exposure time leads to buildup of long term exintction memory but actually the amount of experience encounters with the feared stimuli. In the permanent paradigm (pt) there is only one encounter per session, whereas in the constant (ci) and variable interval (vi) paradigms this happens ten times in one session. It seems that each encounter strengthens the inhibitory memory trace, which finally leads to a long lasting fear decay. Additionally temporal unpredictability seems to increase the inhibitory tendency.

Currently in psychotherapy a more or less massed approach is used to treat patients. Phobia patients are confronted with the feared stimuli for a certain duration of time in order to (i) evoke maximum level of fear and (ii) to reach a maximal decay of fear. On first sight, this procedure is not as time intensive as a spaced approach and therefore maybe more convenient for the patient. Moreover it could be less stressfull for the patient because there is only one maximum inital fear response per session and a fast onset of habituation effects, which leads to fear decay. In a spaced protocol the patient is repeatedly confronted with the feared stimuli and moreover there are consolidation periods inbetween, which can raise stress levels significantly, because of the ecxptected reexposure. Taken together, spaced exposure training could raise stress levels for patients significantly. However we could show in our animal model of extinction (manuscript 2), spaced exposures at variable intervals seem to be most effective

in eliciting long term changes in fear expression levels.

In order to minimize the expenditure of therapy time, which overall lowers stress levels, pharmacotherapy could be added for augumentation of the beneficial outcome of exposure therapy.

2.2.3. Pharmacological augumentation of exposure therapy

Up to now, in most behavioural studies, only single receptors, like the CB1 receptor, were investigated in connection with extinction phenotypes. This can be a promising approach to model certain kinds of diseases, where a "specific defect- specific disease" relationship is given, or where single symptomps of a disease are modeled. But especially psychatric diseases, like phobias, or trauma related anxiety disorders, can have multifactorial causes (Smoller et al., 2008).

It is impossible to target specific receptors in specific brain regions for therapeutical aims in humans. Generally, most pharmactherapies are applied systemically and therefore already the blood brain barrier is a big obstacle and can require huge efforts in pharmaceutical design to be overcome. If the drug passes the barrier all targeted receptors throughout the brain are affected. Neuronal trafficking is limited in time and space to minimize signal to noise ratio. Therefore, manipulations on this level seem to have low success rates in multifactorial diseases, because of low specifity and possible high side effects. For expample, the selective CB1-receptor angatonist rimonabant, which was also used in our work to impair fear adapation, was used in humans to treat unwanted side effects, during antidepressant treatment. It was effective but also led to increased suicidality as a side effect, which resulted in non-approval of the drug on all markets.

Anterograde transmitted signals are integrated in post-synaptic potenial changes and molecular cascades in the target neurons. Major molecular cascades in signal transduction are the second messengers which serve as coincidence detectors, upon which various extracellular signals are integrated into one condition. The phosphorylation status of the protein kinase C is determined by extracelluar ligand binding to receptors and also by the intracellular calcium level, which is also largely determined by receptor gated channels. It could be possible that multifactorial phenotypes are reflected by changes in second messenger activity levels. There are already some evidences obtained in animal models that the regulation of second messenger cascades does play a role in anxiety and depression . Fluoxetine, a pharmacon which is widely used for therapy in depression or post traumatic stress syndrom, seems to target beta-arrestin (David et al, 2009), which regulates the activity of G-protein coupled receptors (Lohse et al, 1990).

In our experiments blockade of the CB1 receptors in impairment of within-session extinction but not of between-session extinction. Decreasing the actvitiy of protein kinase C had a dual effect on fear. It not only seemed to decrease within-session extinction, but also decreased between-session extinction and, most importantly, prevented relapse. From this point of view, downstream effector systems seem to be promising targets for long term modulation of behavioural phenotypes. But one also have to keep in mind, that most drugs discovered so far, which modulate memory processes can not be readily adminsistred to humans (Monfils et al., 2009) and specifity can not be ensured.

2.2.4. The fear extinction brain matrix

The amygdala is one of the key brain structures for consolidation of memory (LeDoux, 2000; Maren, 2001; Fanselow and Poulos, 2005). Moreover, it modulates fear related learning in other brain structures, such as the cortex and the hippocampus (McGaugh, 2004). The amygdala consists of several anatomically and functionally distinct nuclei including the lateral (LA) and basolateral (BLA) nuclei and the central nucleus (CEA), which can be divided into the lateral (CEl) and a medial (CEm) part. In addition, there are clusters of GABAergic interneurons (ITC) surrounding the BLA, which are thought to gate interactions between the BLA and the CEA. Corresponding to current models, the LA serves as a sensoric interface (McDonald, 1998), as it receives sensory information from the thalamus and cortex. The CEm serves as the principal output station (LeDoux, 1988) and its projections contact different structures in the brainstem and in the hypothalamus. In addition amygdala nuclei are connected to many cortical and subcortical brain structures, which participate in generating behaviourally relevant outputs (Pitkänen et al., 2000). This suggests that information can be processed by intrinsic mechanisms as well as by interactions with other brain structures to integrate sensory inputs, generate fear response outputs and modulate fear responses, depending on environmental influences (Sah et al., 2003). There is accumulating evidence indicating that local inhibitory circuits in the amygdala contribute or even mediate important aspects of fear conditioning and extinction. Systemic, or local treatments can interfere with the acquisition or expression of conditioned fear (Harris and Westbrook, 1999 and 2001), but also, they can improve learning and retrieval (Tang, 2003). Also, inhibitory neurons in the amygdala are major targets of neuromodulatory systems, which may allow adjustment of networks according to environmental conditions and the behavioural state (Muller et al., 2007; Pinard 2008).

We were able to verify the crucial role of the amygdala in fear adapation and extinction of fear memory. In our second study we showed that an increase of c-Fos activity in the basolateral amygdala accompanied extinction learning. Moreover changes were observed in the cingulate cortex and the dentate gyrus. However, in our third study, inhibition of PKC activity upon reactivation of fear memory failed to elicit consistent activity changes in the amygdala, but rather in the cingulate cortex.

The process by which labile new memories are stabilized into long lasting memories is refered to as consolidation. In principle, three different ideas about consolidation are discussed. One argues that memory representations are gradually transferred over time among brain areas (McClelland et al, 1995; Knowlton and Fanselow, 1998). The second view relates to the way in which the memory strength is modulated in a given brain area as a result of activity from other areas or influences like hormones (Cahill and McGaugh, 1995). These two hypotheses focus largely on interactions between brain areas. The third idea relies on cellular and molecular events, that convert labile memory traces into stable, long term ones (DeZazzo and Tully, 1995; Bailey et al., 1996). These theories are not entirely different but put their emphasis on different processes.

In fear conditioning, inactivation of the amygdala, by infusion of GABA agonists, before acquisition impaires memory formation (Wilensky et al., 1999) and disruption of protein synthesis prevents the formation of long-term memory for fear conditioning (Schafe and

LeDoux, 2000). These observations indicate that the amygdala is important for the consolidation of this kind of memory.

We have shown in our third manuscript, that inhibition of PKC activity impaires reconsolidation, which leads to long term fear decay. Because of abolished renewal and spontaneous recovery, it shares similarities with amnesia. Amnesia for a particular experienced can follow after certain intrusions, such as electronconvulsive shocks (Duncan, 1949), inhibition of protein synthesis (Davis and Squire, 1984) or brain injury, but only if they occur shortly after the learning event (McGaugh, 1966). Another possibility is, when a newly learned association for one task is immediately followed by another association for the same task, the first one could be compromised (retroactive interference). For this process temporal proximity is essential.

Over the years it has been shown the same manipulations can also lead to memory loss after reactivation of the memory. Moreover, it seems that the same temporal restrictions apply. Electro-convulsive stimulation and new learning can affect reactivated memory only immediatly after but not after a certain period of time has passed (Dawson and McGaugh, 1969; Sara, 2000, Nader et al, 2000). Therefore, reactivated and new memories seem to exist in similar states during certain periods of time.

A recent publication dealt with memory representation in brain circuitry after reactivation (Winocur et al., 2009) and showed that after acquisition memories go through a labile phase in which they are vulnerable to disruption. At a later timepoint more brain regions seem to be integrated into memory consolidation, which leads to a stable long term memory. This integrated memory trace is less vulnerable to modifications. But when the stable memory is reactivated by a reminder, it undergoes reconsoliation and again enters a labile, vulnerable phase. This phase is again time restricted.

Taken together, these findings tell us, that consolidation and reconsolidation might be very similar processes. Our results implicate, that consolidation and reconsolidation may share the same brain circuitry, but the core areas and molecular recuritment could be different. In our second manuscript, we showed that mainly the basolateral amygdala, the dentate gyrus and the cingulate cortex are invoveld in consolidation, by increased activation. In our third manuscript it was shown that, the cingulate cortex seem to be the area to mediate reconsolidation processes. By inhibition of PKC activity we were able to decrease the activity in this area and therefore, impair reconsolidation processes. During consolidation of the fear memory this blockade of activity had no effect on acquisition, or latent inhibition.
Our study can still not definitetly answer the question of how consolidation and reconsolidation are organised. At least we can state, that consolidation of extinction memory and reconsolidation, in our hands, are controlled by different brain regions and second messenger systems.

2.3. Outlook

Out of new results, new questions arise.

Are second messengers a new therapeutical target for psychatric disorders? Currently pharmacotherapies, which indirectly modulate the PKC activity level are mainly used in depressive patients, such as Lithium Chloride and Valproic acid (Zarate and Manji, 2009 ;Bowden et al., 2010). Our findings implicate that these pharmacons could find a new use in augumentation of exposure therapy. Animal experiments in this direction should be conducted to find out if this is a valid approach. Positive findings could results in a major gain for patients undergoing exposure treatment.

We have shown that PKC activity mediates memory reconsolodation. However, the details of the mechanism and the molecular targets of PKC involved, are not yet known. To find out more, screens should be performed for regulation of other proteins by PKC. Because we identified the cingulate cortex as a possible brain region to regulate reconsolidation, this structure should be addressed in detail.

Furthermore, other second messengers and their role in memory regulation could be interesting. PKA is known to play a major role in memory consolidation and acquisition (Arsten et al., 2005) and currently also comes in focus as a therapeutic target for memory disorders. But tyrosine kinases have not been addressed yet and could pose an interesting target. It could be that second messengers play differential roles during memory phases, for example PKA in consolidation, PKC in reconsolidation and tyrosine kinases mediate another phase, not yet known.

This brings us to the question, what is really neccessary to produce a long term change in a fear memory. Do patients really have to undergo exposure sessions or is it enough to induce reconsolidation right from the start? Are there early and late phases of reconsolidation? Which phase is most prone to modifications? Do patients have to be conscious at all? Reconsolidation could be an automatic process, which triggers intrusive memories, and does not necessarily involve the awareness of the patient.

Revelation could come while sleeping.

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"SUIT UP!"

4. Statement of own contribution

Manuscript 1:
Endocannabinoids mediate acute fear adaptation via glutamatergic neurons independently of corticotropin-releasing hormone signaling.
(experiments, data analysis, participation in manuscript writing)

Manuscript 2:
Dissociation of within- and between-session extinction of conditioned fear
(concept, design, experiments, data analysis, manuscript)

Manucript 3:
PKC activity is essential for maintenance of aquired fear memory during reconsolidation
(concept, design, experiments, data analysis, manuscript)

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Endocannabinoids mediate acute fear adaptation via glutamatergic neurons independently of corticotropin-releasing hormone signaling.
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Double dissociation of PKC and AC manipulations on operant and classical learning in Drosophila.
Brembs B, Plendl W Curr Biol. 2008 Aug 5;18(15):1168-71

6. Manuscripts

6.1.

Endocannabinoids mediate acute fear adaptation via glutamatergic neurons independently ofcorticotropin-releasing hormone signaling.
Kamprath K, Plendl W, Marsicano G, Deussing JM, Wurst W, Lutz B, Wotjak CT

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Endocannabinoids mediate acute fear adaptation via glutamatergic neurons independently of corticotropin-releasing hormone signaling

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Recent evidence showed that the endocannabinoid system plays an important role in the behavioral adaptation of stress and fear responses. In this study, we chose a behavioral paradigm that includes criteria of both fear and stress responses to assess whether the involvement of endocannabinoids in these two processes rely on common mechanisms. To this end, we delivered a footshock and measured the fear response to a subsequently presented novel tone stimulus. First, we exposed different groups of cannabinoid receptor type 1 (CB_1)-deficient mice ($CB_1^{-/-}$) and their wild-type littermates ($CB_1^{+/+}$) to footshocks of different intensities. Only application of an intense footshock resulted in a sustained fear response to the tone in $CB_1^{-/-}$. Using the intense protocol, we next investigated whether endocannabinoids mediate their effects via an interplay with corticotropin-releasing hormone (CRH) signaling. Pharmacological blockade of CB_1 receptors by rimonabant in mice deficient for the CRH receptor type 1 ($CRHR1^{-/-}$) or type 2 ($CRHR2^{-/-}$), and in respective wild-type littermates, resulted in a sustained fear response in all genotypes. This suggests that CRH is not involved in the fear-alleviating effects of CB_1. As $CRHR1^{-/-}$ are known to be severely impaired in stress-induced corticosterone secretion, our observation also implicates that corticosterone is dispensable for CB_1-mediated acute fear adaptation. Instead, conditional mutants with a specific deletion of CB_1 in principal neurons of the forebrain (CaMK-$CB_1^{-/-}$), or in cortical glutamatergic neurons (Glu-$CB_1^{-/-}$), showed a similar phenotype as $CB_1^{-/-}$, thus indicating that endocannabinoid-controlled glutamatergic transmission plays an essential role in acute fear adaptation.

Keywords: CB_1, corticosterone, CRF, CRH, endocannabinoids, extinction, HPA axis, rimonabant, SR141716, stress

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Endocannabinoids are fatty acid derivatives that exert their effects on emotional and motivational behavior, cognition, pain perception and neuroprotection primarily via central cannabinoid receptor type 1 (CB_1) (Piomelli 2003). They are synthesized and released on demand from postsynaptic sites and act as retrograde messengers at presynaptic terminals where they activate CB_1 and thereby suppress neurotransmitter release (Wilson & Nicoll 2002). CB_1 is expressed by different neuronal subpopulations including GABAergic and glutamatergic neurons (Marsicano & Lutz 1999). Among other functions, CB_1 was shown to mediate extinction of conditioned fear (Chhatwal *et al.* 2005; Marsicano *et al.* 2002; Suzuki *et al.* 2004), whereby it seems to be involved in habituation-like processes (Kamprath *et al.* 2006). The latter observation strikingly resembles the findings of Hillard and coworkers (Patel *et al.* 2005), who showed that endocannabinoids mediate habituation to homotypic stressors. Based on these similarities, we hypothesize that a common mechanism underlies endocannabinoid-mediated fear and stress adaptation.

Recently, Patel and Hillard (2008) proposed a mechanism for endocannabinoid-mediated stress habituation, which centrally involves the modulation of glutamatergic signaling. Upon repeated exposures to restraint stress, which results in habituation of the behavioral response, levels of the endocannabinoid 2-arachidonoyl glycerol (2-AG) in cortical brain regions were found to be increased (Patel *et al.* 2005; Rademacher *et al.* 2008). Cortical glutamate efflux, in contrast, was shown to decrease under similar circumstances (Moghaddam 2002). 2-arachidonoyl glycerol-mediated activation of CB_1 located on glutamatergic terminals might explain the decrease in glutamate signaling. Thus, it is conceivable that unrestrained glutamate release may also account for the sustained fear responses observed in CB_1-deficient mice (Kamprath *et al.* 2006; Marsicano *et al.* 2002), particularly if one considers the importance of glutamatergic transmission in the regulation of defensive behavioral responses (Millan 2003; Nordquist *et al.* 2008).

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In addition to glutamate, corticotropin-releasing hormone (CRH) was described to control behavioral stress coping, including fear and anxiety, via the hypothalamic–pituitary–adrenal (HPA) axis and/or via extrahypothalamic brain areas by activating CRH receptor type 1 (CRHR1) and type 2 (CRHR2) (for reviews, see Bale & Vale 2004; Keck *et al.* 2005; Steckler & Holsboer 1999). Interestingly, both CRH (Cota *et al.* 2003, 2007) and CRHR1 (Hermann & Lutz 2005) colocalize with CB_1 in hypothalamic and extrahypothalamic brain areas, suggesting that CB_1 may inhibit CRH signaling, accounting for the exaggerated fear responses observed in CB_1-deficient mice (Marsicano *et al.* 2002). In addition to CB_1 influencing CRH signaling, corticosterone itself can potently activate the endocannabinoid system in the hypothalamus (Di *et al.* 2003). This, in turn, inhibits glutamatergic afferences, thereby constraining HPA-axis activity and further corticosterone release.

Taken together, likely candidates in a common mechanism for endocannabinoid-mediated adaptation of fear and stress responses include glutamatergic transmission, CRH and the HPA axis with the possibility for multiple interdependences. Noteworthy, the extent of HPA-axis activation is determined by the intensity of the stressor (Armario *et al.* 1986; Hennessy & Levine 1978; Hennessy *et al.* 1979), which parallels recent findings in the endocannabinoid system, namely that the anxiolytic-like actions of endocannabinoids and their effects on stress-coping behavior seem to depend on the aversiveness of the test situation (Haller *et al.* 2004; Naidu *et al.* 2007). However, it remains to be shown that endocannabinoid-mediated fear adaptation is characterized by a similar dependency.

In the present study, we investigated the impact of the aversiveness of the previously encountered stressful experience on the involvement of endocannabinoids in fear adaptation. To this end, we applied inescapable footshocks of different intensities to different groups of CB_1-null mutant mice and their wild-type littermates and measured their fear responses to a tone on the following day. The most effective protocol was then applied to rimonabant-treated CRHR1-deficient (Timpl *et al.* 1998) and CRHR2-deficient (Coste *et al.* 2000) mice to test the hypothesis that an interplay of the endocannabinoid system and CRH signaling is responsible for the sustained fear response observed in animals with impaired CB_1 signaling. Finally, we investigated which neuronal subpopulation expressing CB_1 is involved in the previously observed phenotype by means of conditional mutants lacking CB_1 expression either in principal neurons of the forebrain (CaMK-CB_1; Marsicano *et al.* 2003) or in cortical glutamatergic neurons (Glu-CB_1; Monory *et al.* 2006). The specific deletion of CB_1 in CaMK-$CB_1^{-/-}$ affects, among others, glutamatergic and GABAergic projection neurons of cortical and subcortical brain structures, including the hypothalamus (Marsicano *et al.* 2003). Consequently, in the forebrain of these mice, expression of CB_1 is largely constricted to GABAergic interneurons. Glu-$CB_1^{-/-}$, in contrast, affects a much lower number of neurons because these mice lack CB_1 specifically in cortical glutamatergic neurons, thus maintaining CB_1 expression in subcortical brain structures (including the hypothalamus; Monory *et al.* 2006). If CB_1, indeed, mediates its fear-alleviating effects via restriction of cortical glutamate release, the phenotype of the two conditional mutant lines should resemble that observed in conventional CB_1 knockouts with germ-line deletion of the *CB_1* gene.

Materials and methods

All experiments were approved by the Committee on Animal Health and Care of the State of Bavaria (Regierung von Oberbayern, Germany) and performed in strict compliance with the European community recommendations for the care and use of laboratory animals.

Animals

Male mice at the age of 8–14 weeks were used in all experiments. All mutant mice and their respective wild-type littermate controls were generated and genotyped as described previously ($CB_1^{-/-}$, $n = 61$ and $CB_1^{+/+}$, $n = 68$: Marsicano *et al.* 2002; CaMK-$CB_1^{-/-}$, $n = 13$ and CaMK-$CB_1^{+/+}$, $n = 14$: Marsicano *et al.* 2003; Glu-$CB_1^{-/-}$, $n = 14$ and Glu-$CB_1^{+/+}$, $n = 10$: Monory *et al.* 2006; $CRHR1^{-/-}$, $n = 14$ and $CRHR1^{+/+}$, $n = 14$: Timpl *et al.* 1998; $CRHR2^{-/-}$, $n = 14$ and $CRHR2^{+/+}$, $n = 18$: Coste *et al.* 2000). All CB_1-mutant mice (CB_1, CaMK-CB_1 and Glu-CB_1 mice) were backcrossed to C57BL/6NCrl for six generations. The $CRHR1^{-/-}$ and $CRHR1^{+/+}$ mice were originally generated by Timpl *et al.* using 129/Ola and CD1 mouse strains (see Timpl *et al.* 1998 for detailed description) and not backcrossed to any mouse strain but maintained by means of heterozygous breeding pairs. The $CRHR2^{-/-}$ and $CRHR2^{+/+}$ mice (courtesy of M. Stenzel-Poore, Oregon Health & Science University, Portland, OR, USA) were backcrossed to C57BL/6J for four generations. All conventional mutant mouse lines ($CB_1^{-/-}$/$CB_1^{+/+}$; $CRHR1^{-/-}$/$CRHR1^{+/+}$ and $CRHR2^{-/-}$/$CRHR2^{+/+}$) were maintained by heterozygous breeding pairs. Only homozygous male offspring were used for the experiments. Conditional mutant mouse lines (CaMK-$CB_1^{+/+}$/CaMK-$CB_1^{-/-}$ and Glu-$CB_1^{+/+}$/Glu-$CB_1^{-/-}$) were maintained by breeding pairs consisting of Cre(−)xCB$_1$fl/fl (i.e. wild-type) mothers and Cre(+)xCB$_1$fl/fl (i.e. knockout) fathers to avoid effects of the genetic modulation on maternal care. For the experiments, the male offspring were taken from different breeding pairs with respect to closely matching birth dates (the maximum variation among birth dates was 6 weeks). Because most litters do not contain equal numbers of male mutant and wild-type offspring, most breeding pairs did not contribute equal numbers of mutant and wild-type animals to the experimental groups. However, care was taken that at least one wild-type littermate was tested together with each mutant mouse and vice versa.

All animals were reared at the animal facilities of the Max Planck Institute of Psychiatry, Munich, Germany. Animals were single housed under an inverse 12 h:12 h light–dark cycle (lights off: 0900 h) with food and water *ad libitum* for at least 14 days before starting the experiments.

Experimental procedures

Experiments were performed on two consecutive days with application of the footshock (sensitization) on day 1 and exposure to the tone on day 2. Rimonabant was applied subcutaneously (s.c.) 45 min before tone presentation on day 2. All experiments were performed during the animals' active phase between 0930 and 1700 h.

Sensitization

Mice were placed into the shock context where they received a single inescapable footshock of 2 seconds duration essentially as previously described (Kamprath & Wotjak 2004). Shock sensitization at the individual pain threshold (PT) was achieved by manually raising the shock intensity until the animal showed the first signs of pain and discomfort (jumping and/or vocalization). The respective current intensity was maintained for 2 seconds, before the current was switched off. Naïve (non-shocked) controls were not placed into the shock context.

Tone presentation
On the day following footshock sensitization, mice were placed into a new 'test context' that differed from the shock context in various aspects, including shape, odor, illumination and bedding (see Kamprath & Wotjak 2004 for details). After 3 min, a tone of 9 kHz and 80 dB was presented for 3 min.

Experiment 1: Interrelation between CB_1-deficiency, footshock intensity and subsequent fear response to a tone
$CB1^{-/-}$ and $CB_1^{+/+}$ were randomly assigned to five experimental groups, which differed in the intensity of the stress sensitization procedure as follows: the first group remained non-shocked (0 mA; $CB_1^{-/-}$: $n = 10$; $CB_1^{+/+}$: $n = 11$), the second group received a footshock at the individual PT ($CB_1^{-/-}$: $n = 15$; $CB_1^{+/+}$: $n = 10$), the third group received a footshock with a current intensity of 0.5 mA ($CB_1^{-/-}$: $n = 8$; $CB_1^{+/+}$: $n = 9$), the fourth group received a footshock with a current intensity of 0.7 mA ($CB_1^{-/-}$: $n = 20$; $CB_1^{+/+}$: $n = 26$) and the fifth group received a footshock with a current intensity of 1.5 mA ($CB_1^{-/-}$: $n = 8$; $CB_1^{+/+}$: $n = 12$). The extent of sensitization was assessed by measuring the freezing response to a 3-min tone at the next day. Note that the groups of mice that received a 0.7-mA shock are identical to those published before (Kamprath *et al.* 2006).

Experiment 2: Interplay between CB_1 and CRHR1 in fear adaptation following footshock sensitization
The $CRHR1^{-/-}$ and their wild-type littermates received a footshock of 1.5 mA (which proved to be the most effective in experiment 1), and the extent of sensitization was assessed by measuring the freezing response to a 3-min tone on the next day. Half of the $CRHR1^{-/-}$ mice ($n = 7$) and their wild-type littermates ($n = 7$) were treated with rimonabant (10 mg/kg, s.c.) 45 min prior to the tone presentation, and the other half ($CRHR1^{-/-}$: $n = 7$; $CRHR1^{+/+}$: $n = 7$) were treated with vehicle.

Experiment 3: Interplay between CB_1 and CRHR2 in fear adaptation following footshock sensitization
The $CRHR2^{-/-}$ and their wild-type littermates received a footshock of 1.5 mA, and the extent of sensitization was assessed by measuring the freezing response to a 3-min tone on the next day. Half of the $CRHR2^{-/-}$ mice ($n = 7$) and their wild-type littermates ($n = 9$) were treated with rimonabant (10 mg/kg, s.c.) 45 min prior to the tone presentation, and the other half ($CRHR2^{-/-}$: $n = 7$; $CRHR2^{+/+}$: $n = 9$) were treated with vehicle.

Experiment 4: Role of CB_1 expressed by principal neurons of the forebrain in fear adaptation following footshock sensitization
CaMK-$CB_1^{-/-}$ and their wild-type littermates received a footshock of 1.5 mA, and the extent of sensitization was assessed by measuring the freezing response to a 3-min tone on the next day.

Experiment 5: Role of CB_1 expressed by cortical glutamatergic neurons in fear adaptation following footshock sensitization
Glu-$CB_1^{-/-}$ and their wild-type littermates received a footshock of 1.5 mA, and the extent of sensitization was assessed by measuring the freezing response to a 3-min tone on the next day.

Behavioral analysis

The behavioral response to the tone was videotaped. Fear was assessed off-line by a trained observer who scored the freezing response of the animals unaware of the genotype or treatment condition as described before (Kamprath & Wotjak 2004). Freezing was defined as the absence of all movements except for those related to respiration.

Drug treatment

Rimonabant [SR141716; *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; kindly provided by NIMH Chemical Synthesis and Drug Supply Program (Rinaldi-Carmona *et al.* 1994)] was dissolved in vehicle solution (one drop Tween-80 in 3 ml 2.5% dimethylsulfoxide in saline) to a final concentration of 10 mg/kg and injected s.c. at 20 ml per kg body weight under light isoflurane anesthesia 45 min prior to behavioral testing. The subcutaneous route of drug administration was chosen on basis of previous experiments (Kamprath *et al.* 2006; Marsicano *et al.* 2002). The time-point of injection (45 min prior to behavioral testing) was based on the study of Petitet *et al.* (1999), who showed that rimonabant elicits its strongest effects up to 60 min after administration independent of the route of administration. The interval between injection and behavioral testing was maximized within the given temporal range to avoid possible effects of the injection stress on the outcome of the behavioral testing. The dose of rimonabant (10 mg/kg) was chosen on basis of a dose–response experiment in C57BL/6J mice, the background strain of CRHR2 mutant mice (Figure S1) and because of its higher efficiency in terms of promoting stress-induced corticosterone secretion in C57BL/6N mice (Steiner *et al.* 2008a).

Data analysis and statistics

For analysis, the total time of tone presentation was subdivided into 20-second bins with one data point representing one interval. For every interval, the duration of freezing was expressed as a percentage of the total time of the interval ('freezing time' per interval/total interval time $\times 100$). Data were analyzed by two-way or three-way analysis of variance (ANOVA) for repeated measurements as indicated in the text using STATISTICA 5.0 (StatSoft Inc., Tulsa, OK, USA) or GRAPHPAD PRISM 5.0 (GraphPad Software Inc., San Diego, CA, USA). Newman–Keuls test was used as the *post hoc* test if appropriate. Data are presented as mean ± SEM. Statistical significance was accepted if $P < 0.05$.

Results

Experiment 1: Fear-alleviating effects of endocannabinoids depend on the intensity of the previously encountered footshock

To investigate whether CB_1 mediates the adaptation of the fear response in an aversiveness-dependent manner, we applied inescapable footshocks of different intensities to different groups of CB_1-deficient mice ($CB_1^{-/-}$) and their wild-type littermates ($CB_1^{+/+}$). One day later, we assessed the behavioral responses of all groups of mice to the same novel stimulus, an 80-dB tone. Both $CB_1^{-/-}$ and $CB_1^{+/+}$ showed an increase in freezing to the tone with increasing shock intensities (Fig. 1; statistics not shown). Significant genotype differences became evident only after application of a footshock of 0.7 mA (genotype: $F_{1,44} = 9.7$, $P = 0.003$; genotype × interval: $F_{8,352} = 3.5$, $P < 0.001$; Fig. 1) or 1.5 mA (genotype: $F_{1,18} = 10.6$, $P = 0.004$; genotype × interval: $F_{8,144} = 3.6$, $P < 0.001$; Fig. 1) but not in the case of lower footshock intensities (statistics not shown). A significant decrease in the development of the freezing response over the 3-min tone presentation was observed in all groups of $CB_1^{+/+}$ which experienced a footshock, while $CB_1^{-/-}$ shocked with 1.5 mA failed to reach significance ($F_{8,56} = 1.8$, $P = 0.09$, one-way ANOVA), in contrast to their wild-type littermates ($F_{8,88} = 6.5$, $P < 0.0001$). Thus, the strongest footshock protocol (1.5 mA) yielded the most

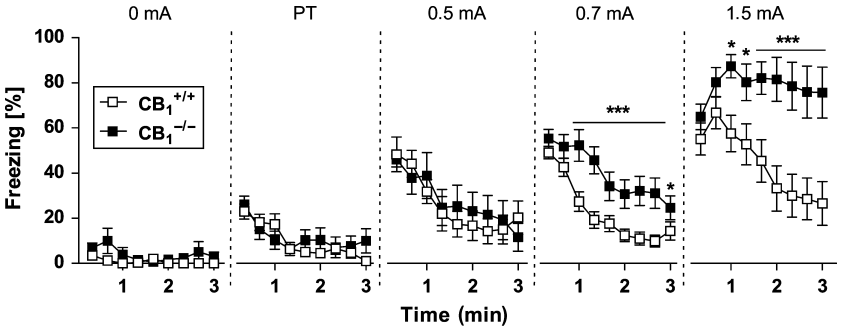

Figure 1: Fear-alleviating effects of endocannabinoids depend on the intensity of the aversive encounter. The freezing responses of both $CB_1^{-/-}$ (■) and $CB_1^{+/+}$ (□) to a 3-min tone presented 24 h after application of an inescapable footshock showed a clear dependency on the intensity of the previously encountered footshock. CB_1 deficiency caused a sustained freezing response after application of an inescapable footshock of high-shock intensities (0.7 and 1.5 mA) only. No significant differences could be detected in the freezing responses of naïve mice (0 mA) and of mice that were shocked either at the individual PT (approximately 0.25 mA) or with 0.5 mA. Data are displayed in 20-second bins. $*P < 0.05$, $***P < 0.001$ (for sample sizes, see *Materials and methods*). Note that the groups of mice that received a 0.7-mA shock are identical to those published in Kamprath *et al.* (2006) (copyright 2006 by the Society for Neuroscience).

pronounced genotype effects. Non-shocked mice showed very little freezing (0 mA, statistics not shown; Fig. 1).

Experiment 2: Interplay between CB_1 and CRHR1 in fear adaptation following footshock sensitization

As both the endocannabinoid system and the CRH signaling play important roles in the adaptation to stressful events, and recent literature suggests an interplay between both systems, our next aim was to investigate whether CRHR1 is involved in CB_1-mediated fear adaptation following sensitization with high footshock intensities. To this end, we used $CRHR1^{-/-}$, which were shown, among others, to be strongly impaired in stress-induced corticotropin (ACTH) and corticosterone responses (Timpl *et al.* 1998). Thus, pharmacological blockade of CB_1 in $CRHR1^{-/-}$ would be expected to be ineffective if CB_1-mediated acute fear adaptation depends on CRHR1 signaling or on stress-induced ACTH or corticosterone release. Based on the previous experiment, a footshock of 1.5 mA was applied to $CRHR1^{+/+}$ and $CRHR1^{-/-}$. On the next day, the freezing response of the animals to the tone was measured. Forty-five minutes prior to tone exposure, half of the animals for each genotype were treated with the CB_1-antagonist rimonabant (10 mg/kg, s.c.) and the other half with vehicle. A three-way ANOVA (drug, genotype and interval) showed that, similar to a genetic CB_1 deficiency, the CB_1 antagonist rimonabant caused an increased freezing response (drug: $F_{1,24} = 8.8$, $P = 0.007$), which was independent of the genotype (drug × genotype: $F_{1,24} = 0.002$, $P = 0.96$; Fig. 2). A significant drug × interval interaction ($F_{8,192} = 2.4$, $P = 0.016$) points to a delayed fear adaptation in rimonabant-treated animals that was independent of the genotype (drug × interval × genotype: $F_{8,192} = 1.56$, $P = 0.14$). Interestingly, $CRHR1^{-/-}$, in general, showed a stronger freezing response than their wild-type littermates (genotype: $F_{8,48} = 18.8$, $P = 0.0002$), irrespective of the treatment (drug × genotype: $F_{1,24} = 0.002$, $P = 0.96$).

Experiment 3: Interplay between CB_1 and CRHR2 in fear adaptation following footshock sensitization

Data of experiment 2 indicate that acute effects of CB_1 on fear adaptation occur independent of CRHR1 signaling and stress-induced corticosterone release. However, the effects of CRH on stress coping depend also on another receptor, CRHR2 (for review, see Bale & Vale 2004). Thus, the various ligands of the CRH family might interact with the endocannabinoid system during the acute fear response via CRHR2. Therefore, we applied a footshock of 1.5 mA to $CRHR2^{+/+}$ and $CRHR2^{-/-}$ and measured their freezing response to the tone on the next day 45 min after treatment with rimonabant (10 mg/kg, s.c.) or vehicle. A three-way ANOVA (drug, interval and genotype) showed that, similar to $CRHR1^{-/-}$, the CB_1 antagonist rimonabant caused an increased freezing response (drug:

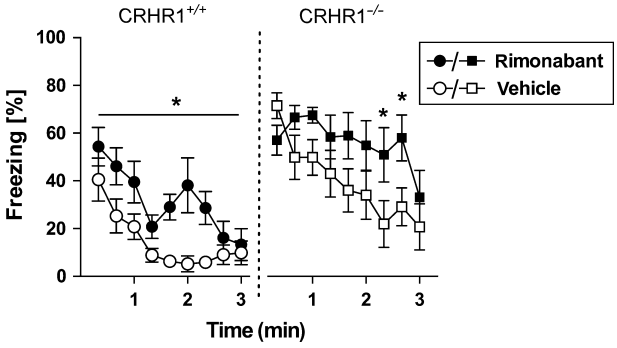

Figure 2: The CB_1-antagonist rimonabant caused increased freezing in both $CRHR1^{+/+}$ and $CRHR1^{-/-}$. $CRHR1^{+/+}$ (●, ○) and $CRHR1^{-/-}$ (■, □) received a footshock of 1.5 mA, followed by exposure to a 3-min tone 24 h later. Before tone presentation, mice were treated either with 10 mg/kg, s.c. rimonabant (●, ■) or with vehicle (○, □). Data are displayed in 20-second bins. $*P < 0.05$ (for sample sizes, see *Materials and methods*).

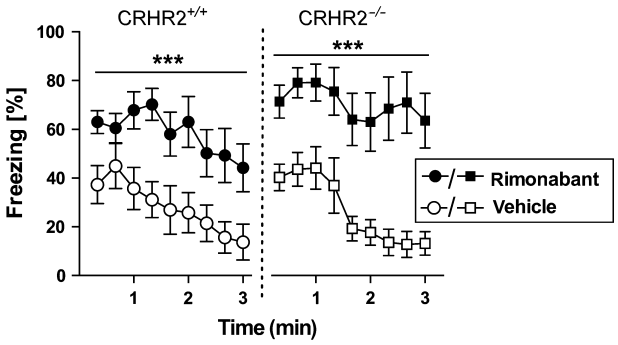

Figure 3: The CB_1-antagonist rimonabant caused increased freezing in both $CRHR2^{+/+}$ and $CRHR2^{-/-}$. $CRHR2^{+/+}$ (●, ○) and $CRHR2^{-/-}$ (■, □) received a footshock of 1.5 mA, followed by exposure to a 3-min tone 24 h later. Before tone presentation, mice were treated either with 10 mg/kg, s.c. rimonabant (●, ■) or with vehicle (○, □). Data are displayed in 20-second bins. $***P < 0.001$ (for sample sizes, see *Materials and methods*).

$F_{1,28} = 26.6$, $P < 0.0001$), which was independent of the genotype (drug × genotype: $F_{1,28} = 0.86$, $P = 0.36$; drug × interval × genotype: $F_{8,224} = 0.94$, $P = 0.48$; Fig. 3). Noteworthy, no significant genotype differences could be detected between $CRHR2^{+/+}$ and $CRHR2^{-/-}$ (genotype: $F_{1,28} = 0.57$, $P = 0.46$; genotype × interval: $F_{8,224} = 0.95$, $P = 0.48$).

Experiment 4: CB_1 deficiency in principal forebrain neurons leads to impaired fear adaptation following footshock sensitization

To investigate which neuronal subpopulation expressing CB_1 is mediating fear adaptation following footshock stress, we next tested CaMK-$CB_1^{-/-}$, a conditional mutant line, which lacks CB_1 expression in principal neurons of the forebrain (Marsicano *et al.* 2003). The specific CB_1 deletion in these mice includes, among others, CB_1 expressed by glutamatergic neurons of cortical and subcortical brain structures and by GABAergic projection neurons. Based on the results of experiment 1, we subjected these animals to the strongest protocol, that is application of a footshock of 1.5 mA and measured the freezing response to a tone 24 h later. CaMK-$CB_1^{-/-}$ showed a significant difference in the freezing response compared with their wild-type littermate controls (genotype: $F_{1,25} = 4.5$, $P = 0.04$; genotype × interval: $F_{8,200} = 3.9$, $P < 0.001$; Fig. 4). This difference was characterized by the feature that CaMK-$CB_1^{-/-}$ were not able to decrease their freezing response over the course of the 3-min tone presentation ($F_{8,96} = 1.2$, $P = 0.29$; one-way ANOVA), in contrast to their wild-type littermates ($F_{8,104} = 6.5$, $P < 0.0001$).

Experiment 5: CB_1 deficiency in cortical glutamatergic neurons leads to impaired fear adaptation following footshock sensitization

In CaMK-$CB_1^{-/-}$, CB_1 expression in the forebrain is restricted to GABAergic interneurons, that is a relatively high number of

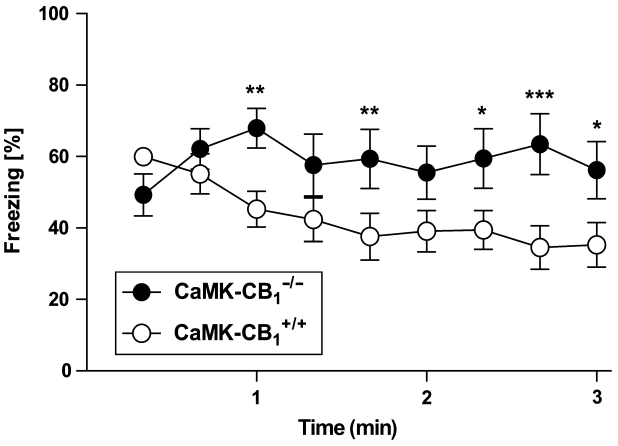

Figure 4: Sustained freezing responses in CaMK-$CB_1^{-/-}$. CaMK-$CB_1^{-/-}$ (●) and CaMK-$CB_1^{+/+}$ (○) received a footshock of 1.5 mA, followed by exposure to a 3-min tone 24 h later. Data are displayed in 20-second bins. $*P < 0.05$, $**P < 0.005$, $***P < 0.001$ (for sample sizes, see *Materials and methods*).

neurons lack CB_1 expression (Monory *et al.* 2006, 2007). To further narrow down which neuronal subpopulation expressing CB_1 mediates fear adaptation following footshock stress, we tested Glu-$CB_1^{-/-}$, another conditional mutant line, which lacks CB_1 expression specifically in cortical glutamatergic neurons (Monory *et al.* 2006). In contrast to CaMK-$CB_1^{-/-}$, the specific CB_1 deletion in Glu-$CB_1^{-/-}$ does not include CB_1 expressed by hypothalamic neurons. Similarly to $CB_1^{-/-}$ and CaMK-$CB_1^{-/-}$, Glu-$CB_1^{-/-}$ showed a stronger freezing response to the tone than their wild-type littermate controls (genotype: $F_{1,22} = 4.3$, $P = 0.049$; Fig. 5). Although we failed to observe a significant genotype × interval interaction ($F_{8,176} = 1.2$, $P = 0.30$), one-way ANOVAs performed

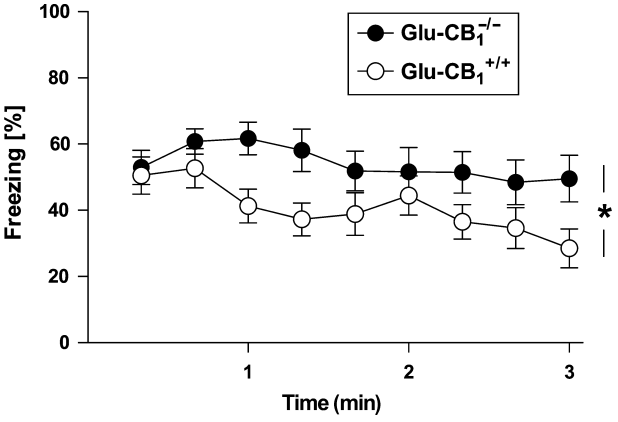

Figure 5: Sustained freezing responses in Glu-$CB_1^{-/-}$. Glu-$CB_1^{-/-}$ (●) and Glu-$CB_1^{+/+}$ (○) received a footshock of 1.5 mA, followed by exposure to a 3-min tone 24 h later. Data are displayed in 20-second bins. $*P < 0.05$ (for sample sizes, see *Materials and methods*).

separately per genotype showed that Glu-$CB_1^{-/-}$ ($F_{8,104} = 1.5$, $P = 0.17$), unlike their wild-type littermates (one-way ANOVA: $F_{8,72} = 2.9$, $P = 0.007$), were not able to significantly decrease their freezing response over the course of the 3-min tone presentation.

It is of note that, after application of a 1.5 mA footshock, none of the CB_1-deficient mouse lines ($CB_1^{-/-}$, CaMK-$CB_1^{-/-}$ and Glu-$CB_1^{-/-}$) were able to decrease their freezing responses over the course of the 3-min tone presentation, whereas the respective wild-type mice did show a decline in freezing. Although this decline of the freezing response appears to be steeper in $CB_1^{+/+}$ (wild-types) than in the wild-types of the conditional mutant lines, CaMK-$CB_1^{+/+}$ and Glu-$CB_1^{+/+}$, these differences between the CB_1-deficient mouse lines failed to reach statistical significance (line: $F_{2,65} = 2.88$, $P = 0.063$) in a three-way ANOVA (genotype, line and interval) and might relate to differences in the genetic background and variations between experiments. In contrast, the three-way ANOVA showed a significant genotype effect ($F_{1,65} = 20.5$, $P < 0.0001$) and a significant genotype × interval interaction ($F_{8,520} = 6.8$, $P < 0.00001$), which were independent of the line (genotype × line: $F_{2,65} = 1.8$, $P = 0.17$; genotype × line × interval: $F_{16,520} = 1.3$, $P = 0.20$).

Discussion

The present study shows that CB_1 only controls acute fear adaptation in the aftermath of highly aversive encounters. This process depends neither on intracerebral CRH signaling nor on stress-induced activation of the HPA axis, but on CB_1-controlled cortical glutamatergic projections.

To investigate whether the involvement of endocannabinoids in fear adaptation depends on the aversiveness of the situation, we applied inescapable footshocks of different intensities to different groups of CB_1-deficient mice and studied their fear responses to a subsequently presented tone. In general, the intensity of the footshock directly modified the intensity of the fear response to the subsequently presented tone in a dose-dependent manner (Fig. 1; see also Kamprath & Wotjak 2004). However, the fear-alleviating effects of endocannabinoids only became evident following the two highest shock intensities (0.7 and 1.5 mA). These data underscore the general importance of intensity-response studies for testing the modulation of fear and stress responses and for evaluating the involvement of different neuronal systems in these processes. The impairments in fear adaptation observed in CB_1-deficient mice did not relate to differences in pain perception because differences between the two genotypes were neither found in the individual pain thresholds (Marsicano *et al.* 2002) nor in the initial fear responses to the subsequently presented tone (Fig. 1). Moreover, data obtained by pharmacological blockade of CB_1 before tone presentation (Figs 2 and 3) indicate that endocannabinoids are acutely involved in the adaptation of the fear response to the tone following sensitization rather than in perception of the footshock (Kamprath *et al.* 2006; Marsicano *et al.* 2002).

The finding that the endocannabinoid system mediates fear adaptation in an aversiveness-dependent manner corroborates recent findings by Haller *et al.* (2004), showing that an anxiogenic-like phenotype of CB_1-deficient mice became only detectable if the illumination of the elevated plus maze (EPM) was increased and, thus, the aversiveness of the test situation was maximized. In line with these results, treatment with the fatty acid amide hydrolase (FAAH) inhibitor URB597, which blocks degradation of the endocannabinoid anandamide, resulted in anxiolytic-like behavior in the EPM test only if a distinct strong illumination was used (Naidu *et al.* 2007) or if the animals were tested during the light phase of the circadian cycle (Moreira *et al.* 2008). Moreover, impairment of FAAH by pharmacological and genetic means led to increased active stress-coping behavior in a tail suspension test only if the aversiveness was increased by a flashlight beam focused to the animals' tail in a dimly lit room (Naidu *et al.* 2007). Our data extend those findings in that the aversiveness of the test situation determines endocannabinoid recruitment not only in terms of anxiety-related and stress-coping behavior but also in terms of behavioral fear responses.

The findings that endocannabinoid involvement in fear, anxiety and stress adaptation depends on the aversiveness of the test situation strikingly resemble the dependency of HPA-axis activation on the intensity of a stressor (Armario *et al.* 1986; Hennessy & Levine 1978; Hennessy *et al.* 1979). In the first steps of stress-induced HPA-axis activation, CRH is released from axon terminals of the hypothalamic paraventricular nucleus into the portal blood at the level of the median eminence, followed by ACTH secretion from the adenohypophysis, which subsequently triggers the release of glucocorticoids (i.e. cortisol or corticosterone) from the adrenal glands. Corticotropin-releasing hormone is also found in extrahypothalamic brain regions, where it is involved in the processing of stress responses, anxiety-like behavior and conditioned fear (Bale & Vale 2004; Keck *et al.* 2005; Steckler & Holsboer 1999). Colocalization of CB_1 and CRHR1 (Hermann & Lutz 2005) and of CB_1 and CRH (Cota *et al.* 2003, 2007) suggest a functional interplay between the two neurotransmitter systems. We therefore assumed that CB_1 might mediate its fear-alleviating effects by restraining CRH signaling within the brain. To address this point, we sensitized $CRHR1^{-/-}$ (Timpl *et al.* 1998) and $CRHR2^{-/-}$ (Coste *et al.* 2000) with a 1.5-mA footshock and treated the animals with 10 mg/kg rimonabant (s.c.) before exposure to the tone 24 h later. The rather high dose of rimonabant was chosen on the basis of a pilot experiment in C57BL6/J mice, the background strain of the CRHR2 mutant mouse strain (Figure S1). In addition, a dose of 10 mg/kg was the most efficient in promoting stress-induced corticosterone secretion in C57BL/6N mice (Steiner *et al.* 2008a). We cannot entirely rule out that the high dose of rimonabant might mediate its effects via its inverse agonist properties. However, the phenotype of CB_1-deficient mice, C57BL/6N mice treated with 3 mg/kg rimonabant (Figure S1; Kamprath *et al.* 2006) and C57BL/6J mice treated with 10 mg/kg rimonabant (Figure S1), appears strikingly similar and is most easily explained by an impairment of CB_1 signaling.

Rimonabant treatment led to impaired fear adaptation in both $CRHR1^{-/-}$ (Fig. 2) and $CRHR2^{-/-}$ (Fig. 3) and the respective wild-type controls, thus indicating that the fear-alleviating effects of endocannabinoids do not involve

intracerebral CRH systems. Although there is still the possibility for a mutual compensation of CRHR1 and CRHR2 deficiency, this seems to be rather unlikely because $CRHR1^{-/-}$ and $CRHR2^{-/-}$ show different phenotypes in anxiety-related behavior when compared with their respective wild-type littermates, with $CRHR1^{-/-}$ showing decreased anxiety (Timpl *et al.* 1998) and different lines of CRHR2-deficient mice showing either increased anxiety (Bale *et al.* 2000; Kishimoto *et al.* 2000) or no changes in anxiety at all (Coste *et al.* 2000). Moreover, pharmacological treatment showed that CRHR1 and CRHR2 seem to exert opposite effects on auditory fear conditioning (Radulovic *et al.* 1999).

Recently, Tasker and co-workers suggested a mechanism for fast glucocorticoid feedback inhibition within the hypothalamus involving endocannabinoid release (Di *et al.* 2003, 2005b), which might account for endocannabinoid-mediated stress adaptation. This model requires glucocorticoid secretion to trigger endocannabinoid release within the paraventricular nucleus of the hypothalamus that, in turn, inhibits glutamatergic afferences to the nucleus and results in CRH secretion. As a similar interaction between the corticosterone and the endocannabinoid system has been described for other parts of the hypothalamus as well (Di *et al.* 2005a), we hypothesized that corticosterone regulates endocannabinoid-controlled fear adaptation in a similar way within the fear matrix of the brain. However, because $CRHR1^{-/-}$, which are severely impaired in stress-induced corticosterone secretion (Timpl *et al.* 1998), still responded to rimonabant, this model may not apply for acute fear adaptation following footshock stress.

The $CRHR1^{-/-}$ showed an increased freezing response to the tone following an inescapable footshock compared with their wild-type littermates, irrespective of the treatment. This observation was unexpected, taking into consideration the increased exploratory activity and the reduced anxiety-related behavior previously reported in these animals (Timpl *et al.* 1998). The data of the present study suggest that CRHR1 is differentially involved in fear and anxiety. In contrast to fear-related paradigms, in which the animal is confronted with an inescapable stressor, the stressor used in anxiety paradigms is avoidable, and risk assessment, that is approach of the stressful situation, is tested. As different neural circuits are involved in controllable vs. uncontrollable stress (Herry *et al.* 2007; Kavushansky *et al.* 2006), CRHR1 might also be differentially involved. Alternatively, compensatory changes in other transmitter systems (e.g. upregulation of vasopressin expression; Muller *et al.* 2000) might be responsible for the increased freezing response of $CRHR1^{-/-}$. Finally, taking into consideration that injections of corticosterone at different learning phases of fear conditioning resulted in a decreased freezing response (Cai *et al.* 2006; Skorzewska *et al.* 2007), the attenuated HPA-axis response in $CRHR1^{-/-}$ with strongly impaired corticosterone release might lead to an increased freezing response not only after conditioning but also after sensitization paradigms. Corticosterone might mediate its fear-alleviating effects, at least in part, via triggering endocannabinoid release. The phenotype of an increased fear response following sensitization in vehicle-treated $CRHR1^{-/-}$ compared with vehicle-treated $CRHR1^{+/+}$ would then relate to impaired corticosterone-induced endocannabinoid signaling in the mutants (Fig. 2). However, the behavioral differences between $CRHR1^{-/-}$ and $CRHR1^{+/+}$ persisted despite rimonabant treatment (Fig. 2), thus rendering it unlikely that corticosterone-induced endocannabinoid signaling via CB_1 is crucially involved in the phenotype of $CRHR1^{-/-}$.

The question remained as to which transmitter systems are involved in CB_1-controlled fear adaptation during highly aversive encounters. Cannabinoid receptor type 1 was shown to be expressed widely throughout the brain by different neuronal subpopulations including GABAergic, glutamatergic and serotonergic neurons (Haring *et al.* 2007; Marsicano & Kuner 2008; Marsicano & Lutz 1999). To investigate whether glutamatergic transmission is involved in CB_1-mediated fear adaptation, we applied the strongest footshock protocol, which was found to yield the strongest effects in $CB_1^{-/-}$ (Fig. 1), to CaMK-$CB_1^{-/-}$ mutants. These mutants lack CB_1 expression in principal forebrain neurons, including glutamatergic and GABAergic projection neurons, but sparing CB_1 expression in GABAergic interneurons. CaMK-$CB_1^{-/-}$ showed a similar impairment of fear adaptation as $CB_1^{-/-}$ (Fig. 4). Consequently, an involvement of CB_1-expressing GABAergic interneurons in this behavioral modulation appears to be rather unlikely. To further narrow down the neuronal subpopulation involved, Glu-$CB_1^{-/-}$ were subjected to the same protocol with similar effects as observed in $CB_1^{-/-}$ and CaMK-$CB_1^{-/-}$ (Fig. 5). These results indicate that CB_1-expressing cortical glutamatergic neurons are involved in endocannabinoid-mediated fear adaptation following inescapable footshock stress. According to a mechanism proposed by Patel and Hillard (2008), endocannabinoid-regulated cortical glutamatergic transmission plays an essential role in the habituation of repeated exposure to a stressor. Although our behavioral paradigm did not involve repeated exposures to the tone, we observed within-session habituation of the behavioral response. Consequently, the mechanism suggested by Patel and Hillard (2008) might partially account for acute fear adaptation, that is endocannabinoid-regulated cortical glutamatergic transmission might play a central role, whereas increases in 2-AG synthetic capacity via upregulation of the synthesizing enzymes, which is also part of the suggested model, may not apply for the current paradigm of acute fear adaptation, but for the pronounced genotype and drug effects observed after repeated tone presentations (Kamprath *et al.* 2006). Recently, we could show that Glu-$CB_1^{-/-}$ were impaired in behavioral stress coping in a forced swim test despite unaltered corticosterone secretion (Steiner *et al.* 2008b). This supports the notion that endocannabinoid-mediated control of both behavioral stress and fear responses may rely on cortical glutamatergic projections, independent of the activity of the CRH/HPA system.

Taken together, the present study emphasizes the importance of intensity-response studies for testing the modulation of fear responses, especially with respect to the endocannabinoid system. We showed that CB_1 mediates fear adaptation following inescapable footshocks of high intensity only. Corticotropin-releasing hormone signaling or corticosterone secretion appears to be dispensable for CB_1-mediated fear adaptation, which, in contrast, critically depends on endocannabinoid-controlled glutamatergic transmission in cortical brain structures. Endocannabinoid-mediated behavioral responses to

stress and fear share similarities in that endocannabinoids are recruited in aversiveness-dependent manner and especially involved in habituation-like processes of stress and fear responses. Moreover, cortical glutamatergic transmission appears to be modulated by endocannabinoids during stress and fear habituation, whereby the exact mechanism underlying short-term and long-term habituation still remains to be determined.

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Supporting Information

The following supporting information are available for this article.

Figure S1: Different doses of rimonabant are required in different mouse strains to cause sustained fear adaptation following sensitization.

Additional Supporting Information may be found in the online version of this article.

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6.2.

Dissociation of within-session and between-session extinction of fear memories.
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Behavioral/Systems/Cognitive

Dissociation of within- and between-Session Extinction of Conditioned Fear

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Recent findings obtained in patients with phobias or trauma-related anxiety disorders raise doubts concerning the interrelation between acute fear relief during an exposure-based therapeutic session and beneficial treatment progress. In a mouse model explicit for exposure therapy, we challenge the view that within-session fear reduction is the turning point for relearning of a stimulus-threat association. Even though within-session extinction of auditory-cued fear memory was identical for prolonged and spaced tone presentations, only the latter caused between-session extinction. Furthermore, spaced tone presentations led to between-session extinction even in the complete absence of within-session extinction, as observed for remote fear memories and in case of abolished cannabinoid receptor type 1 signaling. Induction of between-session extinction was accompanied by an increase in the number of c-Fos-positive neurons within the basolateral amygdala, the cingulate cortex, and the dentate gyrus, independent of the level of within-session extinction. Together, our findings demonstrate that within-session extinction is neither sufficient nor essential for between-session extinction, thus calling for a reconsideration of current concepts underlying exposure-based therapies.

Introduction

Exposure therapy is an effective treatment of phobias and anxiety disorders (Marks and Tobena, 1990; Grös and Antony, 2006; Norton and Price, 2007). Prevailing models interpret the level of fear throughout exposure trials as an index of beneficial learning. It is generally assumed that, during an exposure session, a substantial amount of fear has to be elicited (also called "initial fear activation") and subsequently to wane (in a process called "within-session habituation") to achieve lasting fear alleviation (i.e., between-session habituation) (Craske et al., 2008). Recent findings challenge this view, indicating that performance during training may not be proportional to therapeutic progress (Pitman et al.,1996a,b; Craske et al., 2008).

Extinction of conditioned fear is the laboratory analog of exposure therapy in rodents (Rosen and Schulkin, 1998; Myers and Davis, 2002, 2007; Barad, 2005). Conditioned fear is induced by pairing a neutral environmental stimulus [conditioned stimlus (CS), e.g., tone] with an aversive outcome [unconditioned stimulus (US), e.g., electric footshock]. Re-exposure to the CS elicits a stereotypic, reproducible, and quantifiable behavioral response (conditioned response, e.g., freezing). During extinction training, repeated CS presentations in the absence of the US lead to a decay of the fear response (McSweeney and Swindell, 2002). According to current theories, this decrease does not result from temporal decay or degradation of the original excitatory memory trace but is due to an inhibitory memory trace, which is built up on re-exposure to the CS in the absence of the US. This inhibitory memory trace (CS–no US) competes with and/or suppresses the original excitatory CS–US memory trace. This notion is supported by spontaneous recovery of the fear response with the passage of time after completion of extinction training and its renewal in an environment different from the extinction context (Myers and Davis, 2002, 2007; Ehrlich et al., 2009).

We recently demonstrated that the decrease of conditioned fear over the course of a prolonged tone presentation resembles habituation-like processes (Kamprath and Wotjak, 2004), which crucially involve cannabinoid receptor type 1 (CB1) signaling (Kamprath et al., 2006, 2009). Also with this modality, CB1-deficient mice were strongly impaired, not only in within-session extinction, but also in between-session extinction of aversive memories (Marsicano et al., 2002). These phenotypes support the notion of a causal relationship of within-session (performance) and between-session (progress) extinction.

Analysis of the mechanisms underlying extinction learning and the resulting conditions that facilitate or impair extinction may help to unravel the relationship between extinction performance and extinction progress and to refine exposure-based therapies. Therefore, the present study tried to dissect within-session and between-session extinction in mice with or without intact CB1 signaling in extinction paradigms with different exposure modalities. We provide evidence that spaced extinction trials are most efficient in eliciting between-session extinction. Moreover within-session extinction turned out to be neither sufficient nor essential for between-session extinction. In addition, we demonstrate that CB1 signaling controls within-session extinction but is largely dispensable for between-session extinction. By

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combining these results, we identified anatomical signatures of extinction training.

Materials and Methods

Animals

We used a total of 118 male C57BL/6NCrl (B6N purchased from Charles River Deutschland) and 28 male CB1 receptor-deficient ($CB1^{-/-}$) mice, and 28 male wild-type littermates ($CB1^{+/+}$; sample sizes for the individual experiments are given in the figure legends). Mutant mice and littermate controls were generated/genotyped as described before (Marsicano et al., 2002) and originated from our institutional breeding stock, which had been backcrossed to the B6N strain for six generations. At an age of 6–7 weeks, mice were separated and housed individually with food and water *ad libitum* under an inverse 12:12 h dark/light cycle (light off at 9:00 A.M.). Experiments were performed at an age of 9–12 weeks.

Behavioral procedures

All experimental procedures were approved by the Committee on Animal Health and Care of the State of Bavaria (Regierung von Oberbayern) and performed in strict compliance with the European Union recommendations for the care and use of laboratory animals (86/609/CEE). Experiments were performed during the activity phase (i.e., dark phase) of the animals between 9:30 A.M. and 5:00 P.M. Animals of a given experiment derived from the same batch of mice and were tested simultaneously.

Fear conditioning. For conditioning, mice were placed in the conditioning context (Kamprath and Wotjak, 2004). Three minutes later, a tone (80 dB, 9 kHz sine wave, 10 ms rising and falling time) was presented to the animals for 20 s that coterminated with a 2 s scrambled electric footshock of 0.7 mA. Mice were returned to their home cages (hc) 60 s later.

Extinction training. Extinction procedures were adapted from Kamprath and Wotjak (2004) for permanent tone procedure, and from Herry et al. (2006) for variable interval procedure. Briefly, mice were placed in the test context, which differed from the conditioning context in material, shape, surface texture, and odor of the cleaning solution. After an initial 3 min of habituation, they were confronted either with a permanent 200 s tone [9 kHz, 80 dB, sine-wave; permanent tone (pt)] or with ten 20 s tones of the same characteristics presented with either constant intertone intervals (ci) of 20 s or variable tone intervals (vi) ranging from 20 to 180 s (mean, 80 s). Mice returned to their hc 60 s after the end of the exposure protocol (see also Fig. 2*A*).

Experiments

Experiment 1. B6N mice were conditioned at day 0 (d0) and randomly assigned to one of three groups, which differed in extinction procedures at day 1 and day 2. Group I was exposed to a single pt, group II to ten 20 s tones with ci, and group III to ten 20 s tones with vi per day. At day 3, all animals were exposed to a single 20 s tone (for details, see Fig. 2*A*).

Experiment 2. B6N animals were conditioned at day 0 and randomly assigned to one of two groups, which differed in extinction procedures at day 40 and day 41. Group I was exposed to a single pt, group II to ten 20 s tones with vi. At day 42, all animals were exposed to a single 20 s tone.

Experiment 3. For the first part of the experiment, $CB1^{-/-}$ and $CB1^{+/+}$ mice were conditioned at day 0 and were exposed to a single 200 s tone per day (pt) on days 1, 2, 3, 10, and d40. For the second part, B6N mice were conditioned at d0 and randomly assigned to one of two groups. One group was treated with vehicle (Veh), the other with the CB1 receptor antagonist SR141716 (SR; 3 mg/kg, s.c.) 1 h before exposure to a single 200 s tone (pt) on days 1, 2, 3, 10, and 40.

Experiment 4. For the first part of the experiment, $CB1^{-/-}$ and $CB1^{+/+}$ mice were conditioned at day 0 and exposed to ten 20 s tones with vi on days 1, 2, 3, 10, and 40. For the second part, B6N mice were conditioned at day 0 and randomly assigned to one of two groups. One group was treated with Veh, the other with the CB1 receptor antagonist SR (3 mg/kg, s.c.) 1 h before exposure to ten 20 s tones with vi on days 1, 2, 3, 10, and 40.

Experiment 5. B6N mice were conditioned at day 0 and randomly assigned to one of six groups, which differed in extinction procedures and drug treatment. Injection took place 1 h before exposure on day 1, when group I received a single 200 s tone (pt) after Veh injection, group II a single 200 s tone (pt) after SR injection (3 mg/kg, s.c.), group III ten 20 s tones with vi after Veh injection, and group IV ten 20 s tones with vi after SR injection. Groups V and VI also received Veh and SR injections, respectively, but were left in their hc. All mice were processed for c-Fos immunohistochemistry 70 min after the end of extinction training (or the respective time in the home cage).

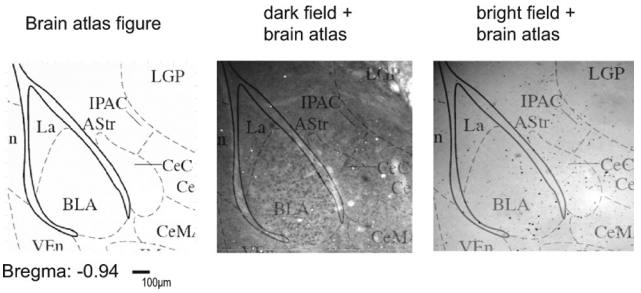


Figure 1. Localization of c-Fos-positive cells in the regions of interest. As shown exemplarily for the basolateral and central amygdala complex, ROIs were selected according to the mouse brain atlas (Franklin and Paxinos, 1997). Dark-field and bright-field pictures of the same slide were taken and overlaid with brain atlas figures to visualize the boundaries of the ROIs together with the c-Fos-positive cells.

Behavioral analysis

The behavior of the mice was videotaped and scored off-line by a trained observer who was blind to the animals' treatment/genotype. Freezing was defined as the absence of all movements, except for those related to respiration.

c-Fos immunohistochemistry

Mice were deeply anesthetized with a mixture of ketamine/xylazine and transcardially perfused. Brains were removed, postfixed in 4% formaldehyde diluted in PBS for 20 h and transferred to 1 M sucrose in PBS for another 20 h. The brains were shock frozen in isobutanol and stored at −80°C until cryosectioning. Floating frontal sections (40 μm) were incubated with an antibody raised against a peptide mapping at the N terminus of human c-Fos p62 (identical to the corresponding mouse sequence; c-Fos sc-42; 1:20000; Santa Cruz Biotechnology) for 4 d. c-Fos-immunoreactive cells were visualized using a biotinylated goat anti-rabbit secondary antibody (1:2000, Jackson Laboratory) and the ABC method (Richter et al., 2005). The number of c-Fos-immunoreactive cells was determined using a Zeiss microscope (Axiophot) and a computer program (ImageJ; http://rsbweb.nih.gov/ij/). The following brain areas were analyzed: dentate gyrus (DG) and CA3 regions of the dorsal hippocampus, basolateral amygdala (BLA), lateral amygdala (LA), central amygdala (CeA), cingulate cortex (Cg1), prelimbic cortex (PrL), and infralimbic cortex (IL) (Franklin and Paxinos, 1997) (see Fig. 6*D*). The total number of c-Fos-positive cells was counted within the regions of interest (ROIs) in both hemispheres on one representative section per mouse and ROI. Sections and ROIs were identified by combining dark-field pictures, bright-field pictures, and schematic drawings from a mouse brain atlas (Franklin and Paxinos, 1997) (for details, see Fig. 1). Sections were chosen and analyses performed unaware of the experimental history. The specificity of the staining procedure was confirmed by omission of the primary antibody (data not shown).

Drug treatment

SR141716 (Rimonabant) (Rinaldi-Carmona et al., 1994), kindly provided by the National Institute of Mental Health Chemical Synthesis and Drug Supply Program, was dissolved in vehicle solution (2.5% DMSO and 1 drop of Tween 80 per 3 ml of saline) and injected subcutaneously at 3 mg/kg body weight 1 h before each extinction training. For all groups, injections were given under light isoflurane anesthesia to avoid differences in coping with the stressful injection procedure between mice with intact and abolished CB1 signaling.

Statistical analysis

Freezing behavior was analyzed in 20 s intervals and expressed as a percentage of the respective analysis interval. The following measures were considered for describing within- and between-session extinction: (1) changes in the total freezing response to the tone(s) over the course of the extinction training days (traditionally used measure of between-session extinction), (2) changes in freezing over the course of tone presentation(s) per day (in 20 s intervals; within-session extinction), and (3) changes in the initial freezing response to the first 20 s of tone presentation per day (defined by the length of the CS during conditioning) over the course of the extinction training days (newly suggested measure of between-session extinction). Data were analyzed by one-way ANOVAs for repeated measures (time), two-way ANOVAs (protocol/genotype/treatment, time) for repeated measures (time = interval) separately per test day (analysis of the freezing data in 20 s intervals), or two-way ANOVAs (protocol/genotype/treatment, time) for repeated measures (time = analysis of the development of the total freezing responses/the initial freezing responses shown during the first 20 s of tone presentation over the course of the repeated training/testing days). c-Fos data were analyzed by two-way ANOVA (protocol, treatment) separately for each brain structure. *Post hoc* comparisons were performed by the Newman–Keuls test, if appropriate. Statistical significance was accepted if $p \leq 0.05$. Statistical analyses were performed using specialized software (GraphPad Prism 5.0, StatSoft Statistica 5.0, and SPSS version 16.0).

Results

Within-session extinction is not sufficient for initiating between-session extinction of recent fear memories

To study the interrelation of within- and between-session extinction, we conditioned male B6N mice with a single pairing of a 20 s tone with a footshock. The next 2 days, mice were re-exposed to the tone for a total of 200 s per day. Tones were presented either as a single 200 s pt or as series of 10 20 s tones with ci or vi (Fig. 2*A*). All mice were exposed to a single 20 s tone at day 3. All three groups showed the same decline in freezing on day 1 of extinction training (time: $F_{(9,243)} = 22.5$, $p < 0.001$), independently of the protocol (protocol: $F_{(2,27)} = 0.81$, $p = 0.921$; protocol × time: $F_{(18,243)} = 0.71$, $p = 0.789$) (Fig. 2*C*). Despite these similarities in within-session extinction, groups differed significantly in between-session extinction (protocol × time: $F_{(2,27)} = 6.37$, $p = 0.005$) (Fig. 2*B*) and in the development of initial freezing responses (protocol × time: $F_{(4,54)} = 6.91$, $p < 0.001$) (Fig. 2*D*). *Post hoc* analyses revealed that between-session decline in freezing was most pronounced in mice with repeated daily tone presentations at variable intervals, significantly retarded following tone presentations at constant intervals, and virtually absent in the case of permanent tone presentations. These data indicate that the predictability of tone presentations retards between-session extinction and that within-session extinction per se is not sufficient for initiating between-session extinction.

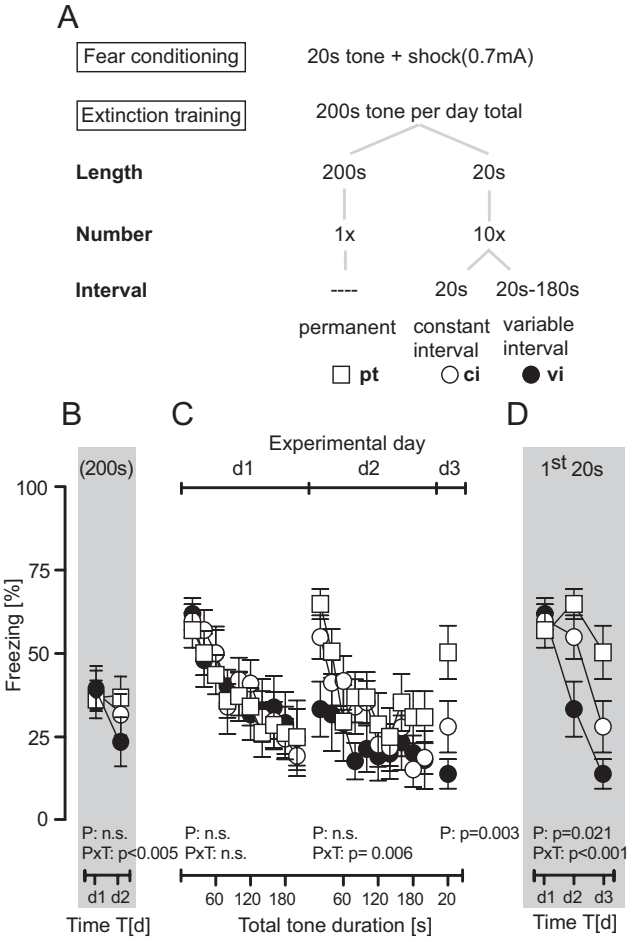


Figure 2. Extinction training as a function of stimulus length, repetition, and predictability. ***A***, Experimental schedule depicting that B6N mice received a single tone–shock pairing at d0 and were subsequently exposed to one of three tone presentation protocols at d1 and d2, followed by a final 20 s tone presentation at d3 ($n = 10$ per group). ***B***, Development of the total freezing responses over the course of repeated extinction training. ***C***, Development of the freezing responses over the course of tone presentations per day (note that freezing to the 200 s tone was analyzed in 20 s bins). ***D***, Development of the freezing responses to the initial 20 s of tone presentations per day. The duration of freezing was normalized to respective observation periods and presented as the mean ± SEM. Results of the two-way ANOVA for repeated measurements with the main factors protocol (P) and time (T) are shown in the graphs (for the sake of clarity, we do not report the results of *post hoc* analyses).

Within-session extinction is not essential for initiating between-session extinction of remote fear memories

To test whether our conclusions also apply to extinction of remote fear memories, we conditioned male B6N mice with a single tone–shock pairing at day 0 and exposed them either to a single pt of 200 s or to series of 10 20 s tone with vi at day 40 and day 41 after conditioning, followed by exposure to a single 20 s tone at day 42. Both groups showed a similar nondecaying freezing response at day 40 (protocol: $F_{(1,14)} = 0.39$, $p = 0.845$; time: $F_{(9,126)} = 1.25$, $p = 0.269$; protocol × time: $F_{(9,126)} = 1.01$, $p = 0.428$) (Fig. 3*B*). At days 41 and 42, however, mice of the vi group showed significantly less freezing than mice of the pt group ($p < 0.005$). This was reflected by reduced total freezing (protocol × time: $F_{(1,14)} = 13.96$, $p = 0.002$) (Fig. 3*A*) and significantly decreased initial freezing responses in the vi group (protocol: $F_{(1,14)} = 22.15$, $p < 0.001$; protocol × time: $F_{(2,28)} = 4.69$, $p = 0.017$) (Fig. 3*C*). Mice of the pt group failed to develop between-session extinction despite prominent within-session extinction at day 41 (Fig. 3*B*,*C*). Thus, within-session extinction is also not sufficient for initiating between-session extinction in cases of remote fear memories. Moreover, the fact that the initial freezing response decreased in the vi group from day 40 to day 41 in the absence of within-session extinction at day 40 demonstrates that within-session extinction is not necessary for initiating between-session extinction.

CB1 receptor signaling mediates within-session but not between-session extinction

To further validate our conclusions, we studied fear extinction in mice with abolished CB1 receptor signaling, an animal model of severe impairments in within-session extinction. In agreement with previous observations (Kamprath et al., 2006, 2009), both

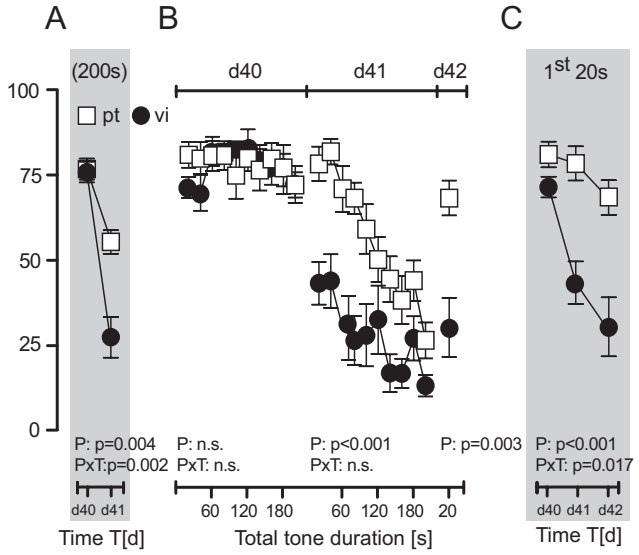


Figure 3. Extinction training after fear incubation. B6N mice received a single tone–shock pairing at d0 and were exposed to either a pt of 200 s duration ($n = 9$) or ten 20 s tones with vi ($n = 7$) at d40 and d41. At d42, all mice were exposed to a single 20 s tone. ***A***, Development of the total freezing responses over the course of repeated extinction training. ***B***, Development of the freezing responses over the course of tone presentations per day. ***C***, Development of the freezing responses to the initial 20 s of tone presentations per day. Data were normalized to the respective observation interval and expressed as means ± SEM. Results of the two-way ANOVA for repeated measurements with the main factors protocol (P) and time (T) are shown in the graphs (for the sake of clarity, we do not report the results of *post hoc* analyses).

genetic (genotype × time: $F_{(9,189)} \geq 1.92, p \leq 0.05$) (Fig. 4*B*) and pharmacological prevention of CB1 receptor signaling (treatment: $F_{(1,19)} > 7.47, p < 0.01$) (Fig. 4*E*) rendered conditioned mice unable to show within-session extinction over the course of a permanent tone presentation. Between-session extinction was considerably more pronounced in wild-type than in CB1-deficient mice if the development of the total freezing responses was considered (time: $F_{(9,84)} = 10.05, p < 0.001$; genotype: $F_{(1,21)} = 6.16, p = 0.021$; two-way ANOVA) (Fig. 4*A*). Also in vehicle-treated controls, the total freezing response was significantly smaller than in B6N mice treated with the CB1 receptor antagonist SR141716 (treatment: $F_{(1,19)} = 21.37, p < 0.001$) (Fig. 4*D*). In contrast to development of the total freezing responses, we failed to observe any significant decrease in the initial freezing responses to the tone, regardless of the genotype (genotype: $F_{(1,21)} = 1.03, p = 0.320$; time: $F_{(9,84)} = 1.68, p = 0.160$; genotype × time: $F_{(9,84)} = 1.30, p = 0.273$) (Fig. 4*C*) or treatment (treatment: $F_{(1,19)} = 7.36, p = 0.013$; time: $F_{(4,76)} = 1.84, p = 0.129$; treatment × time: $F_{(4,76)} = 0.99, p = 0.413$) (Fig. 4*F*) of the animals. These data corroborate our previous findings that within-session extinction is not sufficient for between-session extinction even under circumstances of more intensified extinction training.

Genetic deletion or pharmacological blockade of CB1 also prevented within-session extinction in the case of extinction training by repeated 20 s tone presentations at variable intervals (day 1: $F_{(9,135)} = 1.43, p = 0.179$, in the case of CB1$^{-/-}$ mice; day 1 and day 2: $F_{(9,81)} < 1.98, p > 0.05$, in the case of treatment with SR141716), except for day 2 in CB1$^{-/-}$ mice, when within-session extinction was attenuated but not abolished ($F_{(9,135)} = 2.10, p = 0.038$, one-way ANOVAs performed separately per group) (Fig. 5*B*,*E*). In contrast, wild-type and vehicle-treated controls showed prominent within-session extinction at day 1 and day 2 ($F_{(9,144)} > 9.69, p < 0.0001$, in the case of CB1$^{+/+}$ mice; $F_{(9,99)} > 3.62, p < 0.001$, in the case of vehicle treatment), before they reached floor levels of freezing behavior (d3). Accordingly, freezing responses were always significantly more pronounced in wild-type (genotype: $F_{(1,31)} > 5.93, p < 0.020$, d1, d2, d3, and d40) (Fig. 5*B*) and vehicle-treated mice (treatment: $F_{(1,20)} > 9.89, p < 0.005$, d1 to d40) (Fig. 5*E*). Despite the impairments in within-session extinction, genetic deletion of CB1 failed to affect between-session extinction. This became evident if the development of the total freezing responses (genotype: $F_{(1,31)} = 12.13, p = 0.001$; time: $F_{(4,124)} = 111.5, p < 0.001$; genotype × time: $F_{(4,124)} = 0.89, p = 0.470$) (Fig. 5*A*) or the initial freezing responses (genotype: $F_{(1,31)} = 3.44, p = 0.072$; time: $F_{(4,124)} = 74.98, p < 0.001$; genotype × time: $F_{(4,124)} = 1.89, p = 0.115$) (Fig. 5*C*) were considered. Essentially, the same was the case after pharmacological blockade of CB1 (Fig. 5*D*,*F*) (statistics not shown). All animals showed spontaneous recovery of the freezing response from day 10 to day 40, which was more pronounced in antagonist-treated mice, if development of the total freezing response was considered (treatment × time: $F_{(4,72)} = 3.81, p = 0.007$) (Fig. 5*D*). Together, these data demonstrate that CB1 receptors play an important role in within-session extinction not only during exposure to permanent tones, but also with repeated tone presentations at variable intervals, without affecting between-session extinction. This strengthens our previous conclusion that within-session extinction is neither necessary nor sufficient for between-session extinction.

Neuroanatomical basis of relearning

By combining pharmacological blockade of CB1 receptors with the pt and vi protocols, we ought to dissociate anatomical substrates of within-session and between-session extinction. In agreement with our previous observations, a three-way ANOVA (protocol, drug, time) for repeated measures (time) confirmed that mice failed to show within-session extinction after treatment with SR141716, whereas vehicle-treated controls showed a rapid decline in freezing (drug: $F_{(1,36)} = 98.88, p < 0.001$; drug × time: $F_{(9,324)} = 34.51, p < 0.001$), with no differences between the two extinction protocols (protocol: $F_{(1,36)} = 0.043, p = 0.835$; protocol × drug: $F_{(1,36)} = 0.044, p = 0.834$; protocol × time: $F_{(9,324)} = 1.33, p = 0.216$; protocol × drug × time: $F_{(9,324)} = 1.19, p = 0.300$) (Fig. 6, insets). Freezing behavior before tone presentations was negligible and unaffected by pharmacological treatment (Fig. 6, insets) (statistics not shown).

Consequences of permanent versus repeated tone presentations and pharmacological treatment on neuronal activity were assessed by counting the number of c-Fos-immunoreactive neurons (Fos-ir). We focused our attention on those brain areas that are known to be involved in fear and relearning processes, namely, the hippocampal formation, the amygdala complex, and the prefrontal cortex (Singewald, 2007). Two-way ANOVAs with the factors protocol (presentation of permanent tone vs repeated tone presentations vs home-cage controls) and treatment (SR141716 vs vehicle) revealed highly significant main effects of protocol for most of the brain structures under study ($F_{(2,27)} > 3.84, p < 0.05$) (Fig. 6*C*). *Post hoc* analyses failed to detect any significant differences in Fos-ir between mice exposed to the permanent tone and home-cage controls, except for the CA3 region of the hippocampus and the lateral amygdala (Fig. 6*A*,*B*). Repeated exposure to 20 s tones, in contrast, led to a significant increase in Fos-ir in the dentate gyrus, the basolateral and lateral amygdala, the cingulate cortex, and the prelimbic cortex, compared with mice exposed to a single 200 s tone and to home-cage controls. As indicated by the lack of protocol × treatment interactions in most of the brain structures ($F_{(2,27)} < 2.17, p > 0.133$,

except for IL) (Fig. 6*C*), these activity patterns were largely independent of treatment with the CB1 receptor antagonist. However, pharmacological blockade of CB1 led to a general increase in Fos-ir within the CA3 region (weak effect; $F_{(1,27)} = 5.98$, $p = 0.021$), lateral amygdala, central amygdala, and prelimbic cortex (strong effects; $F_{(1,27)} > 21.7$, $p < 0.001$) (Fig. 6*A*,*B*), independently of the protocol used (i.e., even in home-cage controls). Only in the case of the infralimbic cortex was there a significant interaction between protocol and treatment ($F_{(2,27)} = 16.95, p < 0.001$). Whereas vehicle-treated mice with repeated tone presentation showed increased Fos-ir compared with the two other groups, this difference was abolished by treatment with the CB1 receptor antagonist. Together, freezing to the tone in vehicle-treated mice and precipitation of exaggerated freezing by pharmacological blockade of CB1 failed to reveal significant differences in Fos-ir between mice exposed to the permanent tone and respective home-cage controls. Consequently, expression of freezing per se was not reflected by Fos-ir in the brain structures under study. Repeated tone presentations at variable intervals led to a significant increase in Fos-ir in a number of brain structures, compared with mice exposed to a permanent tone. In the dentate gyrus, basolateral amygdala, and cingulate cortex, these differences were largely independent of the pharmacological blockade of CB1 and within-session extinction of freezing behavior, suggesting those brain structures as primary places of between-session extinction. Finally, some brain structures, such as the prelimbic cortex and central amygdala, showed a general increase in Fos-ir on pharmacological blockade of CB1 even in home-cage controls, thus indicating their general involvement in processing of stressful encounters (e.g., injection procedure).

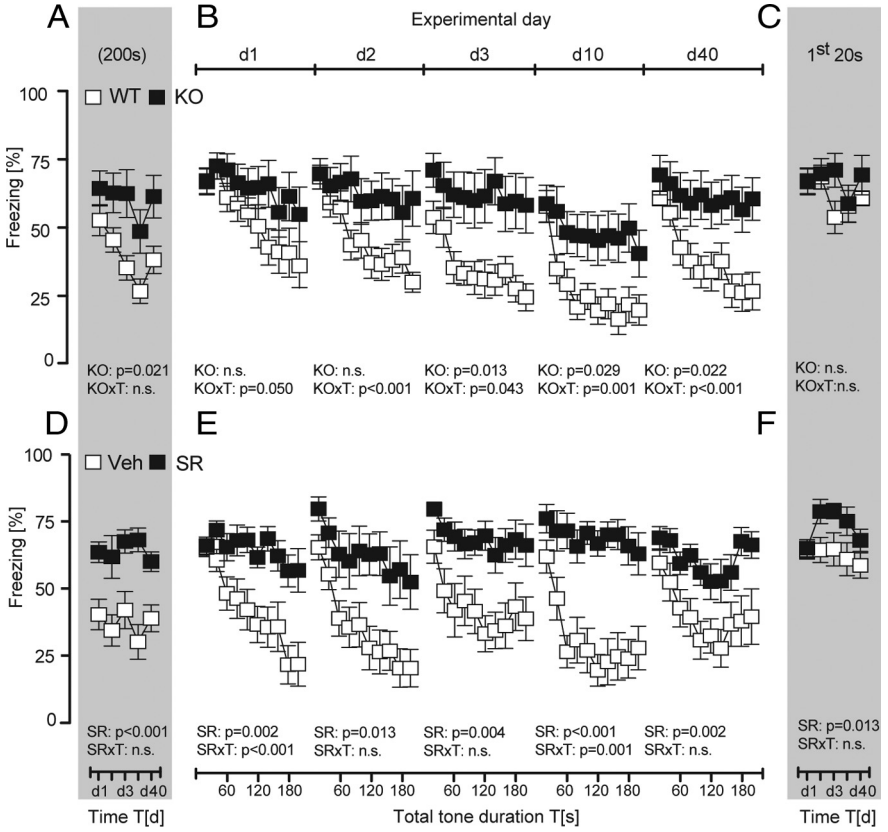


Figure 4. Role of CB1 in extinction training with permanent tones. All animals received a single tone–shock pairing at d0 and were subsequently exposed to the pt protocol (compare Fig. 2*A*) from d1 to d40. ***A–F***, CB1 receptor signaling was abolished by either genetic means (***A–C***) or the CB1 receptor antagonist SR141716 (3 mg/kg, s.c.), which was administered to B6N mice 1 h before tone presentations at d1 to d40 (***D–F***). Analysis of the development of the total freezing responses over the course of repeated extinction training (***A***, ***D***), the development of the freezing responses over the course of tone presentations per day (analyzed in 20 s bins) (***B***, ***E***), and the development of the freezing responses to the initial 20 s of tone presentations per day (***C***, ***F***) of CB1-deficient mice (KO; $n = 12$), wild-type littermates (WT; $n = 11$), and B6N mice with pharmacological blockade of CB1 (SR; $n = 11$) or vehicle treatment ($n = 10$). Data were normalized to the respective observation interval and expressed as means ± SEM. Results of the two-way ANOVA for repeated measurements with the main factors genotype (KO), treatment (SR), and time (T) are shown in the graphs (for the sake of clarity, we do not report the results of *post hoc* analyses).

Discussion

The present study investigated the interrelation between within-session and between-session extinction in an auditory-cued fear-conditioning paradigm. We showed that within-session extinction was the same for permanent and spaced tone presentations. It required intact CB1 signaling and was neither sufficient nor necessary for initiating between-session extinction. Between-session extinction, in contrast, depended on the pattern and predictability of tone presentations. It was maintained in mice with disrupted CB1 receptor signaling and developed independently of within-session extinction. The dissociation of within- and between-session extinction could also be seen at the neuroanatomical level in terms of c-Fos expression.

The striking similarities in within-session extinction in response to permanent (pt) and spaced (ci and vi) tone presentations at day 1 indicate that the decline in freezing over the course of a given extinction session likely relates to the total duration and not to the pattern of tone presentation. It might result from the same short-term habituation processes (Kamprath and Wotjak, 2004). Only spaced tone presentations led to between-session extinction. Apparently, repeated experience of the tone–no-shock association (vi and ci) is more efficient in establishing an inhibitory memory trace than a single tone presentation per day (pt). Consequently, the absence of the predicted punishment rather than acute fear relief per se seems to cause the formation of a CS–no-US association.

Repeated tone presentations at vi were more efficient in eliciting between-session extinction than tone presentations at ci. This might be explained by the role of attention in extinction learning. For instance, some authors propose that focusing attention on the fear stimulus favors fear reduction (Kamphuis and Telch, 2000), which, however, is disputed by others (Oliver and Page, 2003). In the present study, unexpected stimulus presentation may raise attention toward the tone, whereas tone presentations at regular intervals may favor stimulus expectancy and, thus, reduce attention. In this context, it is of note that temporal predictability could promote habituation of neuronal activity in brain structures such as the lateral and basolateral amygdala

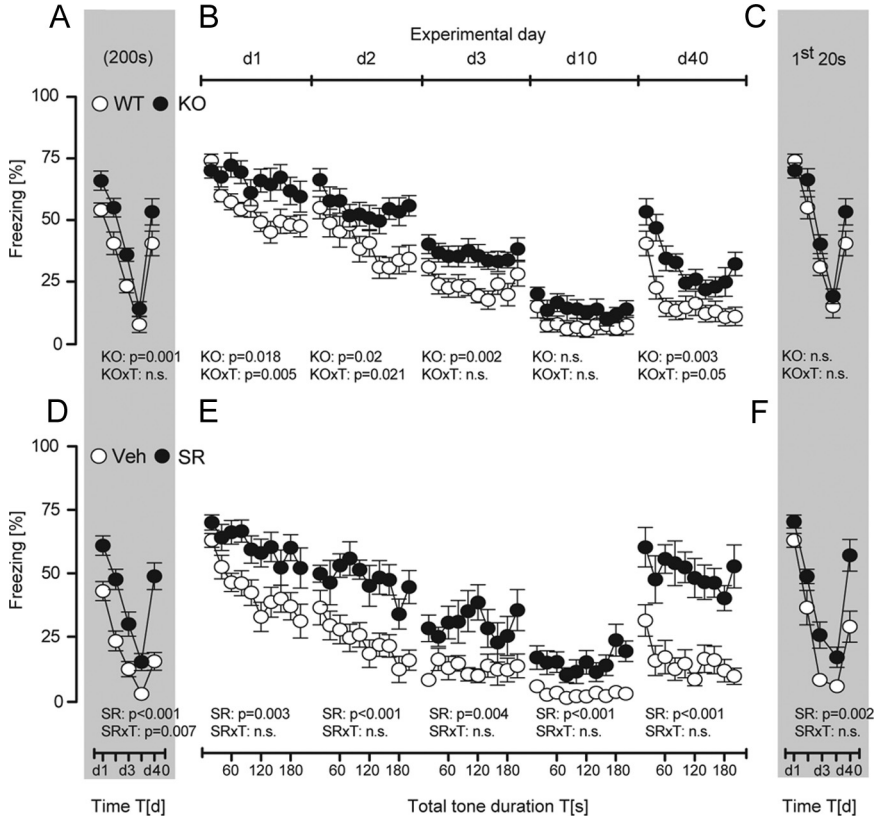


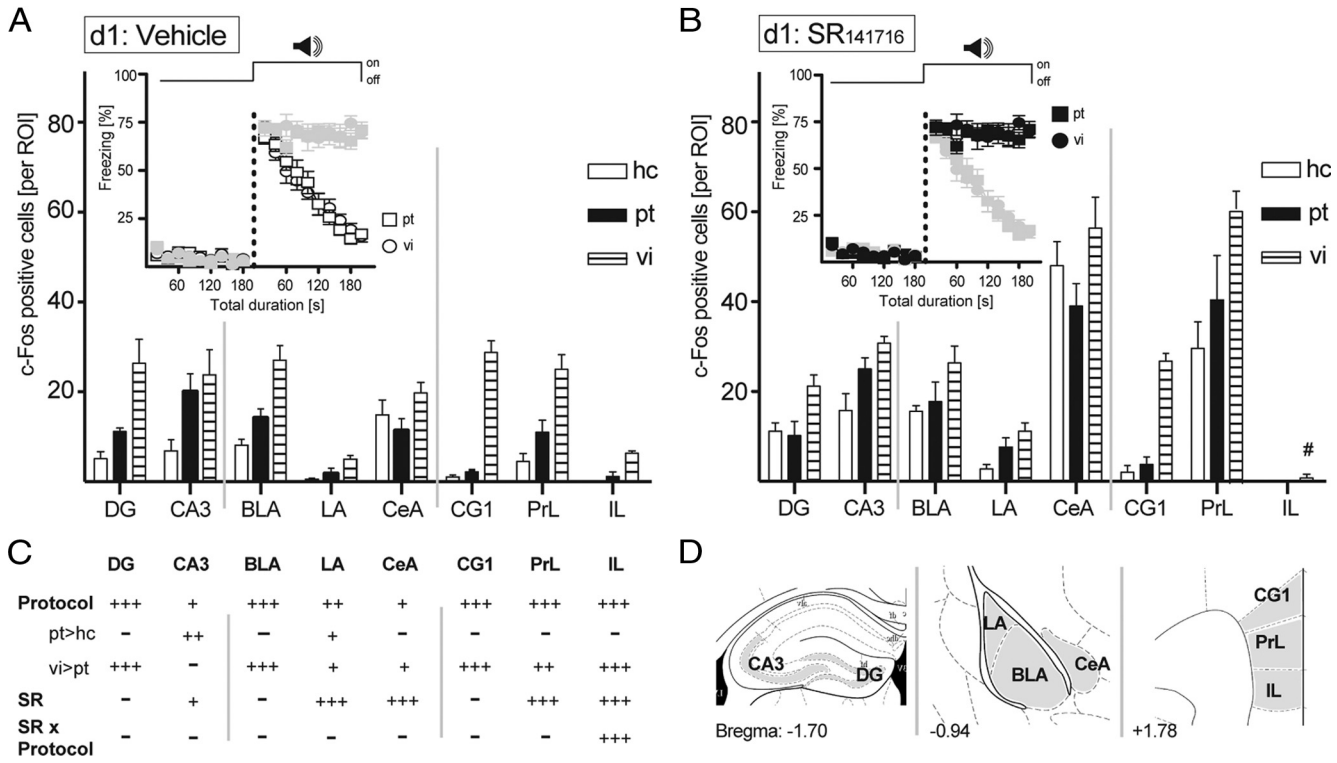
Figure 5. Role of CB1 in extinction training with repeated tone presentations at variable intervals. All animals received a single tone–shock pairing at d0 and were subsequently exposed to ten 20 s tones at vi (compare Fig. 2*A*) from d1 to d40. ***A–F***, CB1 receptor signaling was abolished either by genetic means (***A–C***) or by the CB1 receptor antagonist SR141716 (3 mg/kg, s.c.), which was administered to B6N mice 1 h before the first tone presentation at d1 to d40 (***D–F***). Analysis of the development of the total freezing responses over the course of repeated extinction training (***A***, ***D***), the development of the freezing responses over the course of tone presentations per day (***B***, ***E***), and the development of the freezing responses to the first 20 s tone per day (***C***, ***F***) of CB1-deficient mice (KO; $n = 16$), wild-type littermates (WT; $n = 17$), and B6N mice with pharmacological blockade of CB1 (SR, $n = 10$) or vehicle treatment ($n = 12$). Data were normalized to the respective observation interval and expressed as means ± SEM. Results of the two-way ANOVA for repeated measurements with the main factors genotype (KO), treatment (SR), and time (T) are shown in the graphs (for the sake of clarity, we do not report the results of *post hoc* analyses).

(Herry et al., 2007), which had been implicated in extinction learning (Falls et al., 1992; Herry et al., 2008).

Fear incubation for several weeks led to a sustained freezing response to the tone. Other than for contextual fear memory (Woods and Bouton, 2008), little is known about the dependency of fear extinction on the age of cued fear memories. The only exceptions are studies investigating consequences of extinction training initiated early after conditioning (e.g., Myers et al., 2006; Kim and Richardson, 2009). Here, we demonstrate that mice showed decaying freezing 1 d after but sustained freezing 40 d after the tone–shock pairing, regardless of the tone presentation protocol (pt vs vi). Contextual fear memory is known to undergo major reorganization between recent and remote stages (Frankland et al., 2004). For instance, remote contextual memories are stored in distributed cortical networks (Woods and Bouton, 2008). It remains to be shown whether auditory-cued fear memory undergoes similar changes and whether these changes are the cause for the less efficient within-session extinction at late time points after conditioning.

Despite the complete absence of within-session extinction at day 40, tone presentation at variable intervals readily elicited between-session extinction, thus indicating that within-session extinction is not only insufficient (as discussed before), but also dispensable for initiation of between-session extinction. This finding points to an uncoupling of fear expression and cognitive appraisal of changes in CS–US contingencies, which is supported by the results obtained in mice with genetic deletions or pharmacological blockade of CB1 receptors. Those interventions abolished or severely attenuated within-session extinction but failed to affect between-session extinction triggered by repeated tone presentations at variable intervals. The phenotype seen in within-session extinction precludes that the lack of involvement of CB1 in between-session extinction relates to inefficient activation of the endocannabinoid system (Kamprath et al., 2009). It rather implies that CB1 signaling is dispensable for relearning of the CS–US contingency, similarly to the situation in appetitive conditioning tasks (Hölter et al., 2005). This conclusion seemingly contradicts numerous studies reporting a significant role of the endocannabinoid system in between-session extinction (Viveros et al., 2007; Moreira and Lutz, 2008). To resolve this issue, we propose that extinction of elemental fear memories follows different rules than extinction of contextual/hippocampus-dependent memories. Furthermore, the impairments of CB1-deficient mice in between-session extinction of auditory-cued fear memories reported before (Marsicano et al., 2002) might be explained by an involvement of the endocannabinoid system in habituation to homotypic stressors (Patel and Hillard, 2008). Repeated confrontation with the same aversive encounter is accompanied by increased endocannabinoid signaling, which facilitates acute habituation to the stressor (Patel et al., 2005b). A similar scenario may apply to fear extinction (Kamprath et al., 2006), where group differences between CB1-deficient mice and wild-type controls became stronger on repeated tone presentation (Fig. 4*B*). This accelerated within-session extinction seemingly results in between-session extinction, if development of the total freezing responses is considered (Fig. 4*A*), but does not affect the initial freezing responses per day (Fig. 4*C*). Our failure to observe a similar acceleration of within-session extinction in vehicle-treated controls (Fig. 4*D*) might be explained by summative effects of exposure to the tone and stressful injection procedure, which may result in an almost maximal activation of the endocannabinoid system right from the beginning of extinction training.

If extinction training indeed leads to relearning of the tone–shock contingency and the formation of a new inhibitory tone–no-shock association that suppresses the expression of the original memory trace (Myers and Davis, 2002, 2007; Ehrlich et al., 2009), we should expect decreased fear responses right from the beginning of the tone presentation. Therefore, we propose that the development of the initial fear responses to the tone over the course of repeated extinction training represents the most direct measure of between-session extinction. This measure is superior to analysis of total fear responses, since it is not con-



	DG	CA3	BLA	LA	CeA	CG1	PrL	IL
Protocol	+++	+	+++	++	+	+++	+++	+++
pt>hc	–	++	–	+	–	–	–	–
vi>pt	+++	–	+++	+	+	+++	++	+++
SR	–	+	–	+++	+++	–	+++	+++
SR x Protocol	–	–	–	–	–	–	–	+++

Figure 6. Neuroanatomical signature of extinction training. B6N mice received a single tone–shock pairing at d0 and were exposed to either a pt of 200 s duration (compare Fig. 2*A*) or to ten 20 s tones at vi (compare Fig. 2*A*), or stayed in their hc 24 h later (d1). ***A***, ***B***, Half of the animals of each group were treated with vehicle (***A***), and the other half were treated with the CB1 receptor antagonist SR141716 (3 mg/kg) (***B***) 1 h before tone presentation (in the case of pt and vi). All animals were killed 70 min after the end of the exposure (or after the same time in the home cage), and the brains were processed for c-Fos immunohistochemistry (***A***, ***B***). Insets show the development of the freezing responses before and during tone presentations, whereby freezing data of the animals of the same treatment groups as used for immunohistochemistry in the respective figure panel are shown in black and those of the other treatment groups in gray. ***C***, Results of the two-way ANOVAs with the main factors protocol (P) and treatment (SR), performed separately per brain structure ($^{-}p > 0.05$; $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$; $^{\#}$significant SR–protocol interaction). ***D***, The total number of c-Fos-positive neurons was counted bilaterally in the ROIs in one section per mouse. ROIs were defined according to the mouse brain atlas (Franklin and Paxinos, 1997; compare Fig. 1). Values are expressed as the mean ± SEM ($n = 5$ per group).

founded by differences in within-session extinction (as discussed before). Accordingly, the reoccurrence of the freezing response with the passage of time (i.e., spontaneous recovery) should be assessed by means of the initial fear responses rather than the total fear responses. In any case, comparisons of freezing responses shown at the end of an extinction session and the beginning of the next have to be avoided because of the apparent independence of within-session and between-session extinction revealed by the present study.

We could dissociate within-session and between-session extinction not only at the behavioral and molecular levels (in terms of CB1 signaling), but also at the anatomical level. Exposure to tones at variable intervals caused increased Fos-ir in the lateral and basolateral amygdala, compared to mice with exposure to permanent tones and to home-cage controls. A similar pattern of Fos-ir was observed in the dentate gyrus, the cingulate cortex, and the prelimbic cortex. However, only in the basolateral amygdala, dentate gyrus, and cingulate cortex, Fos-ir was largely independent of pharmacological blockade of CB1 receptors and/or differences in expression of conditioned fear. Given the fact that between-session extinction was unaffected by impairments in CB1 signaling (Fig. 5*C*,*F*), these three brain structures might represent hot spots for relearning processes, which involve plastic changes in neuronal activity. This conclusion is supported by pharmacological studies reporting, for instance, that local inhibition of NMDA receptors (Falls et al., 1992) or mitogen-activated protein kinase activity (Herry et al., 2006) within the basolateral amygdala impairs between-session extinction. Recently, Hefner et al. (2008) performed a thorough investigation of the neuronal matrix underlying extinction recall. They studied Fos-ir in extinction-prone and extinction-resistant mouse strains and reported significant strain differences primarily within the basolateral amygdala and the infralimbic cortex. Consequently, the formation of a new tone–no-shock association and recall of this inhibitory memory trace seems to involve, at least in part, the same brain structures.

Pharmacological blockade of CB1 led to a large increase in Fos-ir primarily within the central amygdala and prelimbic cortex, regardless of the exposure protocol, i.e., even in home-cage controls. These data provide compelling evidence that not only freezing responses elicited by electrical stimulation or pharmacological manipulation of distinct brain structures (Vianna et al., 2003; Borelli et al., 2006), but also expression of conditioned fear fails to induce c-Fos expression in a variety of brain structures known to be implicated in fear and anxiety (Singewald, 2007). Other markers of neuronal activity, such as phosphorylation of kinases, may better reflect differences in freezing behavior (in particular if analyzed in the absence of injection stress in CB1-KO and CB1-WT mice) (Cannich et al., 2004). Previous studies reported an increase in Fos-ir in the central amygdala of naive mice following pharmacological blockade of CB1 (Patel et al., 2005a). In addition, CB1 plays a complex role in the regulation of fear and anxiety within the medial prefrontal cortex (Lin et al., 2009), and neuronal activity within the prelimbic cortex seems to directly

relate to acute fear expression (Vidal-Gonzalez et al., 2006; Burgos-Robles et al., 2009). Therefore, the most parsimonious interpretation of our data is that c-Fos expression at the level of the CeA and PrL reflects impaired endocannabinoid signaling, likely because of exaggerated stress responses and discomfort resulting from the injection procedure (Steiner and Wotjak, 2008). Freezing performance per se is not mirrored by c-Fos. At the level of the BLA, DG, and Cg1, finally, increased c-Fos expression reflects relearning of tone–shock contingencies (i.e., between-session extinction), independently of the acute freezing response (i.e., within-session extinction) and/or disturbed CB1 receptor functioning.

The infralimbic cortex was the only brain structure where we observed a significant interaction between tone presentation and antagonist treatment. Induction of between-session extinction by repeated exposure to tones at variable intervals caused a significant increase in Fos-ir within that brain structure, compared with home-cage controls and exposure to a permanent tone. Pharmacological blockade of CB1 abolished these effects. This is a striking observation, since the infralimbic cortex undergoes plastic changes during relearning/extinction training (Santini et al., 2008), which are thought to underlie its subsequent involvement in suppression of conditioned fear during extinction recall (Milad and Quirk, 2002). However, in light of the largely intact between-session extinction observed in mice with pharmacological blockade of CB1 (Fig. 5*F*), attenuation of Fos expression following treatment with SR141716 might be of minor biological significance for the behavioral phenotype in the present study. It remains to be shown to what extent the differences in Fos-ir in the infralimbic cortex contribute to the reduced retention of fear extinction observed in these animals if alterations in spontaneous recovery of freezing from day 10 to day 40 are considered (Fig. 5*D*,*F*).

Together, our data reveal an uncoupling between the expression of conditioned fear and the relearning of tone–shock contingencies at molecular, anatomical, and behavioral levels. This asks for reconsideration of current strategies in exposure-based therapies in human subjects. As proposed by Craske et al. (2008), within-session fear reduction does not predict therapy success. The apparent dissociation of emotional and cognitive processing disproves exposure paradigms in which the length of an exposure session is defined by the time point of maximal acute fear relief. Protocols should rather favor fast and effective buildup of inhibitory memory traces regardless of acute fear levels. Pharmacological prolongation of endocannabinoid signaling (Di Marzo, 2008) may not directly affect the inhibitory learning process, but may reduce the emotional load during an exposure session and, thus, increase compliance rates.

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6.3.

PKC activity is essential for maintenance of
aquired fear memory during reconsolidation

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PKC activity is essential for maintenance of a fear memory trace during reconsolidation

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Abstract

When retrieved, well consolidated memories are returned to a fragile state and need to be consolidated again in order to be maintained. This process is refered to as reconsolidation. Recently it was shown that during this period fear memories can be edited out. However most drugs interfering with reconsolidation have severe side effects and cannot be readily used in humans. In our study we inhibited PKC activity during extinction training and showed that it caused a prolonged increase of between-session extinction and abolished spontaneous recovery. By c-Fos immunohistochemistry we could show a specific, drug dependent, down-regulation of the cingulate cortex, irrespective of exposure protocols. The results suggest, that PKC activity in the CG1 could mediate mechanisms, which lead to relapse after fear extinction.

Introduction

A newly formed memory is stabilized for long term storage through a process called memory consolidation. During consolidation transcription and *de novo* protein synthesis occur and inhibition of either of these processes specifically blocks long term memory. It was long considered that a memory undergoes consolidation only once and that subsequently it would be permanent and unmodifiable. However this theory has been challenged by the finding that reactivation of a consolidatied memory can return it to a labile state. Thus, memory reactivation induces a second consolidation process which has been named reconsolidation (Alberini, 2005; Alberini et al., 2006; Monfils et al., 2009; Nader and Einarrson, 2010).

We all have memories that we would rather forget. Most of us learn to cope with these memories but some of us cannot. Research on changing fears has highlited several techniques with most relying on the inhibition of the learned fear response. A problem with theses techniques is that the fear is likely to return, even after a significant fear reduction at the end of an exposure paradigm. Recent research showed that the return of fear can be prevented by update mechanisms which induce reconsolidation (Schiller et al., 2010). These findings demonstrate the role of reconsolidation as a window of opportunity to rewrite emotional memories.

A likely canidate for beeing involved in reconsolidation processes is the PKC familiy of the Ser/Thr kinases. Protein Kinase C (PKC) is involved in synaptic remodelling, induction of protein synthesis and many other processes important in learning and memory (Olds et al., 1989; Alkon, 1998; Bonini 2005 and 2007). Tronson et al. reported that if mice were injected with a PKC inhibitor after context fear conditioning but before extinction, fear decrease was facilitated. The effect was more pronounced in extinction prone mice (Tronson et al., 2007). Also PKC is reported to modulate NMDA receptor translocation. Animal studies have identified N-methyl-D-aspartate (NMDA) receptors as crucial in fear memory acquisition and consolidation, as well as in its extinction and reconsolidation. Based on this, the NMDA receptor partial agonist D-cycloserine, which facilitates fear extinction in rodents, has been shown to increase the effect of exposure therapy in psychiatric patients for conditions such as phobias, social anxiety and obsessive-compulsive disorder (Amaral and Roesler, 2008).

Understanding the influences of PKC activity during fear extinction consolidation and reconsolidation could led a step further in treatment of anxiety disorders.

In a recent study (Plendl and Wotjak, 2010) we compared two extinction paradigms. Both

produced within session extinction, but only one elicited between extinction consolidation and led to a decrease of initial fear level in further extinction sessions. Reconsolidation, which is definded as a reactivation of the original memory trace, should occur in both paradigms. So we should be able to differentiate between two mechanisms. First, the extinction consolidation process, which occurs only after an appropriate extinction training, and secondly, the reconsolidation process, which occures independently of the extinction training effectivity. For inhibition of PKC activity we used GF109203X, which is reported to be highly specific (8-20 nM IC_{50}). By using the above mentioned two different extinction protocols we should be able to dissociate the influence of PKC activity on consolidation and reconsolidation .

Materials and methods

Animals

We used male C57BL/6NCrl (B6N, purchased from Charles River Deutschland, Bad Sulzfeld, Germany). At an age of 6-7 weeks, mice were separated and housed individually with food and water ad libitum under an inverse 12:12 h dark-light cycle (light off at 09:00 a.m.). Experiments were performed at an age of 9-12 week.

Behavioural procedures

All experimental procedures were approved by the Committee on Animal Health and Care of the State of Bavaria (Regierung von Oberbayern) and performed in strict compliance with the European Union recommendations for the care and use of laboratory animals (86/609/CEE). Experiments were performed during the activity phase (i.e. dark phase) of the animals between 9:30 a.m. and 5:00 p.m. Animals of a given experiment derived from the same batch of mice and were tested simultaneously.

Fear Conditioning: For conditioning, mice were placed in the conditioning context. Three minutes later, a 20s tone (80 dB, 9 kHz sine wave, 10 ms rising and falling time) was presented to the animals that co-terminated with a 2s scrambled electric foot shock of 0.7 mA. Mice were returned to their home cages 60s later.

Extinction training: Extinction procedures were adapted from Plendl and Wotjak (2010). Briefly, mice were placed in the test context, which differed from the conditioning context in material, shape, surface texture and odor of the cleaning solution. After initial 3 minutes of habituation, they were confronted either with a permanent 200s tone (9kHz, 80dB, sine-wave; pt, see Figure 1) or ten 20s tones, of the same characteristics, with varibale inter-tone intervals of 20 to 180s (vi; see Figure 2).

Experiments

For a detailed description of behavioural experiment procedures please refer to figure legends.

Behavioral analysis

The behavior of the mice was videotaped and scored off-line by a trained observer who was blind to the animals' treatment. Freezing was defined as the absence of all

movements, except for those related to respiration.

c-Fos immunohistochemistry

For detailed description of method see Plendl and Wotjak (2010) and figure legend (Figure 3). Briefly, floating frontal cryosections (40µm) were incubated with c-Fos antibody (1:20000; Santa Cruz Biotechnology Inc., Santa Cruz, CF, USA) for 4 days. C-Fos immunoreactive cells were visualised using a biotinylated goat-anti rabbit secondary antibody (1:2000, Jackson Lab) and the ABC-method (Richter et al., 2005).

Drug treatment

The PKC inhibitor GF109203X-hydrochloride (Sigma, Aldrich) was freshly aliquotet before each experiment in DMSO. Aliquots were freshly diluted before each experimental day in Ringer´s solution to reach the final concentration of 10 pmol/µl. A volume of 2µl of stock solution or control (2µl DMSO in Ringer´s solution) was injected 1h before experiments. The concentration for i.c.v. injection was based on previous reports (Stemmelin et al., 1999; Blank et al., 2003).

Surgery

During surgery mice were deeply anaesthetised by inhalation of isoflurane. 1 hour before the procedure they recived aN injection of Ketamin. Body temperature was kept at a constant 36°C by a heating pad. The skull of the animal was exposed and a guide canula was implanted (1.0mm lateral to bregma, anterior 0.3mm, ventral 1.2mm). Additonally a screw was inserted into the skull. The guide cannula was fixed to the skull and the screw by dental cement. The wound was desinfected and closed with sutures. Mice were allowed to recover for 10-14 days before experiment and recieved analgetic treatment (Metacam) for 7 days after surgery via drinking water.

Statistical analysis

Freezing behavior was analyzed in 20s intervals or for the entire tone presentation(s) per day and expressed as a percentage of the respective analysis interval. c-Fos data were analyzed by 2-way ANOVA (Protocol, Drug) separately for each brain structure and 3-way MANOVA (Protocol, Drug, Tone). Statistical significance was accepted if $p < 0.05$. Statistical analyses were performed by specialized software [GraphPad Prism 5.0 (GraphPad, San Diego, CA), Statistica 5.0 (StatSoft, Tulsa, OK), and SPSS version 16.0 (SPSS, Chicago, IL)].

Results

Inhibition of PKC-activity specifically promotes fear exinction in a time dependent manner and prevents spontaneous recovery and renewal

Even though vehicle-treated mice showed prominent within-session exinction upon exposure to a permanent tone at d1 and d2 after conditioning as well as on subsequent days in absence of treatment (d3, d40; $F_{9,45}$<2.3; p<0.025; Figure 1A), between-session extinction (i.e. a decrease in the freezing response to the initial 20s of tone presentations per day over the course of repeated training) was virtually absend ($F_{4,24}$=0.75; p=0,56). Treatment with GF109203X-hydrochloride (GF) before presentation of the permanent tone at d1 and d2 not only accelerated within-session extinction (d1: *Drug x Time*: F9,117=2.72; p=0.006), but also established between-session extinction ($F_{4,28}$=9.96, p<0.005; *Drug x Day*: $F_{4,28}$=9.96; p<0.005). Interestingly, there was no spontaneous recovery of the freezing response from d10 to d40 ($F_{1,11}$=0.40, p=0.53).

We repeated this experiment with different groups of mice, but exposed the animals to the permanent tone in the original conditiong context at day 11 after conditiong (Figure 1B). Again, treatment with GF triggered between-session extinction (*Drug x Day*: $F_{6,54}$=8.18, p<0.005), which became most prominent at day 10 (*Drug x Time*: $F_{9,126}$=34.7, p<0.005). However, we failed to aberve renewal of the fear response at the next day upon testing in the conditioning context (*Day:* $F_{1,7}$=0.162, p=0.7; *Day x Time*: $F_{9,63}$=0.716, p=0.693; Figure 1B). Contextual fear (as assessed by the freezing levels before tone presentation at d11) was indistinguishable between the two groups (GF: 28.4±8.5 vs. Veh: 38.02±7.4; $F_{1,14}$=0.73, p=0,41). Compared to the last 20s of baseline freezing, vehicle controls showed a significant increase in freezing druing the first 20s of presentation ($F_{1,8}$=23.51, p<0.005), whereas GF treated mice failed to do so ($F_{1,6}$=0.75, p=0.48; Drug x Tone: $F_{1,14}$=7.4, p=0.02; data not shown), indicating that GF treated mice failed to show renewal of the fear response.

Drug treatment without re-activation of the fear memory at d1 and d2 had no consequences on expression of conditioned fear at d3 after conditioning, except for a slightly higher initial freezing response (*Drug x Time*: $F_{9,243}$=1.86, p=0.057; Figure 1C). The same thing happenend, if mice were treated before exposure to a single 20s instead of the 200s at d1 and d2 (d3; F1,9=0.86, p=0.37; Figure 1D). Acquisition of conditioned fear was unaffected by GF treatment (d1; *Drug x Time*: $F_{9,171}$=0.94, p=0.84; Figure 1 E). Finally, GF failed to promote irrelevance learning (d1; *Drug x Time*: $F_{9,180}$=1.6, p=0.12; Figure 1F).

Inhibition of PKC activity before extinction training causes persistent attenuation of fear memories.

In accordacne with our previous observations (Plendl and Wotjak, 2010), extinction training of vehicle controls with ten 20s tone presentations per day at variable intervals was highly efficient in triggering between-session extinction (d1-d10; $F_{3,24}$=55.66, p<0.005). GF treatment before extinction training at d1 and d2 failed to affect wihtin- and between-session extinctoin (statistics not shown; Figure 2). However, whereas vehicle controls showed prominent spontaneous recovery of the freezing response to the initial 20s of tone presentation from d10 to d40 ($F_{1,12}$=37.4, p<0.005), GF treated mice failed to do so ($F_{1,12}$=0.008, p=0.92; Drug x Day: $F_{1,16}$=66.26, p<0.005).

Activity in CG1 is decreased by PKC inhibition, independent of exposure protocol.

We employed c-Fos immunohistochemistry to identify the brain regions involved in the GF effects on extinction of conditioned fear (Figure 3). Conditioned mice were treated with GF or vehicle (Factor *Drug*) 1 h prior to exposure to a permanent 200s tone (series 1) or to ten 20s tones at variable intervals (series 2; Factor *Protocol*). Exposure controls were treated in a similar manner, but exposed to the extinction context without tone presentations (Factor *Tone*). Since experiments were performed in two independent series (permanent vs. variable tone presentation), each series included its own exposure controls. All mice were killed 70 minutes after the end of the exposure and the brains processed for immunohistochemistry. To enable comparability of the results of the two series, we always expressed the number of c-Fos positive cells of a given brain structure as a perentage of the mean expression levels of the respective vehicle-treated exposure controls.
There was no difference in the freezing to the tone between the groups, irrespective of protocol or treatment during tone exposure (*Protocol x Time*: $F_{9,207}$=1.83, p=0.064; *Drug x Time*: $F_{9,207}$=0.423, p=0.92; *Protocol x Drug x Time*: $F_{9,207}$=0.91, p=0.5; data not shown). A MANOVA (*Protocol*, *Drug*, *Tone*) over all Regions of Interest (ROI) revealed significant *Protocol x Tone* interactions in the LA, BLA, CEc, CEm, ITC, CG1 and PrL ($F_{1,52}$>7.993, p<0.007; Figure 3A), indicating that exposure to the tones at variable intervals led to increased and exposure to the permanent tone to decreased or unchanged number of c-Fos positive cells (see Figure 3 A for results and details of statistical analysis). Moreover significant *Drug* effects and *Drug x Tone* interactions were apparent exclusively in the CG1 (*Drug*: $F_{1,45}$=9.370, p=0.004; *Drug x Tone*: $F_{1,45}$=5.756, p=0.021; Figure 3A+B).

Discussion

Repeated exposure to the permanent tones failed to induce between-session extinction. This is in accordance with our previous findings (Plendl and Wotjak, 2010). Treatment with GF turned a non-decaying fear response into a decaying fear response, which did not show spontaneous recovery or renewal. This process required activation of memory, since injections in the absence of reexposures of the tone did not lead to decreased fear. These findings are reminiscent of disturbed reconsolidation rather than improved extinction processes (Nader and Hardt, 2009). Importantly the fact that the decrease of fear outlastet a period of 30 days after the last extinction training speaks against transient impariments in memory retrieval (Abel and Lattal, 2001). Noteworthy GF effects became evident in particular in the training sessions without further treatment (d3 and following). We cannot entirely rule out that GF was still active for days after treatment, however it would not be expected to last until day 40.

GF treatment failed to affect within- and between-session extinction following training with ten 20s tones at variable intervals. However, spontaneous recovery shown by vehicle-treated controls was completely absent. This behavioural pattern is very similar to the recently described exposure protocol, where pre-exposure to the tone before extinction training caused a permanent loss of fear memory (Monfils, 2009). Again, the most parsimonious explanation of the data would be to assume impaired reconsolidation. This may also apply to the conversion of non-decaying to decaying contextual fear, which has been described as evidence of improved extinction learning following PKC inhibition (Tronson et al., 2008).

GF seems to specifically interfere with reconsolidation, since it failed to affect memory acquisition, irrelevance learning and consolidation of extinction memory (Figure 1C-F). Such a specifitiy seems to be exceptional. Most of the known compounds to interfere with reconsolidation also impair memory acquisition or extinction learning (Diergaarde et al., 2008). Some authors aurgue that consolidation and reconsoldation involve the same molecular processes (Nader and Einarrson, 2010). Our data do not support this notion but speak for reconsolidation specific molecular signature. Although, it is of note, that this finding might be task-specific (Bonini et al., 2007).

Analysis of the anatomical correlates of between-session extinction largely confirmed our previous observations (Plendl and Wotjak, 2010). Exposure to 20s tones at variable intervals coincided with increased c-Fos activity in the amygdala and the prefrontal cortex compared to exposure controls and mice exposed to the permanent tone. To our surprise,

GF specifically affected c-Fos activity within the CG1, independent of the extinction training protocol. In each case there was a significant decrease in Fos-immunoreactivity (Figure 3B). The effects in other brain areas were less consistent. These findings suggest the CG1 as a crucial element in reconsolidation of fear memories. This does not imply that CG1 was the direct place of GF action, since changes in afferent brain structures may result in activity changes in the cingulate cortex. Nevertheless even a direct action within the CG1 is concieveable. The prefrontal cortex has a well established role in the inhibition of inappropriate responding (Rhodes and Killcross, 2007). It was shown that prefrontal long term potentiation (LTP), but not long term depression (LTD), is associated with the maintenance of extinction of conditioned fear (Herry and Garcia, 2002). This brings forward the idea that increased LTD could benefit spontaneous recovery. In the mPFC activation of muscarinic acetylcholine, which is known to be critical for cognitive performace, can induce long term depression of excitatory synaptic transmission. For LTD induction, the IP3 second messenger pathway, including PKC activation, is required (Huang and Hsu, 2009). Thus, inhibition of PKC activity could block the expression of LTD based mechanisms and thereby favour LTP processes which strengthen the maintenance of extinction memory and by this spontaneous recovery is abolished.

Most drugs acting on reconsolidation are not specific and also impair acquisition, or extinction learning and may not be readily used in humans (Diergaarde et al., 2008). However, drugs acting on PKC are already in clinical use, though, not for treatment of anxiety disorders (Lithium Chloride,Valproic Acid) (Zarate and Manji, 2009). Our study suggests a new area of application for these drugs.

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Figure legends

Figure 1: Inhibition of PKC activity promotes fear reduction in a paradigm lacking exinction between-session extinction.

All graphs show the development of the freezing responses over the course of the respective exposure days. Mice were treated with either GF109203X-hydrochloride or vehicle 1 hour before testing as indicated in the schemes (filled arrowheads) and were exposed to a 200s permanent tone. (**A**) Mice were injected on d1 and d2 and exposed to the tone on day 1 to d40. (**B**) Test for renewal on d11. Mice were exposed to the tone on d1 to d10 in the extinction context and were treated before tone exposure on d1 and d2. On day 11 the context was changed to the conditioning context. (**C**) On d1 and d2 the animals were injected exposed to the context only, followed by tone presentation on d3. (**D**) Mice were injected on d1 and d2 exposed to only a single 20s tone each day. (**E**) Mice were injected on d0 before conditioning and tested on d1 in a drugfree state. (**F**) Mice were injected on d-2 and d-1 and presented to the 200s permanent tone in the conditioning context. On day1, after conditioning on d0, they were exposed to the tone in the extinction context. The duration of freezing was normalized to respective observation periods and presented as mean ± SEM. Results of the 2-way ANOVA for repeated measurements with the main factors Drug (D) and Time (T) are shown in the graphs.

Figure 2: Inhibition of PKC does not interfere with between-session extinction but prevents spontaneous recovery.

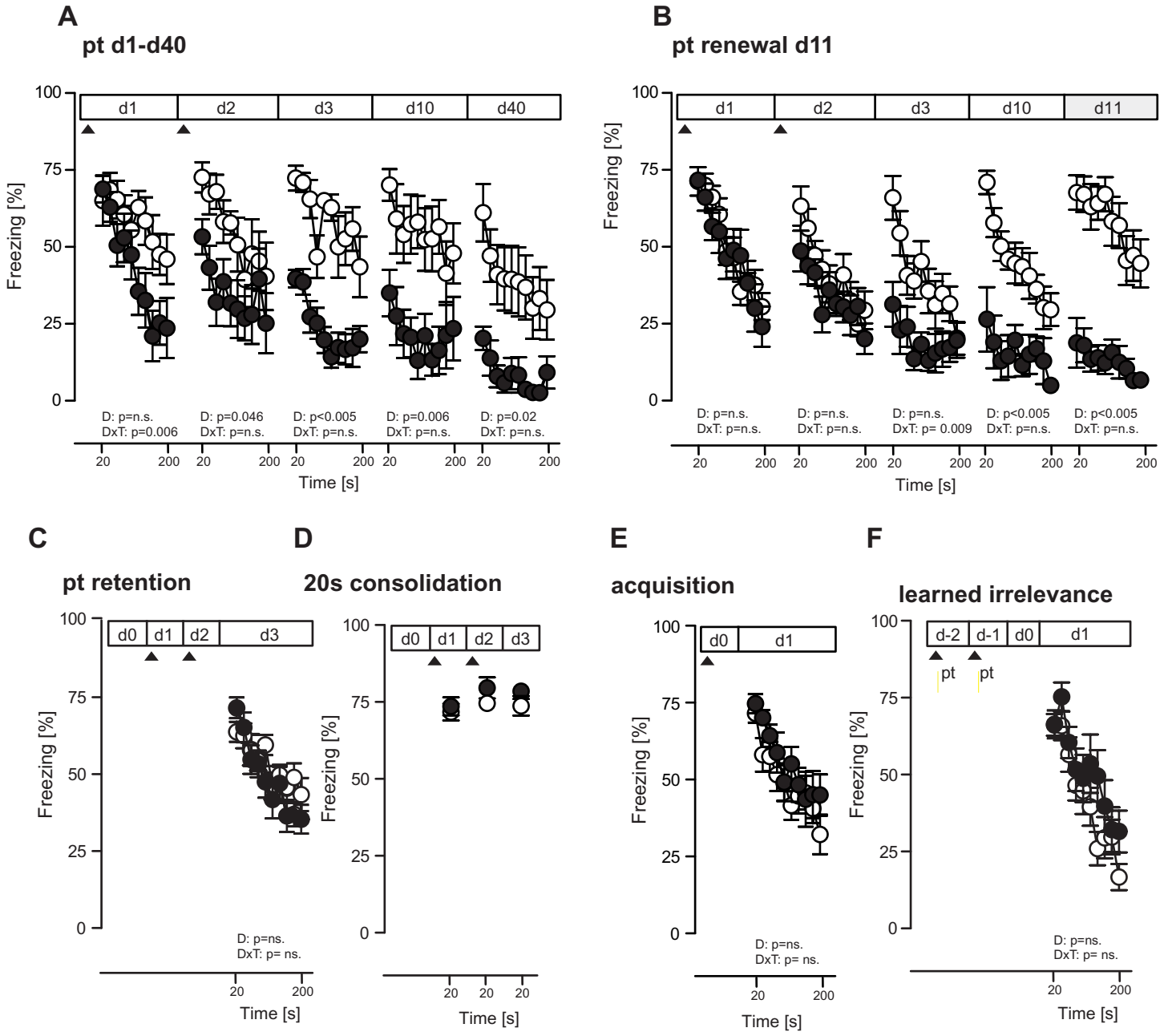
Development of the total freezing responses over the course of repeated extinction training. Animals treated with GF or vehicle, as described in Figure 1, were exposed to ten

single 20s tones with variable inter-tone-intervals on d1 to d40. The duration of freezing was normalized to respective observation periods and presented as mean ± SEM. Results of the 2-way ANOVA for repeated measurements with the main factors Drug (D) and Time (T) are shown in the graphs.

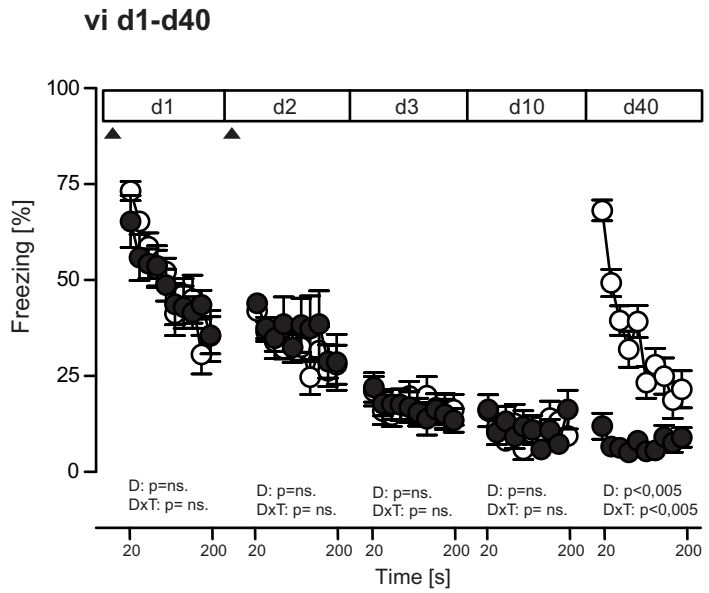
Figure 3: Brain regions modulated by PKC inhibition.

B6N mice received a single tone-shock pairing at d0 and were exposed either to a permanent tone of 200s (**pt**) or ten 20s at variable interval (**vi**) (*Protocol*). Controls were exposed to the context only, without tone presentation (*Tone*: **-** (light exposure only), **+** (light + tone)). One hour prior exposure mice were injected either with Vehicle (veh, **-**) or GF109203X-hydrochloride (GF, **+**) (*Drug*). All animals were killed 70 minutes after the end of the exposur and the brains were processed for c-Fos immunohistochemistry.
The total number of c-Fos positive neurons was counted bilaterally in the Regions of Interest (ROI) from one section per mouse. ROIs were definded according to the mouse brain atlas (Franklin and Paxinos, 1997). LA, lateral amygdala; BLA, basolateral amygdala; CEl, latero-capsular subdivision of the central amygdala; CEm, medial subdivision of the central amygdala; ITC, intercalated cell cluster; CG1, cingulate cortex; PrL, prelimbic cortex; IL, infralimbic cortex; DG, dentate gyrus; CA3, CA3 region of the dorsal hippocampus
(**A**) Percentage of c-Fos immunoreactivity (IR) normalized to the mean of the vehicle/no-tone group (veh-) ± SEM per ROI.
(**B**) c-Fos immunoreactivity in the CG1 area of the medial prefrontal cortex (mPFC). Graphical presentation of the grey highlighted numerical values of Figure 3 A.

Plendl et al., 2010 **Figure 1**



Plendl et al., 2010 **Figure 2**



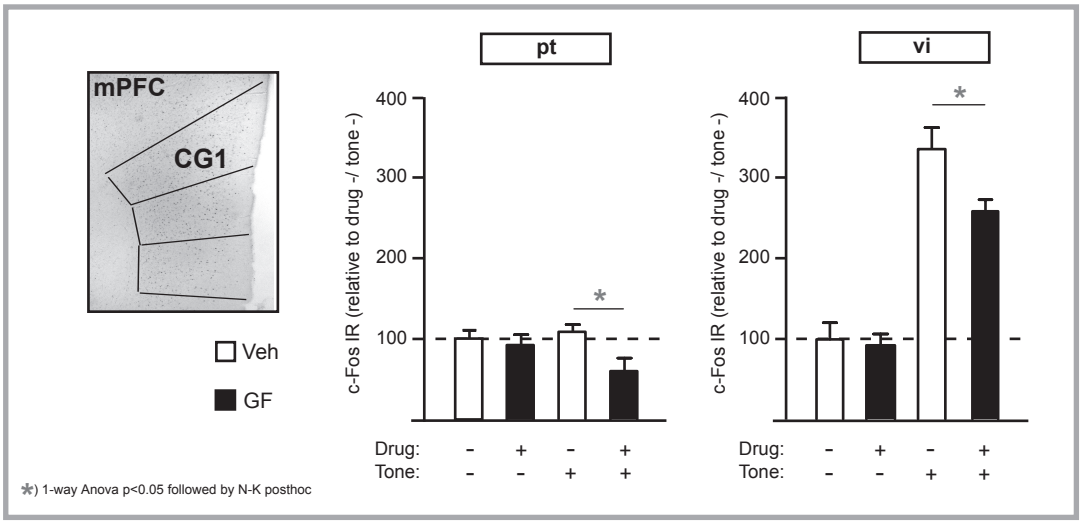
Plendl et al., **Figure 3**

A

	pt				vi				
	Veh-	GF-	Veh+	GF+	Veh-	GF-	Veh+	GF+	MANOVA[1)]
LA	100 ± 17	97 ± 21	69 ± 11	36 ± 8	100 ± 30	114 ± 22	230 ± 21 t	283 ± 36	PxT
BLA	100 ± 13	107 ± 20	63 ± 10 t	45 ± 10	100 ± 19	137 ± 20	200 ± 17 t	218 ± 23	PxT
CEl	100 ± 24	84 ± 32	56 ± 13	36 ± 16	100 ± 20	134 ± 44	291 ± 66 t	232 ± 123	PxT
CEm	100 ± 19	99 ± 26	47 ± 14 t	33 ± 10	100 ± 12	127 ± 33	188 ± 29 t	179 ± 44	PxT
ITC	100 ± 22	180 ± 71 d	104 ± 36	39 ± 10	100 ± 41	216 ± 42 d	277 ± 47 t	323 ± 108	PxT
CG1	100 ± 10	92 ± 12	108 ± 8 *	60 ± 16	100 ± 21	93 ± 14	336 ± 26 t*	259 ± 14	PxT; D; DxT
PrL	100 ± 10	99 ± 4	99 ± 6	70 ± 15	100 ± 17	99 ± 11	169 ± 15 t	142 ± 16	PxT
IL	100 ± 16	94 ± 16	92 ± 17	46 ± 12	100 ± 26	77 ± 19	186 ± 31	190 ± 31	PxT
DG	100 ± 28	66 ± 16	80 ± 14	64 ± 14	100 ± 11	116 ± 9	101 ± 12	80 ± 12	
CA3	100 ± 31	73 ± 16	88 ± 12	74 ± 20	100 ± 11	94 ± 13	118 ± 10	127±16	

1) significant main effects and factorial interactions ($p<0.05$) of the MANOVA performed over all the ROIs with the factors Protocol (P; pt vs. vi), Drug (D; veh vs NP) and Tone presentations (T; - vs +) followed by between subjects and comparisons.
t: $p<0.05$ vs veh- of the same protocol (effect of the tone presentations)
d: $p<0.05$ vs veh- of the same protocol (effect of drug treatment)

B



*) 1-way Anova $p<0.05$ followed by N-K posthoc