Fear conditioning- and extinction-induced neuronal plasticity in the mouse amygdala

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel
von

Stéphane Ciocchi
Aus Bärschwil/ SO

Basel, 2009

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von:

Prof. Dr Andreas Lüthi
(Dissertationleitung)

Prof. Dr Silvia Arber
(Korreferentin)

Prof. Dr Pierre Veinante
(Externer Experte)

Basel, den 24. März 2009

Prof. Dr Eberhard Parlow
(Dekan)

"Matti quelli che cercano, ancora piu mati quelli che non cercano"

Licia Ciocchi

Table of contents   Page

List of abbreviations

AB	Accessory basal amygdala
BA	Basal amygdala
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
CEA	Central amygdala
CEc	Central capsular amygdala
CEl	Central lateral amygdala
CEl$_{on}$	CS-excited neurons in CEl
CEl$_{off}$	CS-inhibited neurons in CEl
CElc	Central latero-capsular amygdala
CEm	Central medial amygdala, CS-excited neurons in CEm
CS	Conditioned stimulus
CS+	Conditioned stimulus paired with the US
CS-	Conditioned stimulus not paired with the US
GABA	γ-aminobutyric acid
HC	Hippocampus
IN	interneurons
LA	Lateral amygdala
NMDA	N-methyl-D-aspartate
MAPK/ERK	Mitogen-activated protein kinase/ extracellular regulated kinase
mPFC	medial prefrontal cortex
PN	Projection neurons
US	Unconditioned stimulus

Abstract

Experience-dependent changes in behavior are mediated by long-term functional modifications in brain circuits. To study the underlying mechanisms, our lab is using classical auditory fear conditioning, a simple and robust form of associative learning. In classical fear conditioning, the subject is exposed to a noxious unconditioned stimulus (US), such as a foot-shock, in conjunction with a neutral conditioned stimulus (CS), such as a tone or a light. As a result of the training, the tone acquires aversive properties and when subsequently presented alone, will elicit a fear response. In rodents, such responses include freezing behavior, alterations in autonomic nervous system activity, release of stress hormones, analgesia, and facilitation of reflexes. Subsequently, conditioned fear can be suppressed when the conditioned stimulus is repeatedly presented alone, a phenomenon called fear extinction.

It emerges from a large number of studies in animals and humans that the amygdala is a key brain structure mediating fear conditioning. The amygdala consists of several distinct nuclei, including the lateral (LA) and basal (BA) nuclei, and the central nucleus (CEA). In the classical circuit model of fear conditioning, the LA is thought of as the primary site where CS-US associations are formed and stored. The formation of CS-US associations in the LA is mediated by N-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation (LTP) at glutamatergic sensory inputs originating from auditory thalamus and cortex. In contrast to the LA, the CEA has been considered to be primarily involved in the behavioral expression of conditioned fear responses.

While the mechanisms and the circuitry underlying fear conditioning in the LA have been extensively studied, much less is known about the neuronal substrates underlying fear extinction. The question of how conditioned fear can be inhibited by extinction is attracting increasing interest because of its clinical importance for the therapy of anxiety disorders. The amygdala is also a potential site of extinction-associated plasticity since intra-amygdala blockade of NMDA receptors or the MAPK signaling pathway prevents extinction.

In the first part of this thesis, a combination of behavioral, pharmacological and *in vivo* electrophysiological approaches was used to study the role of distinct amygdala sub-nuclei in fear exinction. Single unit recordings in behaving mice revealed that the BA contains distinct types of neurons that are specifically activated upon fear conditioning or extinction, respectively. During acquisition of extinction, the activity of "fear neurons" gradually declines, while "extinction neurons" increase their activity. Conversely, when extinguished fear responses are recovered by placing the animal in an unsafe environment, "extinction neurons" switch off, while "fear neurons" switch on. Using local micro-iontophoretic injection of the $GABA_A$ receptor agonist muscimol, we found that inactivation of the BA completely prevents the acquisition of extinction or context-dependent fear recovery, depending on the injection time point. Finally, we could show that "fear neurons" and "extinction neurons" are differentially connected with the medial prefrontal cortex (mPFC) and the ventral hippocampus (vHC), two brain areas involved in context-dependent extinction. In contrast to previous models suggesting that amygdala neurons are active during states of high fear and inactive during states of low fear, our findings indicate that activity in specific neuronal circuits within the amygdala may cause opposite behavioral outcomes, thus providing a new framework for understanding context-dependent expression and extinction of fear behavior.

In the second part of the thesis, I examined how inhibitory circuits in the central nucleus of the amygdala (CEA) contribute to fear conditioning. While many studies have demonstrated that neuronal plasticity in the LA is necessary for fear conditioning, the role of the CEA, which is mainly composed of GABAergic inhibitory neurons, is poorly understood. In the classical circuit model, the CEA has been thought of as a passive relay station conveying LA output to downstream targets in the hypothalamus and in the brain stem. However, recent *in vivo* pharmacological experiments suggest a more active role for the CEA during fear conditioning. To address the role of CEA inhibitory circuits in fear conditioning, we obtained single unit recordings from neurons located in the lateral (CEl) and medial (CEm) subdivisions of the CEA in behaving mice. We found that CEm output neurons, that control fear behavior via projections to brainstem targets, are under tight inhibitory control from a subpopulation of

neurons located in CEl. Fear conditioning induced opposite changes in phasic and tonic inhibition in the CEl to CEm pathway. Targeted pharmacological inactivation of CEl and CEm revealed that whereas plasticity of phasic inhibition is necessary for gating CEm output during fear learning and expression, changes in tonic inhibitory network activity control signal-to-noise ratio and stimulus discrimination. Our results identify CEA inhibitory circuits as a major site of plasticity in fear conditioning, and suggest that regulation of tonic activity of inhibitory circuits may be an important mechanism for controlling sensitivity and specificity in associative learning.

Taken together, these findings suggest that the amygdala is not a functionally homogeneous structure. Rather, our results reveal that the BA and the CEA contain specialized and discrete neuronal populations that contribute to distinct aspects of fear conditioning and extinction. Ultimately, elucidating these mechanisms is fundamental for an understanding of memory processes in the brain in general, and should also inform novel therapeutic strategies for psychiatric disorders involving excessive fear responses associated with amygdala hypersensitivity such as post-traumatic stress disorder and other anxiety disorders.

Introduction

In order to survive, animals must learn to rapidly adapt their behavior to environmental changes. Moreover, to remember appropriate behavioral responses a memory of previously learned information has to be formed. Learning is mediated by functional and structural changes in neuronal circuits in the brain – so called neuronal plasticity. Memory reflects the persistence of such changes over time.

Neuronal circuits are comprised of excitatory and inhibitory components. The integration of sensory input and the control of behavioral output is a function of the activity of these excitatory and inhibitory components and of their connectivity within a circuit. It is thus of particular interest to understand how changes in excitatory and inhibitory circuit elements contribute to learning and memory and to elucidate the underlying mechanisms.

In my doctoral thesis I address this question by studying how experience-dependent plasticity of excitatory and inhibitory circuits in the mouse amygdala mediates a simple form of learning: classical fear conditioning

Classical fear conditioning as a behavioral model system

In classical fear conditioning (FC) an initially neutral sensory stimulus, the conditioned stimulus is paired with a noxious stimulus, the unconditioned stimulus during a training procedure. As a result of the training, the CS acquires aversive properties and when subsequently presented alone, will trigger a multifaceted fear response. In rodents, the conditioned fear response is characterized by freezing, alterations in autonomic nervous system activity, release of stress hormones, analgesia, and facilitation of reflexes (LeDoux, Iwata et al. 1988; Davis 1992; LeDoux 2000).

If, after fear conditioning, the CS is repeatedly presented in the absence of the US, conditioned fear responses decrease, a process known as fear extinction. Behavioral studies in animals demonstrate that fear extinction is not simply the forgetting of previously learned fear, but rather a new, active learning process (Rescorla 2001; Myers and Davis 2007). Fear extinction is context-dependent, and fear responses can still be expressed if the CS is presented in a different

context than the one in which extinction was acquired. Moreover, fear extinction is generally not permanent, as the original CS-evoked fear behavior can spontaneously recover over time, or can be recovered by exposing animals to simple US presentations(Myers and Davis 2007). Thus, fear and extinction memory traces co-exist and can be retrieved dependent on the environmental context and on the state of the animal.

The amygdala and fear conditioning
Discovery of the amygdala and associated functions in humans

The term amygdala was first used by the anatomist Burdach in the 19th century to describe an almond-shape structure located in the human temporal lobe. However, at that time, the amygdala was solely defined by anatomical criteria. The function of the amygdala has only been discovered during the 20th century. Since then, the amygdala has been found in Mammals and supposedly the evolutionary emergence of this brain structure appeared in Amphibians (Moreno and Gonzalez 2007). The amygdala is composed of more than 10 nuclei among which the lateral (LA), basal (BA) and accessory basal (AB) nuclei are of a cortical origin, whereas the extended amygdala, which comprises the central nucleus (Siggins, Martin et al.) and the bed nucleus of the stria terminalis (BNST) are of a striatal origin.

The first ideas on amygdala function date back to 1937 when Klüver and Bucy described in monkeys that damage of the medial temporal lobe led to a variety of symptoms such as hyperphagia or visual agnosia. Importantly, temporal lobe lesions were associated with emotional blunting characterized by a flat affect, inappropriate response to stimuli and loss of fear. Furthermore, the specific functional contribution of human amygdala has been discovered in patients suffering from a rare bilateral amygdala calcification (Urbach-Wiethe Syndrome); notably, Urbach-Wiethe patients suffer from life-long inadequacy in social and emotional behaviors. Most intriguingly, such patients are profoundly impaired in facial recognition of fear expressions (facial expression of happiness is perfectly recognized) as well as in fear conditioning (Adolphs, Tranel et al. 1994). These human behavioral studies pointed to an important role for the human amygdala in the processing and learning of fear-related information.

Neuronal circuitry of fear conditioning

While the first indications of amygdala function have been obtained from human and primate studies, most of our knowledge about the neuronal circuitry underlying fear conditioning and extinction stems from research on rodents. In the next paragraphs, I will first introduce the basolateral complex in terms of its connectivity, neuronal populations, synaptic plasticity and its role in fear conditioning. Next, I will focus on the central complex, a group of amygdala nuclei that have recently been suggested to be involved in fear conditioning.

### *Anatomy and connectivity of the basolateral amygdala*

The basolateral complex (BLA) comprises the lateral nucleus (LA), the basal nucleus (BA), and the accessory basal nucleus (AB). Afferents to the BLA can be divided into those arising from cortical and thalamic nuclei and those arising from hippocampus, rhinal and prefrontal cortices (Canteras and Swanson 1992; McDonald, Mascagni et al. 1996; McDonald 1998; Pitkanen, Pikkarainen et al. 2000).

Cortical and thalamic inputs supply information from sensory areas and structures related with memory systems. The BLA receives sensory inputs from all modalities: olfactory, gustatory, somatosensory, auditory and visual (Luskin and Price 1983; LeDoux, Farb et al. 1991; Shi and Cassell 1998; Shi and Cassell 1998; Shi and Davis 2001). Sensory inputs reach the LA via two main pathways: a direct pathway from thalamic nuclei feeds the LA through the internal capsule, located medially to the LA (LeDoux, Farb et al. 1990). An indirect pathway conveys sensory information to the LA through a thalamo-cortico-amygdala pathway via the external capsule, located laterally to the LA (LeDoux, Farb et al. 1991; Amaral and Insausti 1992)(Fig.1). The major source of cortical sensory information to the BLA is predominantly originating from layer V glutamatergic pyramidal neurons (Amaral and Insausti 1992).

In addition, there are several sources of polymodal inputs to the BLA. These include afferents from the prefrontal cortex (Rosenkranz and Grace), the rhinal cortices and the hippocampus (Canteras and Swanson 1992; McDonald, Mascagni et al. 1996; McDonald 1998; Pitkanen, Pikkarainen et al. 2000). Prefrontal inputs to the BLA are thought to be implicated in behavioral inhibition and behavioral flexibility (Sotres-Bayon, Bush et al. 2004; Stalnaker, Roesch et

al. 2007). Rhinal and hippocampal inputs transmit information about contextual memories (Corcoran and Maren 2001; Corcoran, Desmond et al. 2005). Importantly, prefrontal, rhinal and hippocampal inputs to the BLA are organized in a reciprocal manner, suggesting that the BLA might function upstream or downstream of processes that are important for context coding or behavioral inhibition.

Tracing studies have revealed that the BLA is also making important intrinsic connections in the amygdala. First, the LA has been reported to project to the BA (Smith and Pare 1994). Furthermore, the BLA sends projections to the central amygdala (Siggins, Martin et al.) (Fig.1). In particular, the LA targets the latero-capsular division (CEIc) of the CEA (Pitkanen, Stefanacci et al. 1995), while the BA targets both CEIc and the medial division (CEm) of the CEA (Savander, Go et al. 1996).
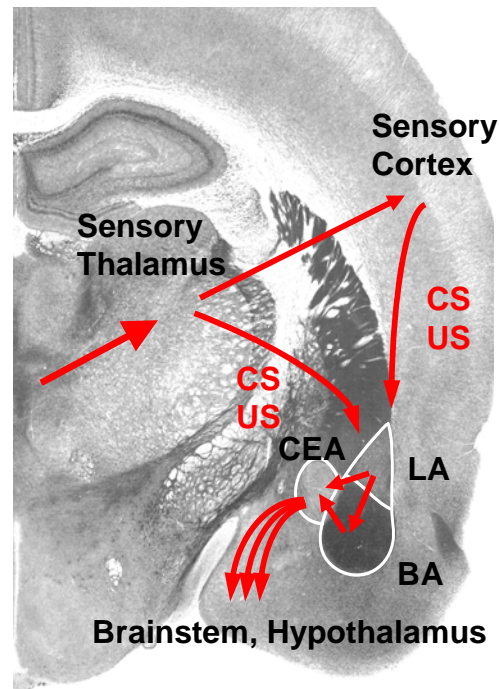
Figure 1. Neuronal circuitry of fear conditioning. During fear conditioning, sensory information about the CS and the US reaches the amygdala through thalamo-LA and cortico-LA pathways. The LA is a critical site for CS-US association during FC. LA sends local projection to the BA. LA and BA projections to brainstem- and hypothalamus-projecting CEA neurons are thought to mediate neuroendocrine, autonomic and motor responses associated with fear conditioning.

Cytoarchitecture of the basolateral amygdala

Based on morphological, neurochemical and physiological features, the BLA contains two main neuronal populations. The first population comprises about 80% of BLA neurons and are described as spiny, glutamatergic projection neurons (PNs)(McDonald 1982; Millhouse and DeOlmos 1983). PNs exhibit several axon collaterals made in the vicinity of the neuron. Antidromic activation of PNs attests that they can indeed project to extra-amygdaloid targets (Herry, Ciocchi et al. 2008). Interestingly, these neurons display a morphological continuity ranging from pyramidal to stellate (Pare, Smith et al. 1995; Faber, Callister et al. 2001). In general, the somata of projection neurons in the LA are smaller than the BA neurons (Millhouse and DeOlmos 1983). These projection neurons exhibit large dendritic arborizations that can cross sub-nuclear boundaries (Pare and Gaudreau 1996).

The second neuronal population described in the BLA consists of aspiny, GABAergic interneurons (INs) (McDonald and Augustine 1993). INs have smaller somata compared to PNs and form a heterogenous population with regard to their dendritic and axonal arborizations (Carlsen 1988; Smith, Pare et al. 1998). Their dendrites have been divided into multipolar, bitufted or bipolar (McDonald 1982). Their axons arise from the soma or from the proximal portion of the primary dendrite and branches several times. Notably, they can form dense pericellular axonal baskets around the somata and axon initial segments of PNs (Millhouse and DeOlmos), thereby efficiently controlling the generation of action potential output of PNs (Lang and Pare 1998). Like interneurons in cortex and hippocampus, basolateral amygdala interneurons can be divided into several subtypes based on the expression of a variety of calcium-binding proteins (parvalbumin (PV), calbindin, calretinin) and neuropeptides (somatostatin (SOM), cholecystokinin, neuropeptide Y, vasointestinal polypeptide)(Kemppainen and Pitkanen 2000; McDonald and Mascagni 2001; Mascagni and McDonald 2003).

It appears that BLA INs represent a diverse population in terms of morphological features, markers expression and physiological properties. PV-expressing INs have been reported to be mainly fast-spiking INs principally targeting PNs somata and proximal dendrites, and possibly the axon initial segment (Muller, Mascagni et al. 2006; Woodruff, Monyer et al. 2006). This suggests that they might strongly control PNs spiking (Lang and Pare). They have been proposed to

be part of BA feedback and feedforward inhibitory circuits (Woodruff, Monyer et al. 2006). In contrast to PV+ INs, SOM+ INs contact mostly distal dendrites and spines of BA PNs (Muller, Mascagni et al. 2007), suggesting that they interact, and perhaps affect plasticity, at distal inputs. Further, INs are instrumental in setting up synchronous activity of PNs, particularly, in generating theta oscillations (Buzsaki 2002; Bartos, Vida et al. 2007). Theta oscillations in the amygdala have been associated with retrieval of aversive memories (Seidenbecher, Laxmi et al. 2003). Overall distinct types of BLA INs likely control separate cellular functions of PNs, such as gating of synaptic plasticity, control of axonal output and oscillations of populations of PNs.

Role of the basolateral amygdala in fear conditioning

The BLA is a key site for the formation of CS-US associations during the fear conditioning. This evidence is based on studies using permanent or reversible lesions of the basolateral amygdala, application of drugs, and electrophysiological recordings of neuronal activity during fear conditioning.

Neurotoxic lesions have demonstrated a key role of the basolateral amygdala in both acquisition and expression of conditioned fear. In fact, selective neurotoxic lesions of the basolateral amygdala before fear conditioning have been shown to impair the formation of CS-US associations (Cousens and Otto 1998). Furthermore, selective neurotoxic lesions of the BLA made after fear learning have been shown to prevent memory retrieval(Campeau and Davis 1995; Cousens and Otto 1998). Notably, these lesions do not affect US (footshock) sensitivity or baseline locomotor activity suggesting a prevalent role of these lesions on learning and memory per se, rather than a deficit in freezing performance (Campeau and Davis 1995; Maren 1998).

The interpretation of experiments involving neurotoxic lesions can be confounded by compensatory effects. To circumvent this problem, acute pharmacological inactivations using, for instance, the GABAA receptor agonist muscimol, have been employed. Inactivating the basolateral amygdala neurons with muscimol immediately before fear conditioning or before memory retrieval impairs both the acquisition and expression of the conditioned fear, respectively (Wilensky, Schafe et al. 1999). Importantly, muscimol only prevents conditioning when infused before training, while immediate post-training infusions do not impair fear

learning (Wilensky, Schafe et al. 1999). This suggests that activity during the conditioning procedure is required for fear conditioning to occur.

To examine the role of the BLA in fear conditioning, extracellular recording techniques have been used to record changes in neuronal activity during fear conditioning. Fear conditioning was found to be associated with an increase in short-latency CS-evoked action potentials (spikes) of LA neurons (Quirk, Repa et al. 1995; Quirk, Armony et al. 1997). The plasticity of the earliest component of the response strongly supports learning-induced changes in thalamo-LA projections, which are the most direct auditory projections to LA leading to the fastest CS-evoked latencies of LA neurons. Importantly, changes in CS-evoked spike firing in LA neurons appear earlier during training compared with cortical neurons. This indicates that direct thalamo-LA inputs, rather than the indirect thalamo-cortico-LA pathway support neuronal spike plasticity in the LA. Furthermore, lateral amygdala neurons have been shown to exhibit discriminative spike plasticity by specifically responding to the CS+ (CS paired with the US) as compared to the CS- (CS unpaired with the US) over the training trials in a differential fear conditioning paradigm (Collins and Pare 2000).

The crucial role of the BLA in acquisition and expression of conditioned fear implies that learning-induced spike plasticity is mediated by local plasticity in the BLA rather than passive propagation of plasticity from other brains areas. Still, altered CS-evoked firing in the LA could reflect plasticity occurring upstream in the medial geniculate nucleus of the thalamus, the primary origin of auditory afferents to the amygdala. Indeed, the medial geniculate nucleus neurons can exhibit long-term potentiation (Gerren and Weinberger 1983), and synaptic plasticity has been demonstrated to occur in the medial geniculate nucleus during fear conditioning (McEchron, Bouwmeester et al. 1998). However, inactivation of the BLA has been demonstrated to be essential for the development of neuronal plasticity in the medial geniculate nucleus (and in cortex) during auditory fear conditioning in rats (Maren, Yap et al. 2001). This demonstrates that changes in CS-evoked spike firing in the LA do not merely reflect plasticity in upstream brain areas, but are mediated by neuronal plasticity processes within the LA, thus further supporting an essential role of the basolateral amygdala in fear conditioning.

### *Synaptic plasticity in the basolateral amygdala*

A major focus in understanding the neural mechanisms of acquisition and expression of fear conditioning has been the study of sensory inputs from thalamus and cortex to the LA. Many studies support the notion that the lateral amygdala is an essential site where early, NMDA-receptor dependent changes in neuronal activity occur that are required for the acquisition of conditioned fear (Miserendino, Sananes et al. 1990; Gewirtz and Davis 1997; Quirk, Armony et al. 1997; Goosens and Maren 2004). This has led to the idea that NMDA receptor-dependent long-term potentiation (LTP) at sensory afferents to LA projection neurons underlies this process (LeDoux 2000; Goosens and Maren 2004). In line with this, blocking and occlusion experiments have consistently supported the notion that LTP, of sensory evoked activity, and acquisition of conditioned fear share the same mechanisms (Rogan and LeDoux 1995; McKernan and Shinnick-Gallagher 1997; Rumpel, LeDoux et al. 2005). This represents one of the strongest established links between synaptic plasticity (i.e. LTP) and behavioral learning. While there is substantial evidence that thalamo-lateral amygdala synapses change rapidly during fear acquisition, the relative importance of the cortico-LA pathway is still poorly understood, though recent studies suggest that theses synapses might be involved in stimulus discrimination (Shaban, Humeau et al. 2006).

The basolateral amygdala and fear extinction

In addition to understanding the neuronal substrates of fear learning and expression, there is considerable interest in unraveling the neuronal circuits underlying fear extinction. Understanding the mechanisms by which fear is inhibited may lead to important clinical applications for treatment of psychiatric conditions in humans such as in posttraumatic stress disorders and panic disorders.

Fear extinction occurs when consecutively presenting the CS alone in the absence of the US. Fear extinction does not reflect the forgetting of the previously learned CS-US association, since passage of time (spontaneous recovery) (Quirk 2002), placing the mouse back in the fear conditioning context (renewal) (Bouton and King 1983) or reexposure to the US (reinstatement) (Rescorla and Heth 1975) induce fear recovery. Accordingly, fear extinction is

thought to be a new learning for which an association is made between the CS and the absence of US presentation. In this regard, it has been shown that fear extinction is leading to the activation of the amgydala using both fMRI studies in humans (Phelps, Delgado et al. 2004) and expression of the immediate early gene c-fos in the mouse basal amygdala (Herry and Mons 2004)(Fig.2). Furthermore, evidence gained from in vivo pharmacology in behaving rodents has pointed to a fundamental role of the basolateral amygdala in fear extinction. For instance, NMDA receptors, which are critical in synaptic plasticity and fear acquisition, have been shown to bidirectionnally modulate retrieval of fear extinction. NMDA receptors antagonists prevent (Falls, Miserendino et al. 1992), whereas NMDA receptors agonists facilitates fear extinction (Walker, Ressler et al. 2002). Recently, the contribution of the basolateral amygdala in extinction learning has been investigated by interfering with the NMDA receptors-mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) pathway. Indeed blocking the most calcium permeable subunit of the NMDA receptors, namely NR2B subunit, has been shown to impair extinction learning(Herry, Trifilieff et al. 2006; Sotres-Bayon, Bush et al. 2007).

Extinction   **No Extinction**
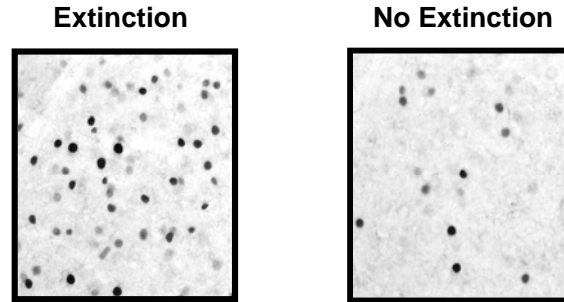
Herry et al., 2004

Figure 2. Increased c-Fos expression in the BA after fear extinction. Acquisition of fear extinction leads to the induction of the immediate early gene c-fos in the BA (left panel). CS presentation in the absence of extinction training (right panel) induces much lower levels of c-Fos.

Moreover, fear extinction is thought to be mediated at the network level by a concerted interaction between the amygdala, the hippocampus and the mPFC which are part of a highly interconnected neuronal network (Canteras and Swanson 1992; McDonald, Mascagni et al. 1996). The hippocampus might contribute to fear extinction by providing contextual information with regard to the animal's environment (Corcoran, Desmond et al. 2005). The mPFC has been largely described to inhibit behavioral responses. Interestingly, CS-evoked

responses of neurons of the infralimbic prefrontal cortex, a subdivision of the mPFC, have been shown to correlate with fear extinction retrieval while infralimbic cortical stimulation leads to the acceleration of extinction learning and enhanced fear extinction retrieval (Milad and Quirk 2002).

Taken together, these data support a role for neuronal plasticity in the basolateral amygdala during extinction learning.

## *Neuronal circuits underlying fear conditioning in the central amygdala*

Most studies on fear conditioning have focused on neuronal plasticity in the basolateral amygdala. However, recent studies indicate that the central amygdala (Siggins, Martin et al.) is likewise involved in fear conditioning. The main difference between the basolateral and the central amygdala is based on their developmental origin. The basolateral amygdala is a cortical-like structure, using glutamate as principal neurotransmitter. The central amygdala is of a striatal-like origin with smaller neurons that are mainly GABAergic (Sun and Cassell 1993; Cassell, Freedman et al. 1999)(Fig.3). In the next chapters, I will describe the anatomy, the connectivity and the neuronal populations of the central amygdala that profoundly differ from the basolateral amygdala. In addition, I will mention pharmacological and electrophysiological results indicating that the central amygdala may actively contribute to fear conditioning.

Anatomy and connectivity of the central amygdala

The CEA is part of a larger anatomical structure termed the extended central amygdala that also comprises the bed nucleus of the stria terminalis (BNST). The CEA is medially adjacent to the BLA and can be subdivided into four different parts: the central capsular (CEc), the central intermediate (CEi), the central lateral (CEl) and the central medial amygdala (CEm) (McDonald 1982; Jolkkonen and Pitkanen 1998)(Fig. 3). Since the CEi has not been identified in mice, I will focus on the three other CEA subdivisions. In a study by Pitkänen and colleagues (Jolkkonen and Pitkanen 1998), the intrinsic connectivity of the central amygdala has been investigated using injections of an anterograde tracer into various CEA subdivisions. This study revealed that intrinsic CEA connectivity is organized topographically and originates primarily in CEl. CEl reciprocally

connects to the CEc while sending unidirectional projections to CEm (Fig. 3). The CEm is providing the main output of the CEA to the hypothalamus and to the brainstem where neuroendocrine, autonomic and motor responses are induced. Therefore, it appears that the flow of information propagates latero-medially, that is from the central latero-capsular amygdala to the central medial amygdala.

Furthermore, there is strong interconnnection within the extended amygdala, in particular between the central latero-capsular amygdala and the bed nucleus of the stria terminalis (Sun, Roberts et al. 1991; Veinante and Freund-Mercier 1998).
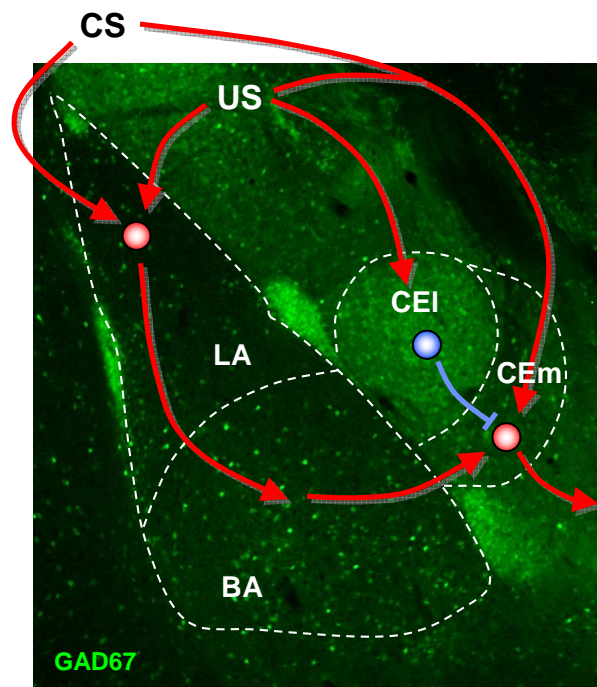
Figure 3. Neuronal circuitry of the central amygdala. CS and US information is thought to reach the CEA in serie or in parallel. In the serial model, CS and US information first transit through the BLA. In the parallel model, sensory information directly reaches the CEA, bypassing the BLA. Staining for GAD67, a marker of GABAergic neurons, reveals dense immunolabelling of the CEA consistent with the knowledge that the CEA is almost exclusively composed of GABAergic neurons. GABAergic neurons connect locally within CEA. In particular, a GABAergic connection from CEl to CEm has been described.

What about external afferents to the CEA? One important input to the CEA originates in the BLA (Pitkanen, Stefanacci et al. 1995)(Fig.3). This is consistent with a serial model in which the CEA is downstream of the BLA (Balleine and Killcross 2006). However, in addition to inputs from the BLA, the CEA also

receives a variety of extra-amygdaloid inputs(Ottersen and Ben-Ari 1979; Veinante and Freund-Mercier 1998), suggesting that it may also function in parallel to and independently from the BLA (Sun, Yi et al. 1994; Balleine and Killcross 2006)(Fig. 3). Cortical inputs arising from the ventral entorhinal cortex and from the insular cortex target CEl, whereas inputs from the mPFC predominantly target CEc (Sun, Yi et al. 1994). The CEA also receives a variety of subcortical inputs from thalamic and brainstem nuclei. The paraventricular nucleus of the thalamus targets all three CEA subdivisions, whereas the auditory thalamus preferentially targets CEm. Visceral and nociceptive inputs arising from the brainstem nuclei (parabrachial nucleus, nucleus of the solitary tract) provide input to both CEl and CEm.

Cyto- and chemoarchitecture of the central amygdala

The main neuronal population in the CEA is GABAergic and exhibits striatum-like, medium-spiny type morphology. This basic feature together with strong dopaminergic and enkephalinergic innervation is reminiscent of the basal ganglia (Cassell, Freedman et al. 1999).

Remarkably, the CEA exhibits high expression levels for a variety of neuropeptides and their receptors (Roberts, Woodhams et al. 1982; Veinante and Freund-Mercier 1997; Veinante, Stoeckel et al. 1997). Moreover, extrinsic afferents containing neuropeptides can target specific CEA subdivisions or even subpopulations of neurons within a subdivision. A vast literature exists linking CEA neuropeptides and their receptors to modulation of neuronal activity and behavior. The following list summarizes key references for the main neuropeptidergic systems described in the CEA:

- Corticotrophin-releasing factor (CRF) and CRF receptors (Yu and Shinnick-Gallagher 1998; Bouret, Duvel et al. 2003; Nie, Schweitzer et al. 2004)
- Dynorphin (Zardetto-Smith, Moga et al. 1988)
- Kappa opioid receptors (Chieng, Christie et al. 2006)
- Enkephalin (Gray, Cassell et al. 1984)
- Oxytocin and oxytocin receptors (Veinante and Freund-Mercier 1995; Veinante and Freund-Mercier 1997)(Fig. 4)

- Mu-opioid receptors and delta-opioid receptors (Chieng, Christie et al. 2006)

- Vasopressin and vasopressin receptors (Veinante and Freund-Mercier 1995; Veinante and Freund-Mercier 1997)(Fig.4)

- Glucocorticoid receptors (Honkaniemi, Pelto-Huikko et al. 1992)

- Calcitonin- gene related peptide (CGRP)(Honkaniemi, Pelto-Huikko et al. 1992)

- Galanin and galanin receptors (Waters and Krause 2000)

- Somatostatin (Roberts, Woodhams et al. 1982)

- Substance P (Roberts, Woodhams et al. 1982)

- Neurotensin (Roberts, Woodhams et al. 1982)

- Cholecystokinin (Roberts, Woodhams et al. 1982)

- Orexin/hypocretin (Ciriello, Rosas-Arellano et al. 2003)

There is emerging evidence that neuronal subpopulations within the CEA may be classified based on specific combinations of neuropeptides and receptors (Roberts, Woodhams et al. 1982; Veinante and Freund-Mercier 1997; Huber, Veinante et al. 2005). According to these experiments, CEIc contains two rather well defined neuronal populations. The first population expresses GABA, corticotrophin releasing factor and dynorphin, and is thought to make intrinsic connections within CEA and extrinsic connections to the parabrachial nucleus (Veinante and Freund-Mercier 1998; Marchant, Densmore et al. 2007). CRF-positive neurons are also densely innervated by dopaminergic afferents (Asan 1998).

The second neuronal population is positive for GABA, enkephalin and oxytocin receptors (Veinante and Freund-Mercier 1997; Huber, Veinante et al. 2005; Marchant, Densmore et al. 2007)(Fig.4). These neurons connect locally within the extended central amygdala. They send projections to CEm and to the BNST (Veening, Swanson et al. 1984; Huber, Veinante et al. 2005)(Fig. 4) and receive input from the parabrachial nucleus (Shimada, Inagaki et al. 1992). Notably, these two neuronal populations appear to be part of separate opioidergic systems(Chieng, Christie et al. 2006). Importantly, this dichotomy of CRH- and enkephalin-expressing neuronal populations in CEI is reflected by functional studies that broadly implicate amygdalar CRH in stress, fear and anxiety

responses and enkephalin/oxytocin as having anxiolytic activity (Uvnas-Moberg, Ahlenius et al. 1994; Bale, Davis et al. 2001; Nie, Schweitzer et al. 2004; Schulkin, Morgan et al. 2005; Kolber, Roberts et al. 2008). Overall, this suggests that distinct neuronal populations in CEl might differentially contribute to fear and anxiety behaviors.
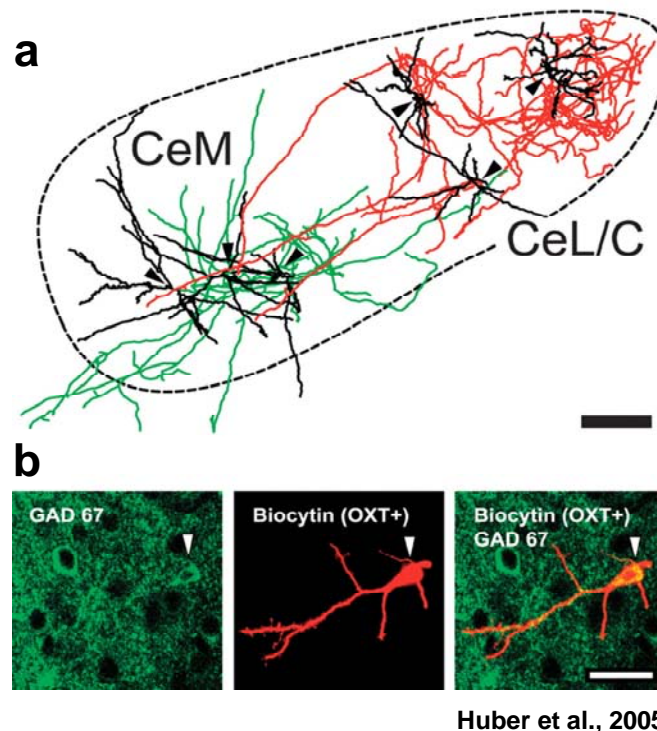
Figure 4. GABAergic projection from CEl to CEm. a, A population of CEl oxytocin-excited neurons (3 neurons indicated with an arrow, red axon collaterals, dendrites in black) send projection to vasopressin-excited neurons (3 neurons, green axon collaterals) in CEm. **b,** Neurochemical charactrization reveals that oxytocin-excited neuron are positive for GAD67 and oxytocin.

At the electrophysiological level, neurons in the CEA have been classified as regular spiking (RS) or low-threshold bursting neurons (LTB)(Martina, Royer et al. 1999; Schiess, Callahan et al. 1999). Low-threshold bursting has been linked to the expression of low-threshold activated-calcium channels (Martina, Royer et al. 1999; Schiess, Callahan et al. 1999) In the CEl, most of the neurons are regular spiking (65%), and a minority are low-threshold bursting (26%), whereas in the CEm the distribution is different (71% LTB vs 27% RS). A recent cross-species comparison revealed correlations between the ratios of these neuronal populations and the manifestation of species-specific physiological responses such as changes in heart rate and blood pressure in response to frightening stimuli (Dumont, Martina et al. 2002). This argues for a role of defined

electrophysiological populations in CEl in promoting fear-related behaviors. Moreover, in vivo extracellular recordings in the rat CEA have identified two types of neurons based on their increase or decrease in firing rate during immobilization and stress, whose ratios differed between strains with high and low emotional behavior (Henke, Sullivan et al. 1988). This suggests that the CEA contains different functional neuronal populations that could gate emotional behaviors in an opposite manner.

Role of the central amygdala in fear-related behaviors

Modulation of the neuropeptidergic system in the central amygdala have been implicated in alcoholism (Cowen, Chen et al. 2004; Nie, Schweitzer et al. 2004), addiction (Koob 2003), anxiety (Amico, Mantella et al. 2004; Bielsky, Hu et al. 2004) and depression (Griebel, Simiand et al. 2002; Landgraf and Wigger 2002; Wigger, Sanchez et al. 2004). Therefore, neuropeptides and their receptors in the CEA have become a major target in drug development (Holmes, Heilig et al. 2003). Studies on the behavioral effects of neuropeptides in the CEA have mainly focused on three systems: CRF, vasopression and oxytocin. All three are classical hypothalamic hormones (Bargmann 1951). CRF has been widely associated with behavioral and physiological aspects of stress (Kolber, Roberts et al. 2008). In addition to its activating effects on the hypothalamic-pituitary-adrenal (HPA) axis, intracerebroventricular injections of CRF induces elevated heart rate, blood pressure and behavioral changes that mimic stress responses. The CEA and the BNST have been identified as the most important sites of the central action of CRF (Rainnie, Fernhout et al. 1992) (Walker, Toufexis et al. 2003). The effect of CRF on neuronal activity has been studied; CRF induces a strong hyperpolarization of CEA neurons (Rainnie, Fernhout et al. 1992; Yu and Shinnick-Gallagher 1998). Furthermore, vasopressin and oxytocin have been shown to modulate several CEA-related behaviors. Injection of vasopressin or oxytocin into the CEA induces opposite effects on anxiety-like behavior. Whereas oxytocin has anxiolytic effects (Uvnas-Moberg, Ahlenius et al. 1994), vasopressin enhances autonomic fear reactions (Roozendaal, Schoorlemmer et al. 1992). The mechanisms underlying these opposite effects of vasopressin and oxytocin have recently been studied in CEA slices. Huber and colleagues (2005) demonstrated that oxytocin excites a neuronal population in CEl that sends GABAergic inhibitory projections to vasopressin receptor-positive neurons in

CEm. This inhibitory neuronal circuit intrinsic to the CEA provides a framework to better understand the opposite actions of oxytocin and vasopressin in vivo (Huber, Veinante et al. 2005).

Until recently, the CEA has been thought of as a relay station between the BLA and the hypothalamus/brainstem (LeDoux 1996). Consistent with that, it is generally accepted that the BLA is the key site of CS-US association during fear conditioning (Maren and Quirk 2004). In this model, projections from the BLA to the CEA control the activity of CEm output neurons (LeDoux, Iwata et al. 1988; Davis 1992; Pitkanen, Savander et al. 1997). Consistent with the CEA being the main output structure activated by the BLA, lesion studies have shown that ablation of the CEA interferes with fear expression (Amorapanth, LeDoux et al. 2000).

In addition to the BLA → CEA pathway, it has been proposed that intra CEA inhibitory circuits might also participate in modulating CEm output (Cassell, Freedman et al. 1999). Recently, acute and reversible inactivation experiments utilizing the GABAA receptor agonist muscimol, revealed a more active role of the CEA during fear conditioning (Wilensky, Schafe et al. 2006). Inactivation of the CEA during the acquisition of fear conditioning resulted in a memory deficit when the animals were tested 24 hrs later in drug-free state (Wilensky, Schafe et al. 2006). Similarly, infusion of an NMDA receptor antagonist into the CEA during fear conditioning interferes with learning (Goosens, Hobin et al. 2003). In keeping with the idea that the CEA could be a site where specific CS-US associations might be formed, recordings from CEA neurons during fear conditioning revealed differential changes in CS+ and CS- -evoked activity in a discriminative fear conditioning paradigm (Pascoe and Kapp 1985; Pascoe and Kapp 1985). Taken together, this strongly suggests that the CEA is an additional site that can actively contribute to the formation of CS-US associations. However, the role of excitatory inputs vs. local intra-CEA inhibitory circuitry in driving CEm output after fear conditioning is not known.

Synaptic plasticity in the central amygdala

Based on evidence from electrophysiological studies in slices, different potential mechanisms for the formation of CS-US associations in the central amygdala emerge. A first possibility that does not require intrinsic central amygdala

inhibitory circuits is that plasticity occurs directly at glutamatergic sensory inputs to CEm output neurons. Indeed, CEm output neurons receive monosynaptic excitatory afferents from sensory thalamus (LeDoux, Ruggiero et al. 1985) (Turner and Herkenham 1991). These afferents exhibit input-specific, NMDA receptor-dependent LTP (Samson and Pare 2005). However, it remains to be tested whether LTP at thalamic afferents to CEm output neurons contribute to increased CEm output after fear conditioning. A second possibility is that inputs from different sources impinging onto CEl neurons undergo activity-dependent synaptic plasticity during fear conditioning. Altered drive of CEl neurons could set the level of inhibitory or disinhibitory control in the CEl to CEm circuit. For instance, afferents from the parabrachial nucleus form strong and reliable synapses onto CEl neurons. These synapses exhibit bidirectional activity-dependent plasticity (Lopez de Armentia and Sah 2007). Parabrachial nucleus afferents convey ascending nociceptive information to the CEl (Neugebauer, Li et al. 2004), and their modification may contribute to the emotional and behavioral consequences accompanying states of persistent pain (Neugebauer, Li et al. 2004). A second set of afferents that show input-specific LTP in vitro are inputs from the BLA to the CEl (Fu and Shinnick-Gallagher 2005). However, other important inputs, such as those originating in the insular cortex, have not been examined. A central open question relates to the role of the different forms of activity-dependent plasticity in fear conditioning and how they affect intra-CEA information processing. One interesting property of CEl neurons is that synapses made by their extrinsic inputs continue to express high levels of the NMDA receptor subunit NR2B into adulthood (Lopez de Armentia and Sah 2003). This is very distinct from the BLA, where NR2B expression decreases during development (and NR2A expression increases), suggesting that high NR2B levels may enable CEl to express distinct forms of plasticity throughout life. Moreover, it is intriguing to speculate that the disruption of fear memory acquisition in behavioral pharmacological experiments that interfered with NR2B signaling (Rodrigues, Schafe et al. 2001) could be, at least partially, mediated by the CEl.

Aim of the thesis

This thesis addresses the fundamental question how the function of neuronal microcircuits in the brain relates to learning at the behavioral level. Whereas a lot is known about the role of entire brain areas in distinct forms of learning and about the underlying mechanisms at the molecular and synaptic levels, there is a big gap in our knowledge of how learning is implemented at the level of defined neuronal circuits. In my thesis, I address this question by using classical auditory fear conditioning and extinction as a model paradigm. Classically, extinction of conditioned fear responses has been thought to be mediated by a general inhibition of amygdala neurons. However, based on recent work indicating that fear extinction induces the expression of the activity-dependent immediate early gene product c-Fos in neurons located in the basal nucleus of the amygdala (Herry and Mons 2004), we explored the role of BA circuitry in fear extinction using a combination of single unit recordings and targeted pharmacological inactivation in behaving mice. These experiments revealed that a switch in the activity between two distinct types of BA neurons underlies fear extinction thus providing a new framework for understanding the acquisition and extinction of conditioned fear at the level of defined neuronal circuits.

In the second part of the thesis, I examine how inhibitory circuits in the central nucleus of the amygdala (Siggins, Martin et al.) contribute to fear conditioning. The majority of previous work in this field has focused on the role of glutamatergic principal cells in the lateral amygdala (LA), which is considered to be the primary site where CS-US associations are formed. The CEA, which is predominantly composed of GABAergic inhibitory neurons, has been considered to act as a passive relay structure linking the LA to downstream targets in the brainstem and in the hypothalamus. Recent in vivo pharmacological studies, however, support a more active role for the CEA in fear conditioning (Goosens and Maren 2003; Wilensky, Schafe et al. 2006), yet a detailed neurophysiological analysis of CEA circuitry in behaving animals has not been performed. We therefore explored CEA circuit plasticity during fear conditioning. Our results indicate that inhibitory circuits in the CEA are highly organized, and establish important, but distinct, roles for plasticity of phasic and tonic inhibitory network activity in fear conditioning.

Results Part I

Switching on and off fear by distinct neuronal circuits

Cyril Herry*, **Stephane Ciocchi***, Verena Senn, Lynda Demmou, Christian Müller
& Andreas Lüthi

* Equal contribution to this work. Nature 454, 600-606 (31 July 2008)

Abstract

Switching between exploratory and defensive behaviour is fundamental to survival of many animals, but how this transition is achieved by specific neuronal circuits is not known. Here, using the converse behavioural states of fear extinction and its context-dependent renewal as a model in mice, we show that bi-directional transitions between states of high and low fear are triggered by a rapid switch in the balance of activity between two distinct populations of basal amygdala neurons. These two populations are integrated into discrete neuronal circuits differentially connected with the hippocampus and the medial prefrontal cortex. Targeted and reversible neuronal inactivation of the basal amygdala prevents behavioural changes without affecting memory or expression of behaviour. Our findings indicate that switching between distinct behavioural states can be triggered by selective activation of specific neuronal circuits integrating sensory and contextual information. These observations provide a new framework for understanding context-dependent changes of fear behaviour.

Introduction

The amygdala is a key brain structure mediating defensive behaviour in states of fear and anxiety. Such states can be induced by classical auditory fear conditioning, in which an initially neutral auditory stimulus (the conditioned stimulus, CS) comes to elicit a fear response after pairing with an aversive foot shock (the unconditioned stimulus, US). Subsequent repetitive presentations of the CS alone induce a progressive decrease in the fear response, a phenomenon called extinction. Whereas firing of amygdala neurons is critical for the retrieval of conditioned fear memories (Quirk, Repa et al. 1995; Collins and Pare 2000; Rosenkranz and Grace 2002; Goosens, Hobin et al. 2003) their firing after the extinction of conditioned fear is thought to be constrained by local

inhibitory circuits activated by the medial prefrontal cortex (mPFC) (Milad and Quirk 2002; Maren and Quirk 2004; Pare, Quirk et al. 2004). Converging evidence from animal studies indicates, however, that the basolateral complex of the amygdala (BLA), comprising the lateral (LA) and the basal (BA) nuclei, actively participates in fear extinction (Falls, Miserendino et al. 1992; Herry, Trifilieff et al. 2006; Quirk, Garcia et al. 2006; Sotres-Bayon, Bush et al. 2007). Although fear extinction is an active learning process eventually leading to the formation of a consolidated extinction memory (Myers and Davis 2007), it is a fragile behavioural state that is readily influenced by context18, 19. Changing context results in the immediate recovery of the previously conditioned fear response, a process known as fear renewal (Bouton and King 1983). In vivo pharmacological studies indicate that the hippocampus, which is reciprocally connected to the BLA (Pitkanen, Pikkarainen et al. 2000), processes contextual information during fear conditioning, extinction and renewal (Corcoran and Maren 2001; Corcoran, Desmond et al. 2005; Bouton, Westbrook et al. 2006). Thus, bi-directional changes in fear behaviour during extinction and context-dependent renewal are likely to be encoded within a distributed network containing the BLA, the mPFC and the hippocampus; however, the neuronal circuits mediating such behavioural transitions are not known. In particular, this raises the question of whether there are specialized circuits driving behavioural transitions in opposite directions.

To address this question, we used a combination of in vivo single-unit recordings and targeted pharmacological inactivation in behaving mice. Because the BA is strongly connected to the hippocampus (Pitkanen, Pikkarainen et al. 2000) and to the mPFC (McDonald, Mascagni et al. 1996), and because extinction has previously been shown to induce the expression of the activity-dependent immediate early gene product Fos in BA neurons (Herry and Mons 2004), we focused our study on this sub-nucleus. Here we identify two distinct neuronal circuits differentially connected with the mPFC and the hippocampus, and show that a rapid switch in the balance of activity between those circuits specifically drives behavioural transitions without being necessary for memory storage or behavioural expression.

Results

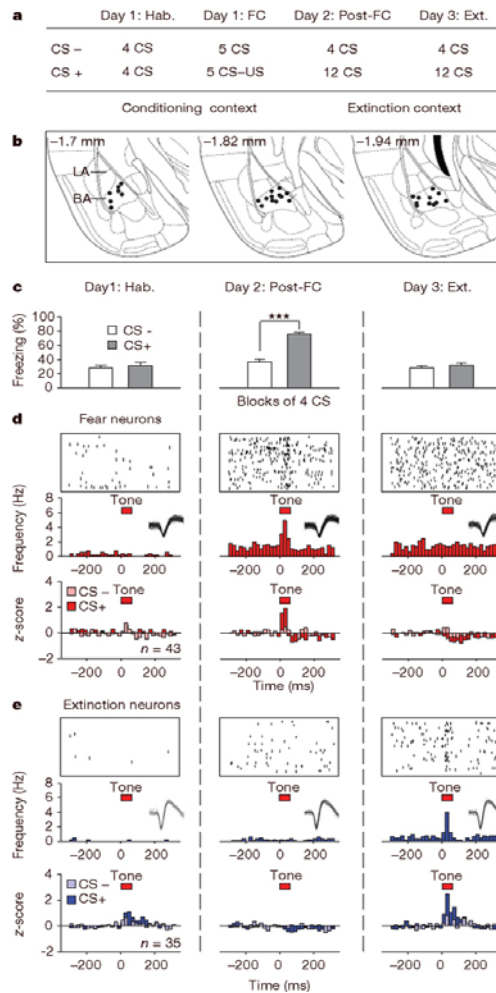### Distinct BA neurons encode fear and extinction

To examine plasticity of spike firing of individual BA neurons, C57Bl/6 mice were implanted with chronic recording electrodes and trained in a discriminative fear-conditioning paradigm (Fig. 1a). During training, mice learned to discriminate two auditory CS of different frequencies. One CS (the CS+) was paired with an aversive foot shock (US), whereas the second CS (CS–) was not paired. Twenty-four hours after fear conditioning, mice (n = 30) exhibited a selective increase in fear behaviour (as measured by freezing) when exposed to the CS+ in a different context (Fig. 1c). Extinction of conditioned fear behaviour was induced by exposing mice to 24 CS+ presentations in the absence of any aversive stimuli. After extinction training, CS+-induced freezing behaviour was reduced back to pre-conditioning levels, and did not differ from CS–-induced freezing (Fig. 1c).

Analysis of changes in CS+- and CS–-evoked spike firing during extinction training revealed that BA neurons (259 recorded units; Fig. 1b) could be divided into distinct functional classes. Consistent with previous reports (Maren, Poremba et al. 1991), we found a class of neurons ('fear neurons'; n = 43 neurons, 22 mice; 17% of recorded units) that exhibited a selective increase in CS+-evoked spike firing during and after fear conditioning (Fig. 1d, Supplementary Fig. 1 and Supplementary Table 1). Subsequent extinction completely abolished this increase and converted it into a CS+-evoked inhibition (Fig. 1d). On average, spontaneous activity of fear neurons was not affected by fear conditioning or extinction (Supplementary Table 1). Thus, fear-conditioning-induced behavioural discrimination between the CS+ and the CS–, and its reversal by extinction, was accurately reflected at the neuronal level by the discriminative and reversible activity of fear neurons. During extinction training, another class of neurons emerged. In contrast to fear neurons, 'extinction neurons' (n = 35 neurons, 20 mice; 14% of recorded units) did not show any increase in CS-evoked responses during or after fear conditioning, but instead showed a slight reduction (Fig. 1e). However, subsequent extinction training induced a marked and selective increase in CS+-evoked activity in these neurons (Fig. 1e), without any changes in spontaneous activity. Plotting extinction-induced changes in z-score for individual fear and extinction neurons revealed that the two populations were separated in a bi-modal distribution

(Supplementary Fig. 2). The remaining neurons did not exhibit any changes in activity during extinction (Supplementary Table 1).
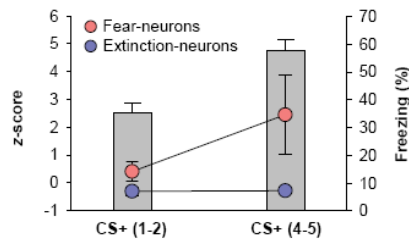
Supplementary Figure 1. Changes in CS-evoked activity during fear conditioning. Summary graph illustrating changes in freezing behavior (grey bars), and CS-evoked activity of fear-neurons (red circles) and extinction-neurons (blue circles). Comparing the first two CSs (CS 1-2) with the last two CSs (CS 4-5) reveals that increased freezing behavior (CS 1-2: 35 ± 4% of time; CS 4-5: 58 ± 4% of time) was associated with enhanced CS-evoked activity in fear neurons (n = 43 neurons from 22 mice, z-score, CS 1-2: 0.41 ± 0.35; CS 4-5: 2.45 ± 1.42), but not in extinction neurons (n = 35 neurons from 20 mice, z-score, CS 1-2: - 0.31 ± 0.15; CS 4-5: - 0.29 ± 0.11).


Figure 1. **Distinct populations of BA neurons encode fear conditioning and extinction. a,** Experimental protocol. Ext., extinction; FC, fear conditioning; Hab., habituation. **b,** Coronal sections through the rostro-caudal extent of the amygdala, showing the location of the recording sites in the BA. **c,** Summary graphs illustrating behavioural data. During habituation, mice (*n* = 30) exhibited equally low freezing levels in response to CS$^+$ and CS$^-$ exposure. Twenty-four hours after fear conditioning, presentation of the CS$^+$ (CS

1–4 on day 2), but not the CS⁻, evoked significantly higher freezing levels. After extinction, both CS⁺ (CS 9–12 on day 3) and CS⁻ elicited low freezing levels. Error bars indicate mean ± s.e.m. **d, e,** Raster plots (top) and peristimulus time histograms (middle) illustrating selective changes in CS⁺-evoked firing of a representative fear (d) and an extinction (e) neuron. The duration of the auditory stimulus is indicated (red bar; tone). Insets show superimposed spike waveforms recorded during habituation, after fear conditioning and after extinction, respectively. Bottom: fear conditioning and extinction-induced changes in CS⁺-evoked firing of fear and extinction neurons. Fear neurons (*n* = 43 neurons from 22 mice) exhibited a selective increase in CS⁺-evoked firing after fear conditioning (*P* < 0.001 versus habituation or versus CS⁻), which was fully reversed on extinction. In contrast, CS⁺-evoked firing of extinction neurons (*n* = 35 neurons from 20 mice) was selectively increased after extinction (*P* < 0.001 versus post-FC or versus CS⁻). ***P < 0.001.

	Habituation			Post-FC			Extinction			Percent of units (%)
	z-score		Spont. Freq. (Hz)	z-score		Spont. Freq. (Hz)	z-score		Spont. Freq. (Hz)	
	CS-	CS+		CS-	CS+		CS-	CS+		
Fear neurons	0.46±0.44	0.38±0.25	2.2±0.5	0.37±0.21	1.78±0.28***	1.8±0.3	0.42±0.21	-0.30±0.10	1.6±0.3	16.6
Extinction neurons	0.61±0.41	0.57±0.20	1.5±0.3	-0.04±0.13	-0.17±0.14	1.1±0.3	0.51±0.18	1.71±0.35***	1.5±0.3	13.5
Extinction-resistant neurons	1.49±0.37	0.84±0.19	2.3±0.5	1.29±0.24	2.64±0.45**	3.6±0.7***	1.54±0.30	2.49±0.44**	4.2±0.8**	25.5

Supplementary Table 1. **Summary of units recorded in BA.** This table summarizes changes in CS-induced neuronal activity (z-scores) and in spontaneous activity across behavioral sessions. Post-fear conditioning (post-FC) values were obtained using the first 4 CS+ presentations on day 2. Post-extinction (extinction) values were obtained using the last 4 CS+ presentations on day 3. Spontaneous activity was measured during the 500 ms preceding CS stimulation. Statistical comparisons: z-scores, CS+ vs. CS– within each behavioral session; spontaneous activity, post-FC and extinction vs. habituation. *P < 0.05, **P < 0.01, ***P < 0.001.
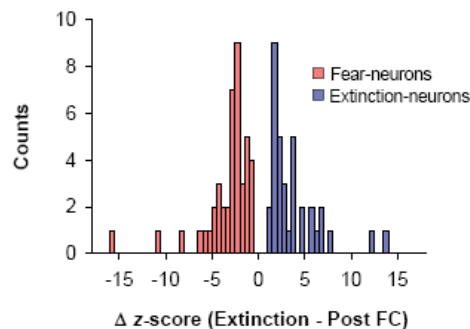

Supplementary Figure 2. Extinction-induced changes in CS-evoked activity reveal a bimodal distribution of fear- and extinction-neurons. Histogram representing the extinction-induced changes in the CS+-evoked neuronal activity (z-score) of individual fear-neurons (n = 43) and extinction-neurons (n = 35). A negative Δ z-score value indicates a preferential activation after fear conditioning, whereas a positive Δ z-score value indicates a preferential activation after extinction. Fear- and extinction-neurons formed two well-separated populations.

Thus, changes in CS+-evoked firing of fear and extinction neurons were oppositely correlated with behavioural extinction.

Although these results demonstrate a specific activation of fear and extinction neurons by a given CS, they do not address the question of whether individual extinction neurons can function as fear neurons for another CS, or vice versa. We therefore trained mice in a discriminative extinction paradigm (Fig. 2a). In this

paradigm, two different CS (CS1 and CS2) were first fear-conditioned, followed by extinction of only one of them (CS1). At the end of extinction, mice exhibited selective freezing behaviour when exposed to the non-extinguished CS2 (Fig. 2b). Fear neurons and extinction neurons were identified during fear conditioning and extinction of CS1 according to the same criteria as described above, and CS1- and CS2-evoked spike firing was compared at the end of extinction. Whereas individual extinction neurons (n = 9 neurons, 3 mice) responded to the extinguished CS (CS1), but not to the non-extinguished CS (CS2), fear neurons (n = 8, 3 mice) only fired during CS2 exposure, but remained unresponsive to the CS1 (Fig. 2c, d).
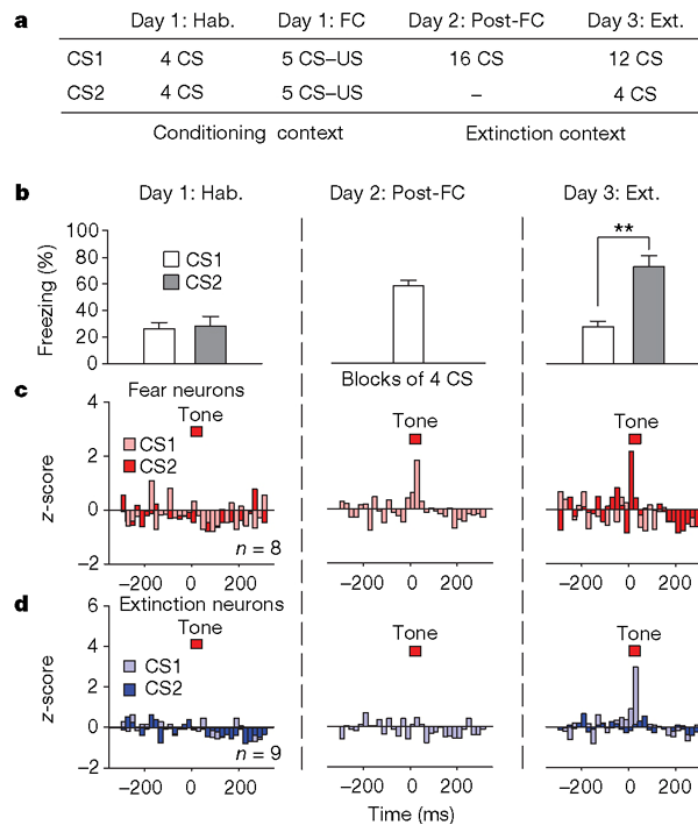
Figure 2. Fear and extinction neurons discriminate stimuli with different emotional significance. a, Experimental design for discriminative extinction training. Initially, animals were fear conditioned to two distinct CS (CS1 and CS2). Both CS were paired with a US (CS–US). Subsequently, only one CS (CS1) was extinguished. **b,** Summary of behavioural data. During habituation, mice (n = 6) exhibited equally low freezing levels in response to CS1 and CS2 exposure. After fear conditioning, presentation of the CS1 (CS 1–4) evoked significantly increased freezing levels. After extinction to CS1, CS1 exposure (CS 9–12) elicited low freezing levels, whereas CS2-evoked freezing behaviour remains high. Error bars indicate mean ± s.e.m. **c,** Fear conditioning- and extinction-induced changes in CS1- and CS2-evoked firing of fear neurons (n = 8 neurons from 3 mice). Twenty-four hours after fear conditioning (day 2), fear neurons exhibited increased firing in response to CS1 stimulation. After extinction of CS1, only CS2 stimulation elicited significant firing (day 3; P < 0.05 versus CS1). **d,** Fear conditioning- and extinction-induced changes in CS1- and CS2-evoked firing of extinction neurons (n = 9 neurons, 3 mice). After fear conditioning (day 2),

extinction neurons did not respond to CS1 stimulation. After extinction of CS1, only CS1 stimulation elicited significant firing (day 3; *P* < 0.05 versus CS2). **P < 0.01.

These observations confirm that individual fear neurons and extinction neurons represent functionally distinct classes of neurons that can discriminate between extinguished and non-extinguished stimuli.

In addition to the BA, we also recorded from 38 neurons in the LA, which represents the main target of sensory afferents from the thalamus and cortex (LeDoux 2000). In keeping with previous studies (Quirk, Repa et al. 1995), we did not observe any LA neuron in which CS+-evoked firing increased during extinction. Although we cannot exclude the existence of such neurons in the LA, this may suggest that extinction neurons are specific for the BA, where they represent 14% of all recorded neurons.

Activity balance predicts behaviour

Comparing the averaged time courses of CS-evoked activity of fear and extinction neurons during the acquisition of behavioural extinction indicated that significant behavioural changes occurred after the activity scores of the two populations of neurons crossed over (Fig. 3a). The largest changes in CS-evoked activity for both fear and extinction neurons occurred between the third and the fourth blocks of extinction training, which are separated by 24 h, suggesting that an overnight consolidation process may be required. To investigate further the exact time point during extinction learning at which fear and extinction neurons displayed a significant change in activity, we applied a change-point analysis algorithm (Gallistel, Fairhurst et al. 2004). Change-point analysis identifies the trial(s) exhibiting a significant change in neuronal activity or freezing behaviour relative to the preceding trials. This analysis confirmed that changes in neuronal activity precede behavioural changes, and revealed that the activity of extinction neurons started to increase one trial before the activity of fear neurons began to decline (Fig. 3b, c). Plotting activity changes of single fear and extinction neurons recorded in the same animal showed that the sequence of events is the same in an individual animal, and that such changes occur abruptly in an all-or-none manner (Fig. 3c). This is consistent with the idea that behavioural changes are driven by sequential switches in the activity of two distinct neuronal circuits.
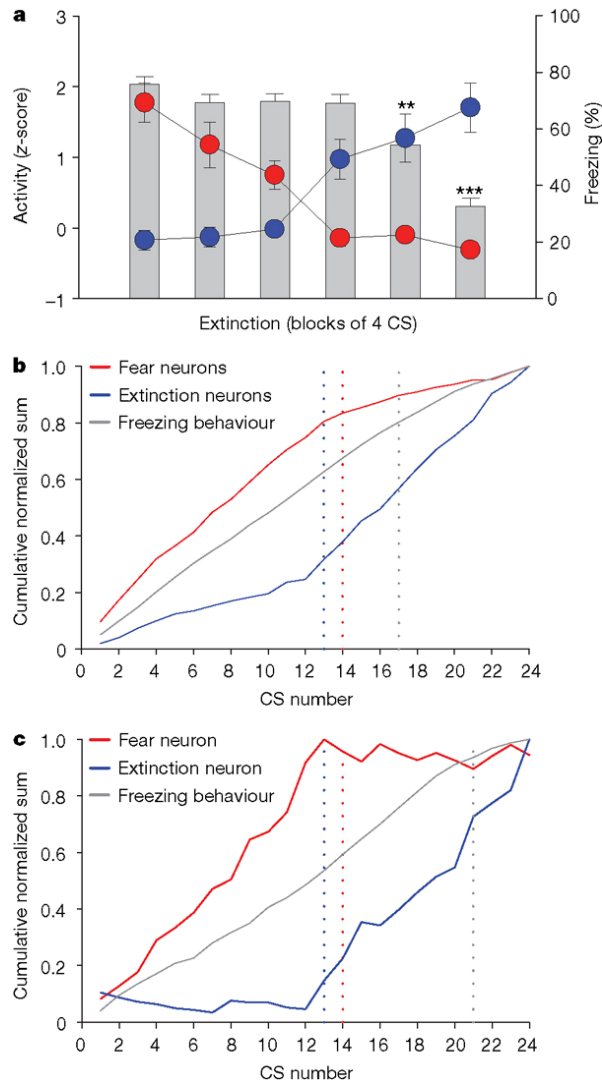
Figure 3. Sequential switches in neuronal activity precede behavioural changes. a, Averaged time courses of freezing behaviour (grey bars; n = 30 mice) and neuronal activity (z-scores) of BA fear neurons (red circles; n = 43) and extinction neurons (blue circles; n = 35) during extinction training. Significant behavioural changes (that is, decreased freezing levels) occurred after activity scores of fear and extinction neurons have crossed over. Error bars indicate mean ± s.e.m. **b,** Change-point analysis confirms that changes in neuronal activity preceded behavioural changes, and demonstrates that the activity of extinction neurons started to increase one trial before the activity of fear neurons changed. The plot represents the cumulative sums of the averaged and normalized z-scores of fear and extinction neurons, and freezing behaviour during extinction training. Change points are indicated by dotted lines. **c,** Normalized cumulative sums of the z-scores of a single fear neuron and a single extinction neuron recorded in the same animal together with the corresponding freezing behaviour during extinction training. Change-point analysis reveals that the extinction neuron abruptly switched on one trial before the fear neuron switched off. Changes in neuronal activity preceded behavioural changes. Change points are indicated by dotted lines. $**P < 0.01$; $***P < 0.001$.

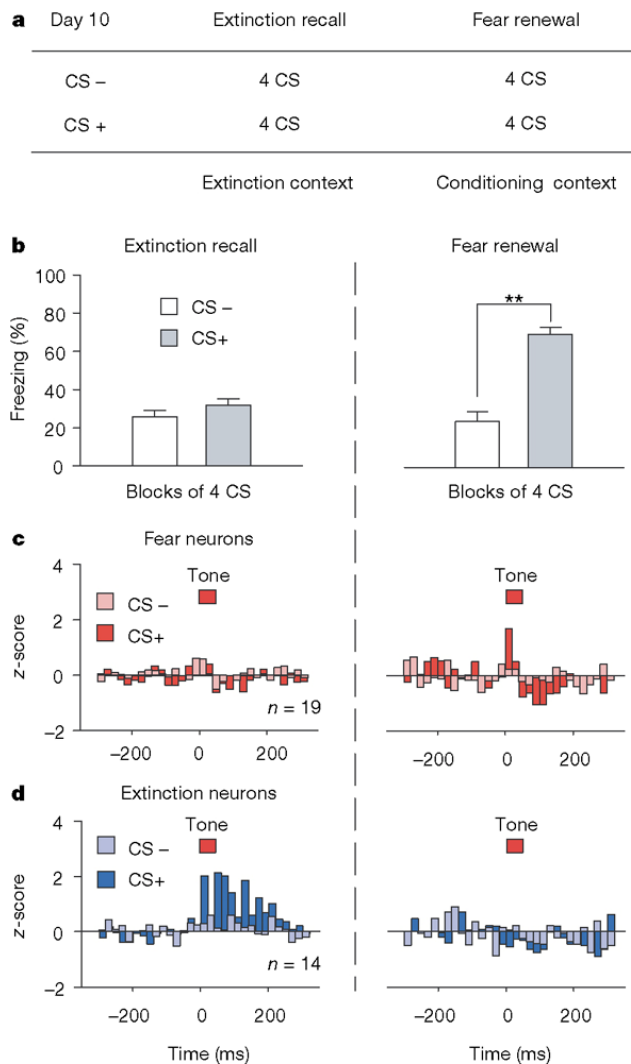## Rapid reversal of activity during fear renewal

To test whether the activity of fear and extinction neurons represents the same behavioural values in a different paradigm, we analysed renewal of extinguished fear behaviour and associated changes in CS-evoked spike firing. To make sure that extinction memory was stably consolidated, mice (n = 15) were tested for extinction memory 7 days after extinction training in the same context in which extinction training occurred (Fig. 4a). After successful recall of extinction memory (Fig. 4b), mice were transferred to the context in which they had been initially fear conditioned. Changing context resulted in a modest, but significant, increase in baseline freezing levels owing to contextual fear conditioning (Supplementary Fig. 3), and in a full renewal of the original cued fear memory (Fig. 4b).
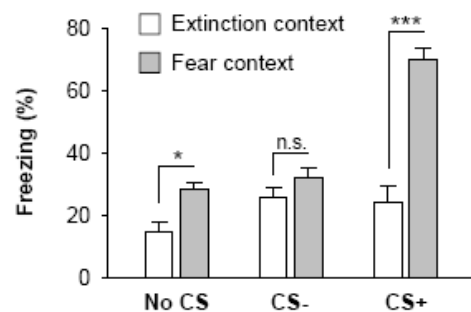
Supplementary Figure 3. Context-dependent freezing during fear renewal. Seven days after extinction, mice (n = 15 animals) were exposed to the CS+ and to the CS- in the extinction context and in the context in which fear conditioning took place. In the extinction context, both the CS+ and the CS– elicited low freezing behavior (CS–: 26 ± 3% of time; CS+: 32 ± 3%, P = 0.128 vs. CS–, P = 0.513 vs. extinction; same data as shown in figure 4). In the fear conditioning context, mice exhibited a modest, but significant increase in baseline freezing levels due to contextual fear conditioning (extinction context: 15 ± 3% of time; fear conditioning context: 28 ± 2%, P < 0.05), which was not significantly different from CS–-induced freezing. In this context, exposure to the CS+ evoked significantly more freezing than CS– stimulation (CS–: 24 ± 5% of time; CS+: 70 ± 4%, P < 0.01 vs. CS–, P < 0.001 vs. extinction recall; same data as shown in figure 4). *P < 0.05, ***P < 0.001.

During recall of extinction memory in the extinction context, presentation of the CS+ induced a selective activation of extinction neurons (n = 14, 8 mice) with no effect on fear neurons (n = 19, 9 mice; Fig. 4c, d). Thus, activation of extinction neurons by an extinguished CS is not a transient phenomenon, but remains stable for at least one week. After placing the animals in the fear-conditioning context, increased CS+-evoked freezing behaviour was associated with a complete reversal of spiking activity at the cellular level. Whereas extinction

neurons stopped responding to CS+ stimulation, fear neurons exhibited a significant and selective increase in CS+-evoked spike firing (Fig. 4d). Extinction-resistant neurons were not significantly activated during renewal (not shown). Thus, a switch in the balance of activity between fear and extinction neurons not only reflects extinction but also parallels rapid context-dependent renewal of conditioned fear responses.


Figure 4. Context-dependent fear renewal induces rapid reversal of neuronal activity patterns. a, Experimental protocol. **b,** Summary of behavioural data. Seven days after extinction, extinction memory was tested in the same context in which extinction training took place (n = 15 animals). Both CS+ and CS– elicited low freezing behaviour. Subsequently, mice were placed back into the context in which fear conditioning took place. In this context, exposure to the CS+ evoked significantly more freezing than CS– stimulation. Error bars indicate mean ± s.e.m. **c,** Context-dependent changes in CS+-evoked firing of fear neurons (n = 19 neurons from 9 mice). Fear neurons exhibited a context-dependent increase in CS+-evoked firing in the fear-conditioning context where freezing levels were high (P < 0.05 versus extinction context and versus CS–). **d,** Extinction neurons (n = 14 neurons, 8 mice) showed the opposite pattern. Whereas CS+-exposure elicited strong firing in the extinction context (P < 0.05 versus fear-conditioning context and versus CS–), extinction neurons did not show any CS+-evoked responses in the fear-conditioning context. **P < 0.01.

Differential long-range connectivity

We next addressed the question of whether fear neurons and extinction neurons are anatomically segregated. Comparing the location of electrolytic lesions made by the electrodes from which fear and extinction neurons were recorded did not provide any evidence for anatomical segregation (Supplementary Fig. 4). As a complementary approach, we compared the anatomical distribution of BA neurons activated during exposure to an extinguished or to a non-extinguished CS using the immediate early gene product Fos as an activity-marker. Given the similar numbers of extinction and fear neurons, one would predict that an extinguished and a non-extinguished CS should induce Fos expression in an equal number of BA neurons with an overlapping anatomical distribution. Consistent with this, we found no difference in the density and anatomical distribution of Fos-positive neurons in animals exposed to an extinguished and a non-extinguished CS (Supplementary Fig. 4). Together, these results suggest that BA fear and extinction neurons are intermingled in a salt-and-pepper-like manner.

Converging evidence supports a role for the mPFC in the consolidation of extinction memory (Milad and Quirk 2002; Quirk, Garcia et al. 2006; Myers and Davis 2007), and for the hippocampus in processing contextual information relevant for the expression and extinction of conditioned fear behaviour (Bouton, Westbrook et al. 2006). This raises the question as to how fear and extinction neurons in the BA communicate with the mPFC and the hippocampus during context-dependent behavioural transitions. We first addressed the possibility that fear neurons might be excitatory projection neurons whereas extinction neurons might be inhibitory interneurons. However, both fear and extinction neurons exhibited low spontaneous firing rates characteristic of BLA projection neurons (Likhtik, Pelletier et al. 2006) (Supplementary Table 1). Consistent with this, analysis of cross-correlations between identified fear or extinction neurons and neighbouring BA neurons revealed short-latency excitatory interactions (Supplementary Fig. 5). To examine whether identified fear and extinction neurons project to, or receive input from, the mPFC and/or the hippocampus, we tested for antidromic activation of BA efferents and orthodromic activation of afferents by using extracellular stimulation electrodes in re-anaesthetized mice (Fig. 5a; see Methods). These experiments revealed that fear neurons received

input from the hippocampus, whereas no connections with the hippocampus were found for extinction neurons (P < 0.05 versus fear neurons; Fig. 5b).
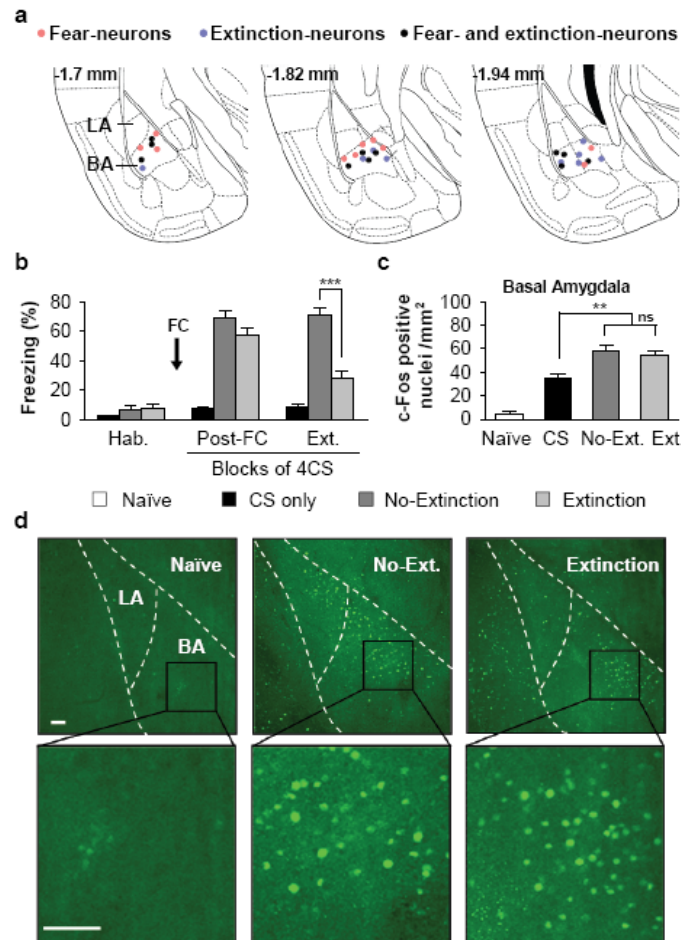

Supplementary Figure 4. **Fear and extinction neurons are intermingled within B**A. **a,** Coronal sections through the rostrocaudal extent of the amygdala showing the location of the recording wires in the BA from which activity of fear and extinction neuron was recorded. BA: basal nucleus of the amygdala; LA: lateral nucleus of the amygdala. **b,** Naïve mice (n = 7) and control animals (n = 21) exposed to the CS and to the context exhibited low freezing levels throughout the experiment. Fear conditioned animals showed high freezing levels at both time points. In mice subjected to extinction training, freezing levels were significantly reduced (Day 3, no-extinction: 71 ± 5% of time, n = 16; extinction: 28 ± 5% of time, n = 13, P < 0.001, two-tailed unpaired t-test). **c,** Averaged data illustrating that even though freezing behavior was significantly different, equal numbers of c-Fos expressing neurons were detected in the BA of mice exposed to an extinguished or to a non-extinguished CS (No-extinction: 58 ± 5 cells per mm2; extinction: 54 ± 4 cells per mm2, P = 0.533; two-tailed unpaired t-test). **d,** Examples of c- Fos expression in BA neurons of a naïve, non-extinguished and extinguished mice. **P < 0.01, ***P < 0.001, scale bar 100 μm

Although these findings cannot exclude that some extinction neurons might be contacted by hippocampal afferents, they demonstrate that the probability of receiving hippocampal input is significantly different for fear and extinction neurons. Likewise, fear and extinction neurons were differentially connected with the mPFC. Whereas extinction neurons were reciprocally connected, fear

neurons projected to the mPFC, but we did not find any inputs (P < 0.001 versus extinction neurons; Fig. 5b). Extinction-resistant neurons were reciprocally connected to both the mPFC and to the hippocampus (Supplementary Fig. 6). Taken together, these findings indicate that fear and extinction neurons, although co-localized within the same nucleus, not only are functionally specialized but also form part of discrete neuronal circuits.
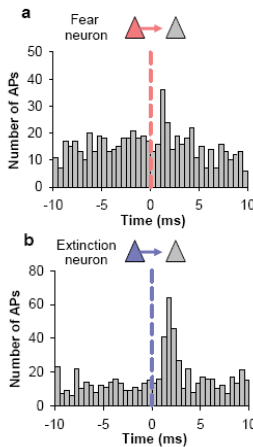

Supplementary Figure 5. Cross-correlation analysis. Consistent with the extracellular stimulation experiments, analysis of cross-correlations between identified fear- or extinction-neurons and neighboring BA neurons indicate that fear- and extinction-neurons are projection neurons. **a,** Cross-correlation between a fear-neuron and a non-identified neuron showing a short-latency, monosynaptic, excitatory interaction. Reference event is the spike of the fear neuron (dotted line at time 0). **b,** Cross-correlation between an extinction-neuron and a non-identified neuron showing a short-latency, monosynaptic, excitatory interaction. Reference event is the spike of the extinction neuron (dotted line at time 0).
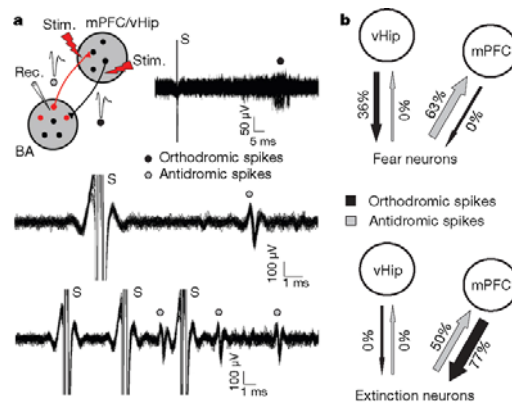
Figure 5. Fear neurons and extinction neurons are part of distinct neuronal circuits. a, Use of extracellular stimulation in anaesthetized mice to identify orthodromic and antidromic connections between BA neurons and the mPFC or the hippocampus. Top left: schematic illustrating the placement of stimulating and recording electrodes. Rec., recording electrode; Stim., stimulation electrode. Top right: orthodromic spikes elicited in a BA fear neuron on stimulation of the ventral hippocampus. Orthodromic spikes exhibited a large temporal jitter and high failure rates. Middle: antidromic spikes recorded from a BA extinction neuron in response to mPFC stimulation. Antidromic spikes exhibited low temporal jitter, and followed high frequency (200 Hz) stimulation (bottom). **b,** Top: fear neurons project to the mPFC (5 out of 8 stimulated neurons) and receive input from the hippocampus (5 out of 14 stimulated neurons). vHip, ventral hippocampus. No antidromic responses from the hippocampus (0 out of 14 stimulated neurons) or orthodormic responses from the mPFC (0 out of 8 stimulated neurons) were observed. The graph depicts

the percentage of all stimulation experiments in which a particular response was observed in identified fear neurons. Bottom: extinction neurons are reciprocally connected with the mPFC (antidromic responses, 3 out of 6 stimulated neurons; orthodromic responses, 7 out of 9 stimulated neurons, P < 0.001 versus fear neurons). No connections with the hippocampus were observed (0 out of 9 stimulated neurons, P < 0.05 versus fear neurons).
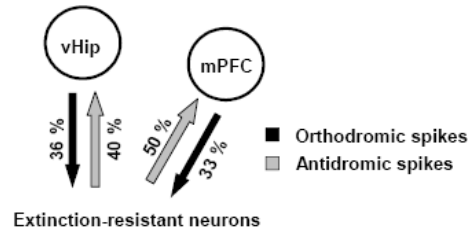
Supplementary Figure 6. Connectivity of extinction-resistant depicts **neurons.** Extinction-resistant neurons are reciprocally connected to the mPFC (orthodromic responses: 3 out of 9 stimulated neurons; antidromic responses: 6 out of 12 neurons) and to the hippocampus (orthodromic responses: 4 out of 11 stimulated neurons; antidromic responses: 2 out of 5 neurons). The graph the percentage of all stimulation experiments in which a particular response was observed in identified extinction-resistant neurons.

BA inactivation prevents behavioural transitions

The observed changes in CS+-evoked spike firing of fear and extinction neurons during the extinction and context-dependent renewal of conditioned fear responses could be necessary for the acquisition, storage and/or behavioural expression of the learned information. To distinguish between these possibilities, we used micro-iontophoresis of a fluorescently labelled GABAA (γ-aminobutyric acid subtype A) receptor agonist (muscimol) to reversibly inactivate neuronal activity in the BA in a targeted and controlled manner (Fig. 6a). Simultaneous iontophoresis and multi-unit recording revealed that muscimol application silenced neuronal activity in the BA for more than 60 min (Fig. 6b). We first tested whether BA activity was necessary for the acquisition of extinction. Inactivation of the BA completely prevented the decrease in freezing behaviour normally observed during extinction training (Fig. 6c), with no effect on pre-CS freezing levels (not shown). Twenty-four hours later, after wash-out of muscimol, the same animals initially exhibited high freezing levels followed by normal fear extinction, demonstrating that BA inactivation did not merely interfere with the behavioural expression of extinction, nor irreversibly damage BA function (Fig. 6d). These results demonstrate that BA activity is necessary for the acquisition of extinction.
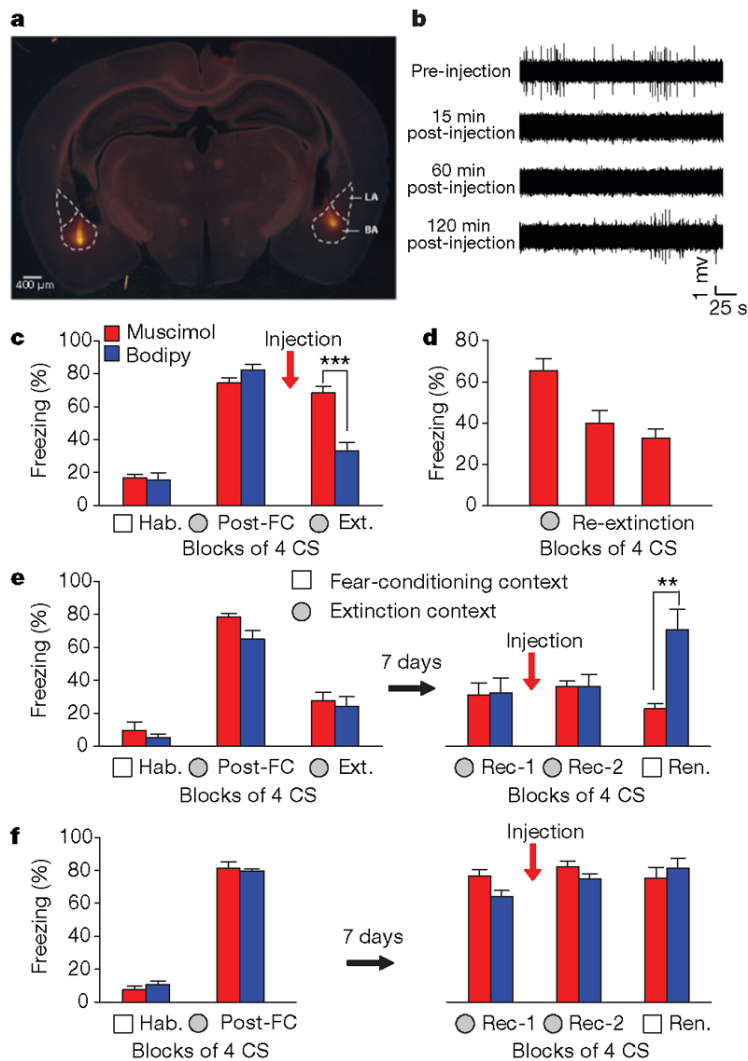
Figure 6: Targeted inactivation of the BA prevents behavioural changes without affecting memory. a, Epifluorescent image illustrating bilateral targeting of the BA with muscimol covalently attached to a fluorescent tag (bodipy, dipyrromethene boron difluoride). **b,** Simultaneous multi-unit recordings revealed silencing of neuronal activity for up to two hours after muscimol iontophoresis. **c,** Inactivation of the BA before extinction training prevented the acquisition of extinction. Control mice injected with fluorophore only (n = 5; blue bars) exhibited significant reduction of freezing levels after extinction training. Muscimol-injected animals (n = 11; red bars) showed high freezing levels after extinction. **d,** Twenty-four hours later, in the absence of muscimol, the same animals showed normal acquisition of extinction ($P < 0.05$). Bars illustrate the progressive decrease in freezing behaviour during extinction learning. **e,** Inactivation of the BA prevented context-dependent renewal. Control mice injected with fluorophore only (n = 5) exhibited a significant increase in freezing levels on change of context ($P < 0.05$). Muscimol-injected animals (n = 5) did not show any context-dependent fear renewal ($P < 0.01$ versus control). **f,** In the absence of extinction training, BA inactivation did not affect fear memory retrieval. Fluorophore-injected mice (n = 4) and muscimol-injected mice (n = 5) exhibited equal freezing levels during CS+ exposure in the fear-conditioning context one week after fear conditioning. Error bars indicate mean ± s.e.m. **$P < 0.01$, ***$P < 0.001$.

Next, we tested whether BA activity was necessary for the context-dependent renewal of previously extinguished fear responses. Mice exhibiting low freezing levels during recall of extinction memory one week after extinction training were injected with muscimol before renewal. In contrast to control animals injected

with the fluorescent label only, muscimol-injected animals exhibited no increase in freezing levels when exposed to the CS+ in the fear-conditioning context (Fig. 6e). These results demonstrate that BA activity is necessary for context-dependent fear renewal.

Because muscimol unselectively silences all neurons in the targeted region, the high fear level observed in muscimol-injected mice during extinction learning cannot be accounted for by activity of fear neurons. Conversely, the low fear level displayed by muscimol-injected mice during context-dependent fear renewal cannot be dependent on the activation of extinction neurons. Thus, whereas animals with inactivated BA are able to express high and low fear states, possibly by activation of other parts of the amygdala and the mPFC, they exhibit emotional perseveration (that is, they remained in the emotional state they were in before BA inactivation). This suggests that the BA is unlikely to be associated with the storage, retrieval or expression of conditioned fear and extinction memories, but may instead mediate context-dependent behavioural transitions between low and high fear states.

Thus, silencing of BA activity should have no effect on the retrieval and expression of conditioned and extinguished fear memories when there is no need to change fear levels in a context-dependent manner. Consistent with this scenario, BA inactivation had no effect on the retrieval or expression of consolidated extinction memories (Fig. 6e). Moreover, in animals that had been fear conditioned one week before, but that did not receive extinction training, muscimol had no effect on the retrieval and expression of the fear memory independently of the context in which they were tested (Fig. 6f).

Discussion

Our data show that the BA contains distinct populations of neurons for which activity is oppositely correlated with high and low fear behaviour—two converse behavioural states. Although fear and extinction neurons represent relatively small sub-populations within the BA, a rapid switch in the balance of their activity is essential for triggering behavioural transitions during extinction and context-dependent fear renewal. Although intermingled within the BA, fear and extinction neurons are differentially connected with the hippocampus and the mPFC, two brain areas previously implicated in extinction and context-dependent renewal of

conditioned fear responses. In keeping with the proposed role of the ventral hippocampus in mediating context-dependent renewal of fear behaviour in animals subjected to extinction (Hobin, Ji et al. 2006), we found that hippocampal input to the BA selectively targets fear neurons over extinction neurons. Thus, hippocampal input to BA fear neurons may override the retrieval of extinction memory allowing for fear expression after a particular CS has undergone extinction. Extinction neurons, in turn, are bi-directionally connected with the mPFC and are switched on during extinction training. This indicates that they may be upstream of a previously identified population of mPFC neurons thought to mediate consolidation of extinction memory, because they are activated by an extinguished CS during recall, but not during the acquisition of extinction (Milad and Quirk 2002)

Previous findings demonstrate that the BLA is not critical for triggering behavioural transitions during reversal learning in a two-odour-discrimination task (Stalnaker, Roesch et al. 2007). Nevertheless, abnormally persistent BLA activity induced by orbitofrontal cortex lesions (Stalnaker, Franz et al. 2007) or repeated cocaine administration34 interferes with reversal learning. This suggests that, whereas the BLA can only veto slow behavioural transitions during more complex reversal learning tasks, it is actively involved in situations requiring rapid context-dependent switching between two converse behavioural states.

How might activity of BA fear and extinction neurons mediate behavioural transitions? In keeping with a role for the amygdala in facilitating network function and memory formation in other parts of the brain (Amorapanth, LeDoux et al. 2000; McGaugh 2004; Paz, Pelletier et al. 2006), a possible interpretation is that BA fear and extinction neurons might drive or facilitate the induction of synaptic plasticity in their respective target areas. Moreover, whereas previous studies using pre-fear-conditioning lesions came to the conclusion that the BA does not contribute to the acquisition or the expression of conditioned fear ((Amorapanth, LeDoux et al. 2000; Nader, Majidishad et al. 2001), but see (Goosens and Maren 2001)), a recent analysis using post-fear-conditioning lesions indicates that the BA also contributes to the consolidation of long-term fear memories (Anglada-Figueroa and Quirk 2005). This suggests that repeated activity of BA fear neurons, over longer-time periods, may be required for fear memory consolidation.

Our findings are consistent with the idea that in mammals, as in invertebrates (Jing and Gillette 2000; Yapici, Kim et al. 2008), switches between appropriate behavioural states can be driven by discrete neuronal circuits. It may be a general principle of the functional micro-architecture of the nervous system in diverse species, that circuits mediating switches between distinct behavioural states are located in close anatomical proximity thereby allowing for local interactions. However, it remains to be shown how fear and extinction neurons interact locally. Finally, our results also suggest that context-dependent recovery of extinguished fear behaviour in humans (Milad, Orr et al. 2005), which represents a major clinical obstacle for the therapy of certain anxiety (Rodriguez, Craske et al. 1999), might be modulated by tipping the balance of activity between specific neuronal circuits.

Results Part II

Dissociable roles for tonic and phasic inhibitory network activity in fear conditioning

Stephane Ciocchi*, Cyril Herry*, Francois Grenier, Steffen B.E. Wolff, Michael Stadler, Christian Müller and Andreas Lüthi.
In preparation. * Equal contribution to this work.

Abstract

While many studies have demonstrated that neuronal plasticity in the lateral amygdala (LA) is necessary for the acquisition of Pavlovian fear conditioning, the role of the central nucleus of the amygdala (Siggins, Martin et al.), which is mainly composed of GABAergic inhibitory neurons, is poorly understood. To address the role of CEA inhibitory circuits in fear conditioning, we obtained single unit recordings from neurons located in the lateral (CEl) and medial (CEm) subdivisions of the CEA in behaving mice. We found that CEm output neurons, that control fear behavior via projections to brainstem targets, are under tight inhibitory control from a subpopulation of neurons located in CEl. Fear conditioning induced opposite changes in phasic and tonic inhibition in the CEl to CEm pathway. Targeted pharmacological inactivation of CEl and CEm revealed that whereas plasticity of phasic inhibition is necessary for gating CEm output during fear learning and expression, changes in tonic inhibitory network activity control signal-to-noise ratio and stimulus discrimination. Our results identify CEA inhibitory circuits as a major site of plasticity in fear conditioning, and suggest that regulation of tonic activity of inhibitory circuits may be an important mechanism for controlling sensitivity and specificity in associative learning.

Introduction

The amygdala is a key brain structure involved in the acquisition and expression of conditioned fear responses (LeDoux 2000; Maren and Quirk 2004). During classical auditory fear conditioning, an initially neutral sensory stimulus (the conditioned stimulus, CS) is paired with a noxious stimulus (the unconditioned stimulus, US). When subsequently presented alone, the CS triggers a fear

response. In the classical circuit model of fear conditioning, the lateral nucleus of the amygdala (LA) is thought of as the primary site where CS-US associations are formed and stored (LeDoux 2000; Maren and Quirk 2004). The formation of CS-US associations in the LA is mediated by N-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation (LTP) at glutamatergic sensory inputs originating from auditory thalamus and cortex (Sigurdsson, Doyere et al. 2007; Sah, Westbrook et al. 2008). NMDA receptor-dependent LTP of glutamatergic sensory inputs eventually results in enhanced CS-evoked firing of LA principal neurons (Maren and Quirk 2004).

In contrast to the LA, the central nucleus of the amygdala (Siggins, Martin et al.) has been considered to be primarily involved in the behavioral expression of conditioned fear responses. Single unit recordings from rabbit CEA revealed specific changes in neuronal activity after discriminative fear conditioning (Pascoe and Kapp 1985). Moreover, CEA output neurons, located in the medial subdivision (CEm), project to downstream targets in the brain stem and in the hypothalamus where they orchestrate conditioned autonomic and motor responses (Krettek and Price 1978; Veening, Swanson et al. 1984). However, in addition to its role in behavioral expression, recent evidence indicates a role of the CEA in learning. For instance, acute and reversible inactivation of the CEA during fear conditioning, or local blockade of NMDA receptors, result in impaired acquisition of conditioned fear responses (Goosens and Maren 2003; Wilensky, Schafe et al. 2006). This strongly suggests that activity-dependent plasticity within CEA is necessary for the acquisition of fear conditioning, yet the neuronal substrates mediating CEA plasticity during fear conditioning are unclear.

Possible sites of plasticity include glutamatergic synaptic inputs from the basolateral complex and from sensory thalamus onto CEm output neurons (Turner and Herkenham 1991; Samson, Duvarci et al. 2005; Samson and Pare 2005). Moreover, since CEm output neurons are under tight inhibitory control originating in the lateral and capsular subdivisions (together referred to as CEl)(Sun, Yi et al. 1994; Veinante and Freund-Mercier 1998; Cassell, Freedman et al. 1999; Huber, Veinante et al. 2005), a reduction in CEl to CEm inhibition might contribute to increased CEm output after fear conditioning. Consistent with this idea, enhancing inhibitory activity in CEl by endogenous neuropeptides and exogenous substances, such as ethanol, has anxiolytic effects (Roberto,

Madamba et al. 2003). However, the role of intra-CEA inhibitory circuitry in the acquisition and expression of conditioned fear responses is not known.

To address this question, we have used a combination of in vivo electrophysiological approaches in behaving and anaesthetized mice, together with targeted pharmacological manipulations of CEl and CEm. We found that fear conditioning induced an increase CS-evoked firing of CEm output neurons. This increase was predominantly gated by a CS-evoked, phasic dis-inhibition originating from a subpopulation of CEl neurons. Moreover, the signal-to-noise ratio and the stimulus-specificity of CEm output were regulated by fear conditioning-associated changes in tonic inhibition. Our study identifies concerted changes in phasic and tonic activity within intra-CEA GABAergic circuits as key events gating the acquisition and shaping the behavioral expression of conditioned fear responses.

Results
Fear conditioning induces differential plasticity in CEl and CEm

To investigate fear conditioning-induced changes in CS-evoked neuronal firing in discrete CEA subnuclei, C57Bl/6 mice were implanted with chronic recording electrodes in CEl or CEm. After habituation to the CS, mice (n= 30) were trained in a discriminative fear conditioning paradigm consisting of five CS+-US pairings (Fig. 1a). A second, unpaired CS (CS–) served as an internal control. Twenty four hours after conditioning, mice exhibited an increase in fear behavior (as measured by freezing) when exposed to the CS+ in a different context (60.9 ± 4.1% of time spent freezing; P < 0.001) (Fig. 1b).

We recorded a total of 176 units, 161 of which were located in CEl and 15 in CEm (Fig. 1c). In contrast to BLA neurons, which generally show very low levels of spontaneous firing (Likhtik, Pelletier et al. 2006; Herry, Ciocchi et al. 2008), the average spontaneous firing rate of CEl and CEm neurons was considerably higher (CEl, 5.01 ± 1.12 Hz; CEm, 9.88 ± 2.99 Hz). To examine whether fear conditioning induced changes in CS-evoked firing, we quantified neuronal activity of each neuron from 0 – 500 ms (50 ms bins) following CS onset as an average z-score (each bin expressed as the number of standard deviations above or below the mean baseline firing rate), and compared CS-evoked firing 24 hrs after

conditioning to baseline levels measured during habituation. Compared to habituation, 87% of neurons (n = 13) located in CEm exhibited a marked increase in CS+-evoked firing (average z-score, 0 – 100 ms, habituation: -0.02 ± 0.31; post fear conditioning: 2.83 ± 0.76, P < 0.001)(Fig. 1d). The remainder of the units did not exhibit any CS+-evoked responses. In contrast to CEm, responses of CEl units could be divided into two classes exhibiting opposite changes in CS-evoked activity after fear conditioning. Whereas 32% of units (n =52) acquired an excitatory response similar to CEm units (CElon neurons; habituation: 0.79 ± 0.28; post fear conditioning: 2.45 ± 0.39, P < 0.001)(Fig. 1e), in 25% of CEl neurons (n = 41) a strong inhibitory response was evoked by the
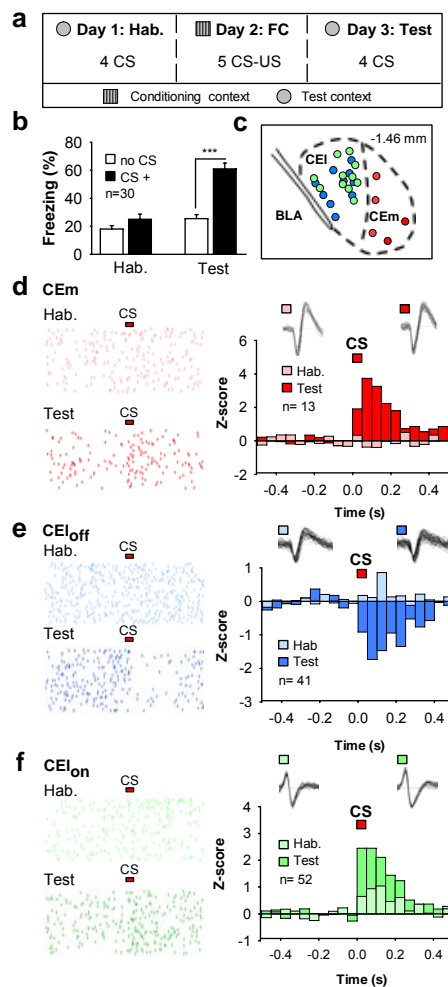
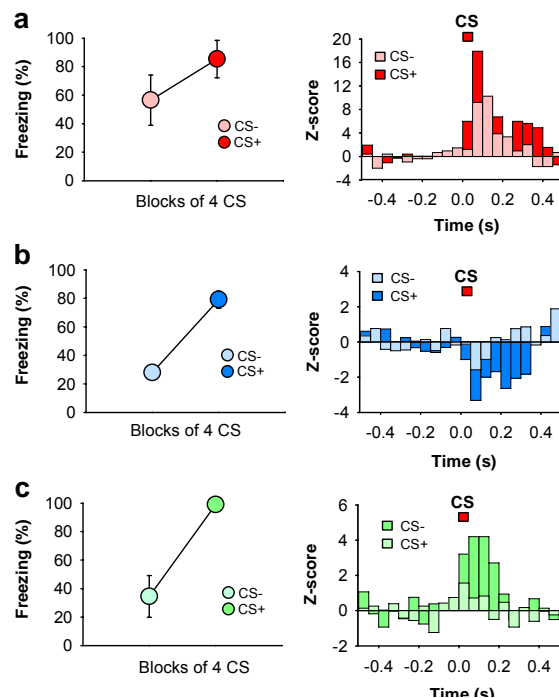

Figure 1. Fear conditioning induces differential plasticity in CEl and CEm. a, Experimental protocol. Hab., habituation; FC, fear conditioning. **b,** Summary graph illustrating behavioral data. During habituation, mice (*n* = 30) exhibited equally low freezing levels in the presence or absence of CS. Twenty-four hours after fear conditioning, presentation of the CS evoked significantly higher freezing levels. **c,** Coronal section of the amygdala showing the location of the recordings sites in CEl/CEm. Numbers indicate the anteroposterior coordinates caudal to bregma. **d – f**, Example raster plots (left panels) and normalized population peristimulus time histograms (right panels) illustrating changes in CS-evoked firing of CEm (d),

CEI$_{off}$ (e) and CEI$_{on}$ (f) neurons after fear conditioning. The duration of the auditory stimulus is indicated (red bar; CS). Insets show superimposed spike waveforms recorded during habituation and test, respectively. CEm neurons (n = 13 neurons from 5 mice) and CEI$_{on}$ neurons (n = 52 neurons from 21 mice) exhibited an increase in CS-evoked firing after fear conditioning. In contrast, CEI$_{off}$ neurons (n = 41 neurons from 21 mice) acquired an inhibitory CS-evoked response after fear conditioning. Error bars indicate mean ± s.e.m. ***P < 0.001.

CS+ after fear conditioning (CEIoff neurons; habituation: 0.14 ± 0.31; post fear conditioning: -1.32 ± 0.23, P < 0.001)(Fig. 1f). The rest of the units (43%) did not exhibit any CS+-evoked responses.

To address the specificity of CS-evoked neuronal firing, we compared CS+ and CS– responses in animals exhibiting behavioral discrimination (CS+: 72.8 ± 3.4% of time spent freezing; CS–, 27.8 ± 4.0%; n = 13; P < 0.001). Both classes of units recorded in CEI (CEIon and CEIoff units) as well as CEm units exhibited discriminating neuronal responses which correlated with freezing levels (Supplementary Fig. 1). Moreover, CS+-evoked responses were not only apparent 24 hrs after fear conditioning, but already started to increase during CS-US pairing (Supplementary Fig. 2).

Supplementary Figure 1   Behavioral and neuronal discrimination between CS$^+$ and CS$^-$.
a, b, c, Discriminative fear conditioning induces specific increases in freezing levels to the CS$^+$ vs CS$^-$ in mice with CEm (a, left) and CEI (b, c, left) recordings. Fear conditioning-induced changes in CS-evoked firing of CEm (a, right), CEI$_{off}$ (b, right) and CEI$_{on}$ (c, right) are larger for the CS$^+$-evoked responses compared to the CS$^-$-evoked responses.

Thus, fear conditioning induces rapid, specific and persistent changes in CS-evoked activity of CEl and CEm neurons. Whereas CEm contains a functionally homogeneous population of neurons that acquires excitatory CS responses, CEl appears to harbor at least two functionally distinct neuronal subpopulations in which CS responses change in opposite directions during conditioning.
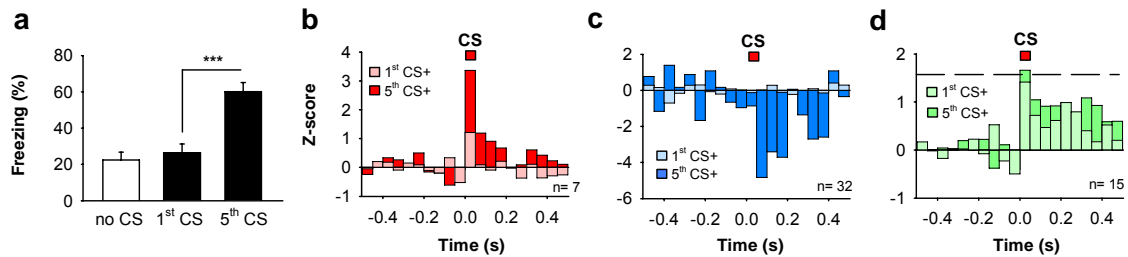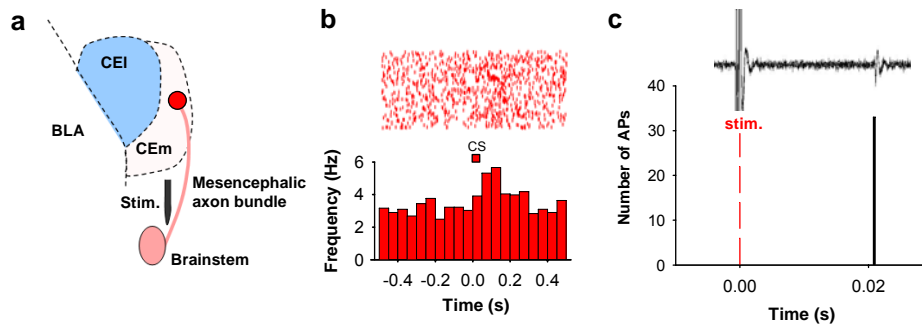

Supplementary Figure 2.   CS-evoked firing of CEl and CEm units changes during fear conditioning.
a, Summary graph illustrating changes in freezing behavior during fear conditioning. Comparing the first CS with the last CS reveals an increase in freezing behavior (CS1: 26.3 ± 4.9% of the time spent freezing; CS5: 60.0 ± 5.1%) during conditioning. **b - d,** Increased in freezing levels were associated with significant increases in CS-evoked activity in CEm neurons (n = 7 neurons from 4 mice), CEl$_{off}$ neurons (n = 32 neurons from 17 mice), and CEl$_{on}$ neurons (n = 15 neurons from 14 mice).

Analysis of intra-CEA circuitry reveals organized inhibitory networks

We first verified whether CEm neurons exhibiting excitatory CS responses were indeed output neurons projecting to the brainstem. After identification of CS-activated units in awake animals, mice were anaesthetized and a stimulation electrode was placed in the mesencephalic axon bundle, a fiber tract containing CEm projections to various targets in the brainstem (Pascoe and Kapp 1985). In 4 out of 6 cases we were able to evoke reliable, time locked antidromic responses in CS-activated CEm units (Supplementary Fig. 3), thus identifying them as output neurons. Next, given the inverse direction of plasticity in CEloff neurons and CEm output neurons, and based on previous anatomical and in vitro electrophysiological studies in rats describing an inhibitory GABAergic projection from CEl to CEm (Sun, Yi et al. 1994; Veinante and Freund-Mercier 1998; Cassell, Freedman et al. 1999; Huber, Veinante et al. 2005), we hypothesized that CEloff neurons project to CEm and disinhibit CEm output neurons. To address the plausibility of this model, we first measured the latencies of CS-evoked responses of CEloff and CEm neurons at higher temporal resolution. Interestingly, after fear conditioning, CEm neurons displayed a bi-phasic CS response; a very brief, short-latency response, followed by a much slower long-latency response (Supplementary Fig. 4). Because the short-latency response

started within 10-15 ms after CS onset, it most likely reflects the activity of direct thalamic inputs to CEm (Turner and Herkenham 1991). Consistent with the hypothesis that the long-latency component of CEm neurons was mediated by disinhibition from CEl, inhibition of CEloff neurons started before the onset of the late excitatory component in CEm neurons (onset latencies: CEloff neurons: 30-35 ms, n = 41; CEm neurons: 40-45 ms, n = 13).


Supplementary Figure 3. **Backfiring of CEm output neurons by stimulation of the mesencephalic axon bundle. a,** Schematic illustrating the placement of a stimulating electrode in the mesencephalic axon bundle to elicit antidromic spikes of CEm neurons. **b,** Peristimulus time histogram showing a CS-excited neuron recorded in CEm. **c,** Stimulation of the mesencephalic axon bundle elicits CEm neuron was observed upon mesencephalic axon bundle stimulation.

We then examined anatomical connectivity between CEl and CEm using two approaches. Firstly, we locally injected a retrogradely transported, replication-defective strain of herpes simplex virus (HSV-1) expressing the fluorescent marker GFP into CEl or CEm. Whereas injections into CEm resulted in intense retrograde labeling of neurons in CEl, CEm remained largely devoid of GFP after injections targeted at CEl (Supplementary Fig. 5). Thus, in keeping with earlier studies in rats (Pitkanen, Savander et al. 1997), CEl unidirectionally projects to CEm. Secondly, to address whether identified CEloff neurons project to CEm, we obtained intracellular recordings from CEl neurons in anaesthetized animals (Fig. 2a). Like in awake and behaving animals, CEl neurons recorded in anaesthetized mice were spontaneously active. A subpopulation of CEl neurons exhibited an inhibitory CS-evoked response (n = 2)(Fig. 2b). Filling CEloff neurons with neurobiotin revealed that their axon locally arborizes within CEl, but also sends collaterals to CEm (Fig. 2c). Axon collaterals targeting CEm exhibited boutons, putative sites of synaptic contacts (Fig. 2c).
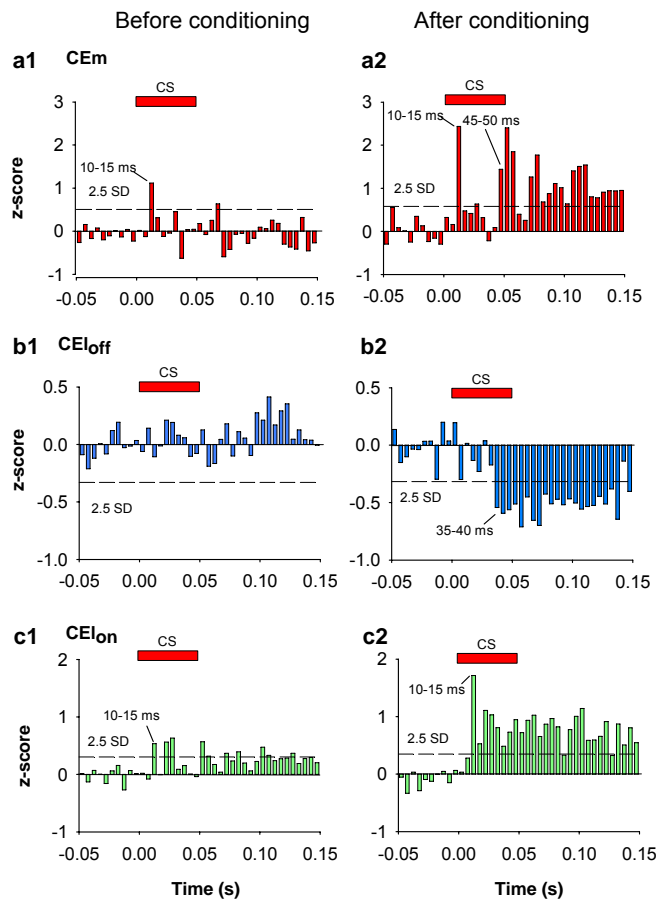
Supplementary Figure 4. Analysis of response latencies in CEl and CEm. a, Normalized population peristimulus time histograms revealed CS-evoked plasticity of a biphasic excitatory response of CEm neurons after fear conditionoing (a2; onset latency of first excitatory component; 10-15 ms; onset latency of second excitatory component; 45-50 ms) compared to before conditioning (a1; onset latency of first excitatory component; 10-15 ms) **b,** Normalized population peristimulus time histograms revealed CS-evoked plasticity of an inhibitory response of CEl$_{off}$ neurons after fear conditionoing (b2, onset latency of inhibition; 35-40 ms) compared to before conditioning (b1) **c,** Normalized population peristimulus time histograms revealed CS-evoked plasticity of an excitatory response of CEl$_{on}$ neurons after fear conditionoing (c2, onset latency of excitation; 10-15 ms) compared to before conditioning (c1, onset latency of excitation; 10-15 ms).
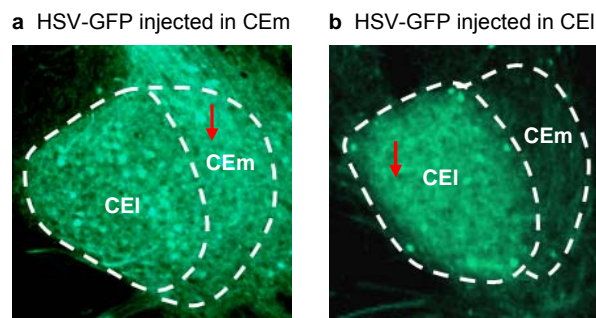
Supplementary Figure 5. Retrograde tracing of CEl to CEm projections. a, HSV-GFP injection to CEm reveals retrograde labelling of CEl neurons detected with anti-GFP immunostaining. **b,** HSV-GFP injection to CEl reveals local labelling in CEl as detected with anti-GFP immunostaining. Very few labelled neurons were observed in CEm, indicating that the CEl to CEm projection is unidirectional. Red arrow indicates injection site of the virus.
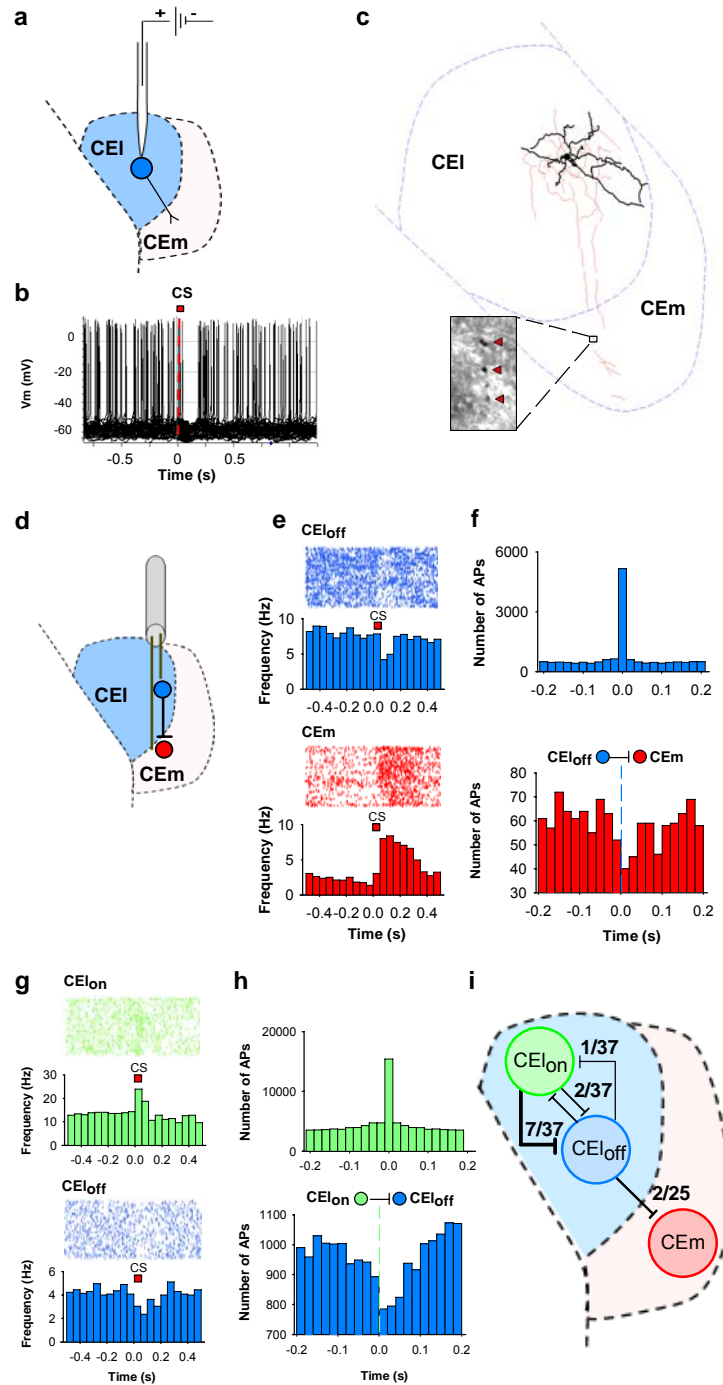
Figure 2. **Analysis of intra-CEA circuitry reveals organized inhibitory networks. a,** Schematic illustrating intracellular recordings of CS-responsive neurons in the CEl of anaesthetised mice. **b,** Example recording of a CEl$_{off}$ neuron spontaneously active at resting membrane potential (V_m). CS presentation induced an inhibition of spike firing. The duration of the auditory stimulus is indicated (red bar; CS). **c,** Reconstruction of the neurobiotin-filled CEl$_{off}$ neuron revealed axonal projections targeting CEm. Inset shows putative synaptic contacts as suggested by the presence of axonal boutons in CEm. **d,** Schematic illustrating simultaneous multi-site extracellular recordings in CEl and CEm. **e,** Example raster plots and non-normalized peristimulus time histograms showing a pair of a simultaneously recorded CEl$_{off}$ neuron (top, in blue) and a CEm neuron (bottom, in red). **f,** Top: Auto-correlogram of the CEl$_{off}$ neuron (top) shows a peak

54

at time 0. Bottom: Cross-correlation analysis reveals a short latency inhibitory interaction between the CEl$_{off}$ neuron taken as a reference and the CEm neuron. APs: action potentials. **g,** Example raster plots and non-normalized peristimulus time histograms showing a pair of a simultaneously recorded CEl$_{on}$ neuron (top, in green) and a CEl$_{off}$ neuron (bottom, in blue). **h,** Top: Auto-correlogram of the CEl$_{on}$ neuron (top) shows a peak at time 0. Bottom: Cross-correlation analysis reveals a short latency inhibitory interaction between the CEl$_{on}$ neuron and the CEl$_{off}$ neuron. **i,** Schematic illustrating the number of connected pairs relative to the total number of possible connections.

To directly test whether CEloff neurons functionally inhibit CEm output neurons, we performed simultaneous multi-site single unit recordings in CEl and CEm and cross-correlated spiking activity between identified CEloff and CS-activated CEm neurons during periods of spontaneous activity (Fig. 2d,e). Out of 25 possible connections, we found two CEm neurons showing a short-latency decrease in firing probability contingent on CEloff neuron spiking (Fig. 2f). No interactions in the other direction (from CEm to CEl) were found. Together, these findings provide strong evidence that a subpopulation of CEl neurons – CEloff neurons – inhibit CEm output neurons in vivo.

Finally, we addressed the question of where inhibitory responses of CEloff neurons might originate from. If inhibitory responses of CEloff neurons were mediated by local inputs from CElon neurons, it should be possible to detect inhibitory cross-correlations between CElon and CEloff neurons. Cross-correlating spontaneous spikes of simultaneously recorded CElon and CEloff neurons revealed substantial, but asymmetric inhibitory interactions between the two classes of neurons (Fig. 2g,e). Out of 37 possible connections, we found 9 cases in which CEloff neurons were inhibited by spikes of CElon units (Fig. 2e,f). Two of these 9 interactions were reciprocal and only 1 CElon neuron was inhibited by a CEloff neuron (Fig. 2f). Furthermore, inhibitory cross-correlations between CElon or between CEloff neurons were very rare (3 out of 37 possible connections). Together, these findings indicate that distinct functional classes of CEl and CEm neurons form a highly organized inhibitory network that ultimately results in the CS-evoked disinhibition of CEm output neurons. This raises the question of whether activity-dependent plasticity of disinhibitory circuits within CEl may be required for the acquisition of conditioned fear responses.

**Differential role of CEl and CEm in fear acquisition and expression**

The observed changes in CS+-evoked spike firing of CEl and CEm neurons during conditioning could be necessary for the acquisition, retrieval and/or

behavioral expression of the fear responses. To address whether neuronal activity in CEl or CEm is required for the acquisition and/or the expression of conditioned fear responses we used micro-iontophoresis of a fluorescently labeled GABAA receptor agonist (muscimol) to reversibly inactivate neuronal activity in CEl, CEm, or the entire CEA in a targeted and controlled manner (Fig. 3a)(Herry, Ciocchi et al. 2008). Consistent with a previous study (Wilensky, Schafe et al. 2006), targeted inactivation of the entire CEA during fear conditioning resulted in a profound memory deficit when measured 24hrs later in the absence of muscimol (control: 59.4 ± 8.6% of time spent freezing, n = 5; muscimol: 27.4 ± 6.4%, n = 7, P < 0.01) (Fig. 3b,c). Whereas inactivation of CEl only resulted in the same memory deficit (24.4 ± 3.7% of time spent freezing, n = 14, P < 0.01), CEm inactivations had no effect (54.3 ± 5.5% of time spent freezing, n = 9, P > 0.05)(Fig. 3c). The observed learning deficit with CEA or CEl inactivations was not caused by a complete inability to detect and process nociceptive (US-related) information. Even though CEl neurons received orthodromic inputs from the PB and exhibited US responses (Supplementary Fig. 6), inactivation of CEA or CEl did not affect the threshold at which mice started to exhibit flinching behavior or vocalizations when exposed to USs of different intensities (Supplementary Fig. 7). Moreover, after wash-out of muscimol, animals in which CEl had been inactivated were able to acquire conditioned indicating that CEl inactivation did not irreversibly damage CEl function (Supplementary Fig. 8). Together, these results demonstrate that neuronal activity in CEl, but not in CEm, is necessary for the acquisition of conditioned fear. We next examined the role of CEl and CEm for memory retrieval or expression by local application of muscimol before memory retrieval (24 hrs after conditioning)(Fig. 3d). In contrast to the acquisition phase, we found that whereas inactivation of the entire CEA or CEm only resulted in a retrieval/expression deficit (CEA: control: 65.3 ± 8.0% of time spent freezing, n = 7; muscimol: 30.1 ± 10.2%, n = 7, P < 0.001; CEm: control: 54.3 ± 5.5%, n = 9; muscimol: 39.2 ± 6.0%, n = 9, P < 0.05), inactivation of CEl resulted in normal freezing levels (control: 57.7 ± 7.6%, n = 8; muscimol: 60.4 ± 6.7%, n = 8, P > 0.05)(Fig. 3d,e).
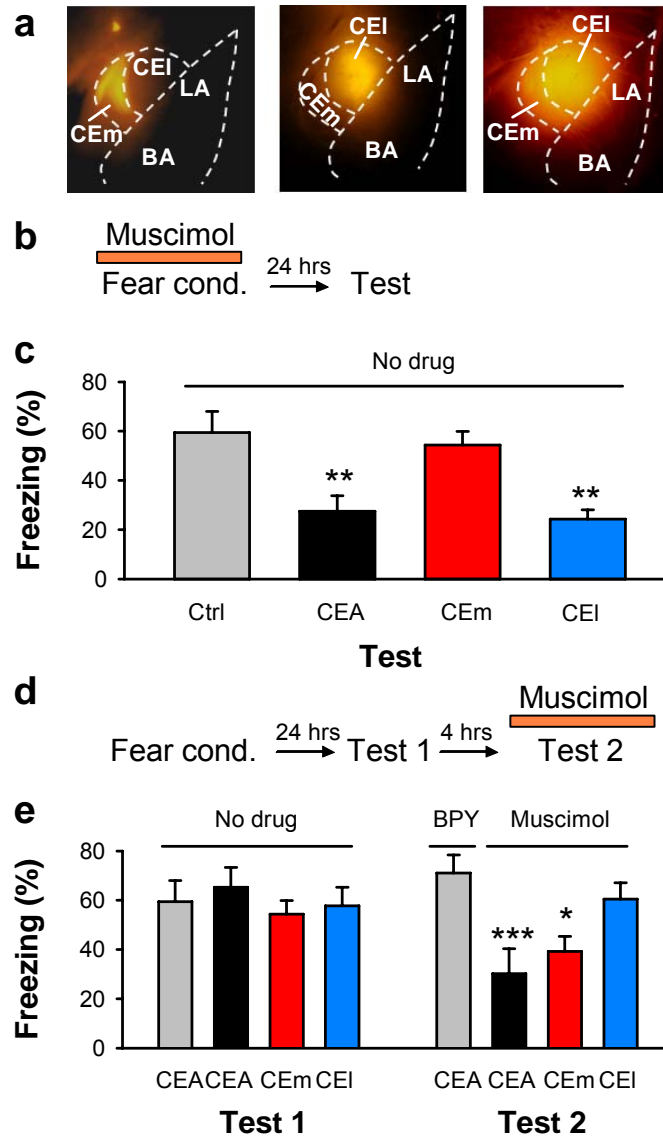
Figure 3. Differential role of CEl and CEm in fear acquisition and expression. a, Epifluorescent image illustrating micro-iontophoretic application of fluorescently labelled muscimol targeted at CEm, CEl, or the entire CEA. **b,** Experimental protocol used to test the role of CEm, CEl or the entire CEA in fear acquisition. Muscimol was applied during fear conditioning. Animals were tested 24 hrs later in the absence of muscimol. **c,** Inactivation of CEl (n = 14) or the entire CEA (n = 7), but not of CEm (n = 9), during fear conditioning prevented fear acquisition. Control mice injected with fluorophore only (n = 5) exhibited normal freezing levels at test. **d,** Experimental protocol used to assess the role of CEm, CEl or the entire CEA in fear expression. Animals were fear conditioned in the absence of muscimol and tested 24 hrs later. Muscimol was applied before animals were retested on the same day. **e,** Left: At test, all experimental groups exhibited equal freezing levels before muscimol application. Right: Inactivation of the CEm (n = 9) or the entire CEA (n = 7), but not of CEl (n = 8), impaired fear expression when tested in the presence of muscimol. Control mice injected with fluorophore only (n = 5) exhibited normal freezing levels. Error bars indicate mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.
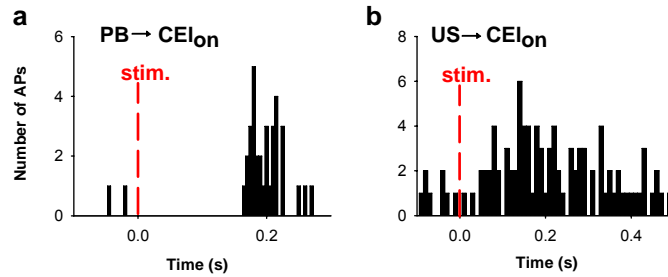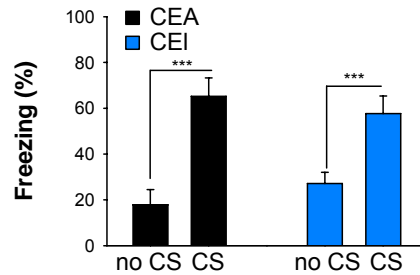
Supplementary Figure 6. Activation of CEI units by PB stimulation and US exposure. a, Orthodromic spikes elicited in a CEI_{on} neuron by extracellular stimulation of the parabrachial nucleus. PB, parabrachial nucleus; Stim., stimulation. Note that orthodromic spikes exhibited a large temporal jitter. **b,** Delivery of a footshock US (0.6-1.3 mA, 3 ms) to the hindlimb of an anesthetized mouse induces firing in CEI_{on} neurons. US, unconditioned stimulus; stim., stimulation.
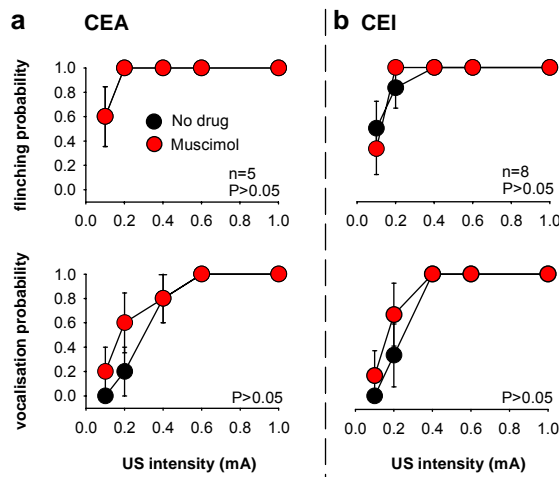
Supplementary Figure 7. Inactivation of CEI does not affect US sensitivity. a, Inactivation of CEA with muscimol does not affect US sensitivity as measured by the US threshold inducing flinching behavior (top, *n* = 5) or vocalization (bottom, *n* = 5). b, Likewise, inactivation of CEI with muscimol does not affect US sensitivity (top: flinching behavior, *n* = 8; bottom: vocalizations, *n* = 8).

Thus, there is a functional dissociation of CEI and CEm during the acquisition and expression of conditioned fear responses. Consistent with a role for activity-dependent neuronal plasticity in CEI for the acquisition of conditioned fear responses, we observed that neuronal activity in CEI was necessary for learning, but not for expression, whereas neuronal activity in CEm was dispensable during learning, but necessary for expression.

Supplementary Figure 8. Re-conditioning of mice after CEA or CEI inactivation. After wash-out of muscimol, mice in which CEA or CEI had been inactivated showed normal acquisition and retrieval of conditioned fear responses. Twenty four hours after muscimol application, mice (*n* = 7 in each group) were reconditioned and tested for fear memory retrieval again 24 hrs later (CEA, 65.3 ± 8.0% during CS exposure vs. 17.9 ± 6.6% before CS exposure; CEI, 57.7 ± 7.6% during CS exposure vs. 27.1 ± 4.9% before CS exposure). **P < 0.01.

Tonic inhibition controls signal-to-noise ratio and stimulus discrimination

CEI and CEm neurons exhibit much greater levels of spontaneous (tonic) activity as compared with BLA neurons (Likhtik, Pelletier et al. 2006; Herry, Ciocchi et al. 2008). This raises the question of whether tonic inhibition of CEm output neurons is behaviorally relevant. If this were the case, inactivation of CEI should induce freezing behavior. Consistent with this hypothesis, we found that during CEI inactivation, mice displayed greatly elevated levels of freezing (52.9± 5.2% of time spent freezing, n = 22)(Fig. 4a). This increase was more pronounced when CEI was inactivated bilaterally as compared with unilateral inactivations (Fig. 4b). Moreover, since freezing levels did not increase when the entire CEA was inactivated (26.8 ± 4.5% of time spent freezing, n = 5, P < 0.01 vs. CEI)(Fig. 4a), we conclude that CEm is necessary to express high freezing levels when CEI is non-functional. Thus, CEm output neurons are under tonic inhibitory control originating in CEI. To address whether tonic activity of CEI and CEm neurons changes with fear conditioning, we analyzed non-normalized firing rates of identified CEIoff and CEm neurons before and after fear conditioning. Similar to fear conditioning-induced changes in CS-evoked activity, tonic activity of CEIoff neurons and CEm neurons changed in opposite directions. However, in contrast to CS responses (which became inhibitory in CEIoff neurons and excitatory in CEm output neurons), tonic activity levels of CEIoff neurons had the tendency to increase with fear conditioning (before conditioning: 4.65 ± 1.12 Hz, after conditioning: 6.11 ± 1.41 Hz, n = 41, P = 0.07)(Fig. 4c), whereas those of CEm neurons decreased (before conditioning: 9.88 ± 2.99 Hz, after conditioning: 6.65 ± 2.22 Hz, n = 13, P < 0.01)(Fig. 4c). Plotting changes in tonic activity vs.

changes in CS-evoked activity for single neurons revealed that the two phenomena were correlated both in CEl and in CEm (Fig. 4b). The more tonic activity increased (in CEloff neurons) or decreased (in CEm neurons) with fear conditioning, the larger were the changes in CS-evoked activity, indicating that the signal-to-noise ratio of CS responses can be enhanced by concomitant changes in tonic activity levels.
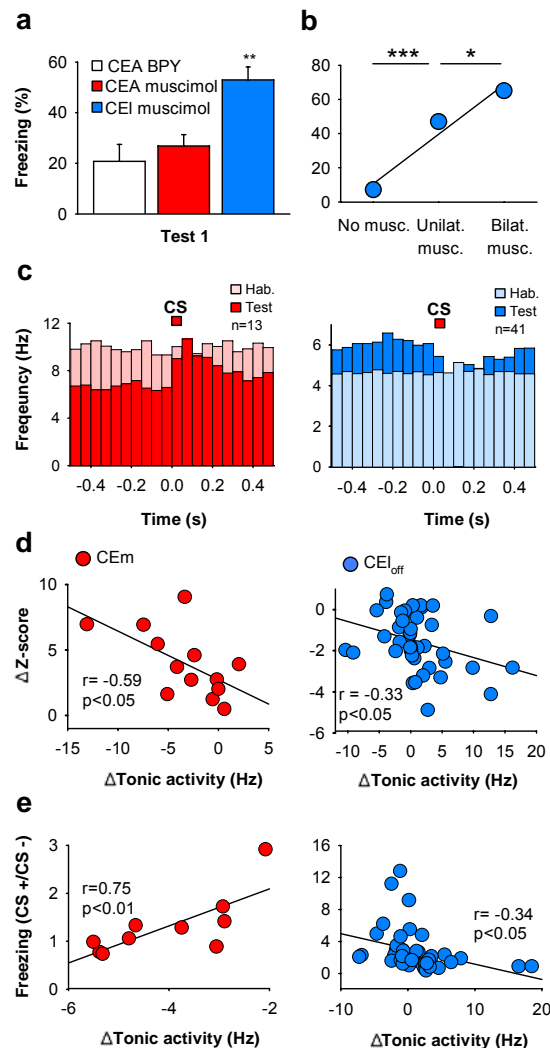
Figure 4 Tonic inhibition of CEm controls fear behavior and regulates generalization. **a,** CEl inactivation (n = 8) induces spontaneous freezing behavior. In contrast, inactivation of the entire CEA (n = 5), or control mice injected with the fluorophore only (BPY; n = 7) exhibit low freezing levels. **b,** Mice with bilateral injection of muscimol in CEl (n = 5) display significantly higher freezing levels compared to mice with unilateral injections (n = 17). Sham control mice were not injected with muscimol and show normal low freezing levels (n = 8). **c,** Non-normalized population peristimulus time histograms of CEm neurons (left) and CEl$_{off}$ neurons (right). Fear conditioning induces opposite changes in tonic activity in CEm and CEl$_{off}$ neurons. Tonic activity is decreased in CEm neurons (n = 13) while it increases in CEl$_{off}$ neurons (n = 41). **d,** Direct correlation between fear conditioning-induced changes in tonic activity and changes in CS-evoked phasic activity (z-scored) in CEm (left) and CEl$_{off}$ (right) indicating an increase in signal-to-noise ratio. **e,** Inverse correlation between fear conditioning-induced changes in tonic activity in CEm (left) and CEl$_{off}$ (right) and behavioral discrimination between the CS^{+} and the CS^{-}. $^{**}P$ < 0.01

Since an increase in the signal-to-noise ratio of stimulus-evoked responses would not be expected to be stimulus-specific, this might alter stimulus discrimination. We therefore examined whether changes in tonic activity affected CS+ vs. CS– discrimination at the behavioral level. Indeed, changes in tonic activity were inversely correlated with CS+ vs. CS– discrimination (Fig. 4e), indicating that stimulus discrimination is sensitive to changes in tonic inhibitory network activity.

Discussion

Our study shows that plasticity of intra-CEA inhibitory network function is a key factor controlling different aspects of fear conditioning. Whereas phasic, CS-evoked disinhibition of CEm output neurons is necessary for the acquisition of conditioned fear responses, modulation of spontaneous activity levels in CEm by means of changes in tonic inhibitory activity of CEIoff neurons shapes important aspects of conditioned fear behavior, such as stimulus sensitivity and discrimination.

Single unit recordings from CEm output neurons revealed CS-evoked bi-phasic responses, consisting of a brief short-latency response followed by a much slower and longer response. Considering the very short onset latency of the first component (11 ms) it can only be mediated by direct excitatory input from sensory thalamus. The observation that this short-latency component increased with fear conditioning suggests that thalamo-CEm synapses might be strengthened, possibly involving NMDA receptor-dependent LTP (Samson and Pare 2005). Interestingly, thalamo-CEm LTP does not require postsynaptic activity (Samson and Pare 2005), which is consistent with our finding that inactivation of CEm does not interfere with the acquisition of conditioned fear responses. The second component of CS-evoked responses of CEm output neurons, which had a much longer duration and contained the majority of the spikes, most likely reflects disinhibitory input from CEl. First, CS-evoked inhibition of CEIoff neurons started before the onset of slow excitation in CEm, and the responses in CEl and CEm exhibited a similar time course. Second, CEIoff neurons project to CEm and functionally inhibit CEm neurons. Third, the amplitude of CS-evoked slow excitation in CEm and CS-evoked inhibition in CEl correlated with conditioned freezing levels. Fourth, pharmacological inactivation

of CEl induced CEm-dependent freezing responses strongly suggesting that CEm is under tonic inhibitory control from CEl. And fifth, pharmacological inactivation of CEl during fear conditioning resulted in a learning deficit, indicating that activity-dependent plasticity in CEl to CEm inhibition is necessary for the acquisition of conditioned fear responses. Together, these findings indicate that fear conditioning-induced changes in CEm output are predominantly mediated by disinhibition from CEl.

What drives inhibitory responses of CEloff neurons? Cross-correlation analysis of local neuronal activity within CEl indicates that a separate subpopulation of CEl neurons (CElon neurons), which acquires CS-evoked excitatory responses during fear conditioning, drives inhibition in CEloff neurons. Since CElon neurons were more likely to inhibit CEloff neurons than other CElon neurons, and since the reverse interaction was less frequent, this suggests that CEl inhibitory circuits are highly organized. Although this does not exclude alternative possibilities, such as input from nearby intercalated interneurons (Millhouse, 1986; Paré and Smith, 1993), this may suggest a role for synaptic plasticity at glutamatergic inputs onto CElon neurons during fear conditioning. Consistent with this scenario, various forms of activity-dependent synaptic plasticity at glutamatergic inputs from the basolateral complex or from the parabrachial nucleus to CEl have been described (Fu and Shinnick-Gallagher, 2005; Lopez de Armentia and Sah, 2007).

In contrast to principal neurons in the basolateral complex, CEA neurons exhibit considerable levels of spontaneous activity. In keeping with the idea that CEm output neurons are under tonic inhibitory control from CEloff neurons, targeted inactivation of CEl resulted in CEm-dependent freezing. Moreover, fear conditioning was associated with opposite changes in spontaneous activity levels in CEloff and CEm neurons. Interestingly, decreased spontaneous activity of CEm output neurons resulted in an increased signal-to-noise ratio of CS-evoked responses. The mechanisms underlying increased tonic inhibition of CEm output neurons might involve diverse processes including changes in synaptic or intrinsic properties of CEloff neurons leading to increased spontaneous activity. Interestingly, decreased the levels of GABAA receptors containing α5 subunits have been described in the CEA after fear conditioning (Heldt and Ressler, 2007). Since extrasynaptic α5 containing GABAA receptors mediate tonic inhibitory currents in other cell types (Farrant and Nusser, 2005), and since α5-

deficient mice perform better in a number of learning paradigms (Collinson et al., 2002; Crestani et al., 2002), this may suggest a role for altered GABAA receptor subunit expression in mediating fear conditioning-induced changes in tonic inhibitory network activity.

Increased signal-to-noise ratio of CS-evoked responses was associated with generalization of conditioned fear responses to a non-conditioned stimulus. Notably, mice deficient for the 65kD isoform of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD65), which is strongly expressed in CEA (Poulin et al., 2008), exhibit reduced extracellular GABA levels and generalization of conditioned fear responses (Stork et al., 2000; Bergado-Acosta et al., 2008). This suggests that enhanced stimulus sensitivity may come at the price of lower stimulus discrimination, and that tonic activity in CEA disinhibitory networks is critical for determining the appropriate balance between stimulus sensitivity and stimulus discrimination.

In keeping with studies on appetitive conditioning paradigms (Cardinal et al., 2002; Balleine and Killcross, 2006), our results support the notion that CEA might process fear-related information in series with the basolateral complex, or independently, in a parallel manner. Moreover, our data reveal that inhibitory circuits in the CEA are highly organized, and establish important, but distinct, roles for plasticity of phasic and tonic inhibitory network activity in fear conditioning. Given that CEA circuitry is thought to be organized similar to striatal circuits (Cassell et al., 1999), this may indicate that coordinated changes in phasic and tonic inhibition are a widespread mechanism underlying associative learning in the CNS.

Outlook

To study experience-dependent plasticity of neuronal circuits, we took advantage of classical auditory fear conditioning, a robust behavioral paradigm that enables a direct link between plasticity of neuronal circuits and behavioral changes. Numerous studies have highlighted to the amygdala as the key brain region mediating CS-US association during fear conditioning (Goosens and Maren 2003; Maren and Quirk 2004).

In my thesis, I describe that the amygdala contains specific neuronal subtypes and circuits that contribute to distinct aspects of fear conditioning. Indeed, our data have shown that the BA contains discrete neuronal populations of neurons, the activity of which is inversely correlated with high and low fear behavior. The so-called fear and extinction neurons were found to be intermingled in the BA, while displaying preferential connectivity with the mPFC and the HC (Canteras and Swanson 1992; McDonald 1998; Pitkanen, Pikkarainen et al. 2000) two brain structures important for behavioral inhibition (Sotres-Bayon, Bush et al. 2004) and context coding (Corcoran and Maren 2001). Fear neurons received inputs from the hippocampus suggesting that this pathway is important with context-dependent fear renewal. Consistent with the idea that ventral hippocampus activates fear neurons in the BA during context dependent fear renewal (Corcoran and Maren 2001), we found that BA inactivation prevents fear renewal. Similarly, extinction neurons were found to be reciprocally connected to the mPFC. The mPFC has been shown to contain a population of neurons important to meditate consolidation of fear extinction memory (Milad and Quirk 2002). Our data show that the extinction neurons in the BA may actually be upstream to the mPFC neurons since inactivation of the BA prevents acquisition of fear extinction. Interestingly, we found that activity of fear neurons and extinction neurons in the BA are not important for memory per se, but rather act as a switch to trigger behavioral transition between states of low and high fear in a context-dependent manner.

In the second part of my thesis, I have investigated whether neuronal microcircuits in the CEA might actively contribute to FC, rather than just being a passive relay between the BLA and downstream structures in the brainstem. Recent studies showed that sensory and somatosensory inputs to the CEA can undergo some forms of activity dependent synaptic plasticity (Samson, Duvarci

et al. 2005; Lopez de Armentia and Sah 2007) and since interfering with activity or plasticity in CEA during FC prevents acquisition of conditioned fear (Wilensky, Schafe et al. 2006), we hypothesized that the CEA might be, in complement to the LA, an important brain structure for CS-US association during fear conditioning. The CEA is, at the cytoarchitectural level, profoundly different to the LA. Indeed, while the LA is a cortical-like structure consisting primarily of excitatory PNs (Millhouse and DeOlmos 1983), the CEA is a striatum-like structure almost exclusively containing GABAergic inhibitory neurons (Sun, Yi et al. 1994) (Cassell, Freedman et al. 1999). This raised the question of how the inhibitory CEA microcircuits control fear behavior. First, we found that intra-CEA inhibitory networks are plastic during fear conditioning. Three populations of CS-responsive neurons connected by inhibitory synapses were found in the CEl/CEm and constituted a (dis)–inhibitory network. Second, phasic, CS-evoked disinhibition of CEm output neurons was shown to be necessary for fear conditioning. In particular, activity in CEl was shown to be necessary for acquisition of conditioned fear, while activity in CEm was necessary for fear expression. Third, we found that CEA neurons have a high tonic activity. The tonic activity levels in CEm neuron was controlled by the tonic inhibitory activity of CEl_{off} neurons. Interestingly, tonic activity was shown to be plastic during fear conditioning and mediate important aspects of the conditioned fear behavior, such as stimulus sensitivity and discrimination.

Taken together, our studies on the microcircuitries of the BA and CEA suggest that FC is supported by a concerted activity of distinct neuronal sub-circuits controlling specific aspects of conditioned fear behavior. In the future, we are interested in understanding the molecular and physiological mechanisms controlling phasic and tonic plasticity in BA/CEA neuronal circuits during FC. Single-unit recordings and pharmacological inactivation have appeared to be a method of choice to discover neuronal circuits fundamental for FC. However, accessing intracellularly these neuronal populations is crucial for a mechanistic understanding. This will rely on finding cell-type specific markers, potentially in combination analysis of immediate early gene expression. Ultimately, these approaches are aiming at the expression of reporters to identify and visualize specific neurons, to obtain patch-clamp recordings in brain slices, and to manipulate their activity using conditionally activated channels/receptors.

In particular, it would be interesting to understand how extinction and fear neurons locally interact within the BA. Local GABAergic INs might be good candidates which could control opposite activity patterns in BA fear and extinction neurons. Amygdala INs are heterogeneous in terms of their marker expression profile (parvalbumin, somatostatin, calretinin) (Kemppainen and Pitkanen 2000; McDonald and Betette 2001). This may allow for using targeted gene insertion techniques in order to specifically express light-activated channels to induce (Channelrhodopsin) or repress (Halorhodopsin) neuronal activity in defined populations of INs. Combining such approaches with single unit recording in behaving mice constitutes a challenging and promising approach aiming at understanding the basis of the opposite activity patterns of fear and extinction neurons. In addition, to identify fear and extinction neurons, and to gain intracellular access allowing for analysis of physiological and morphological properties of BA fear and extinction neurons, mouse lines conditionally expressing GFP under the control of immediate early genes might represent an interesting approach, since immediate early genes have been shown to be specifically regulated during behavior (Herry and Mons 2004).

How would then fear and extinction neurons in the BA mediate behavioral transitions? We consider two main possibilites. First and consistent with a role of the amygdala in facilitating network function and memory formation in other brain areas (McGaugh 2004; Paz, Pelletier et al. 2006), extinction and fear neurons could drive or facilitate the induction of synaptic plasticity in their target brain regions. Second, locally in the BA, afferent inputs to fear and extinction neurons arising from the HC or the mPFC might exhibit some forms of synaptic plasticity that could underlie the rapid switch in CS-evoked activity observed during rapid behavioral transitions.

Evidence from recent literature proposed a role for the amygdala in processing information about rewards. In particular, segregated neuronal circuits have been shown to encode reward or aversive values (Paton, Belova et al. 2006). Thus, it is interesting to speculate that extinction neurons might be "reward neurons", in the sense that they might code for a CS- no US association during acquisition of extinction.as opposed to aversive CS-US coding of fear neurons.

Further, as we found that specific neuronal subpopulations in the CEA display differential plasticity of CS-evoked, phasic and tonic activity, it will be important to

further examine the underlying mechanisms using other approaches. Because CEA neurons express a variety of neuropeptides and neuropetides receptors (Roberts, Woodhams et al. 1982) that would potentially allow for generating mice expressing GFP in specific subpopulations under the control of promoters driving neuropeptides or neuropeptide receptors. In particular, a population of oxytocin receptor-expressing neurons in CEl has been demonstrated to inhibit a population of vasopressin receptor-expressing neurons in CEm (Huber, Veinante et al. 2005), similar to the CEloff neurons inhibiting CEm neurons in our experiments. Thus, ex vivo patch-clamp recordings of GFP-expressing neurons in the CEA in mice generalizing or discriminating between CSs might constitute a powerful model system to unravel the mechanisms underlying plasticity of tonic and phasic activity at the level of identified neuronal subpopulations. In particular, we think that changes in phasic activity in CEA inhibitory circuits might be dependent on synaptic plasticity mechanisms at afferent inputs to the CEA or even intrinsically to CEA, namely occurring at GABAergic inhibitory synapses. We consider that tonic activity changes might rely on the modulation of the CEA neuropeptidergic system or, alternaltively, on the regulation of ion channels that would control the intrinsic excitability of CEA neurons for prolonged period of time.

Finally, we believe that the CEA inhibitory networks might play a role in fear extinction. Fear extinction has been proposed to recruit inhibitory circuits in the amygdala to inhibit behavioral fear responses (Pare and Smith 1993; Pare and Smith 1994). The major candidate inhibitory circuits are thought to be the intercalated cell masses, which consists of clusters of GABAergic interneurons that surround the amygdala (Berretta, Pantazopoulos et al. 2005). However, the CEA inhibitory circuits could potentially lead to inhibition of fear responses, since CEm neurons are directly contacted by inhibitory neurons arising from CEl. Future experiments will have to address the neuronal correlateds of fear extinction in the CEA and their role in fear extinction.

Dysfunction of the amygdala has been suggested to be the cause of psychiatric diseases such as major anxiety disorders or post-traumatic stress disorders. Thus, in the long-term, targeting the activity of defined neuronal circuits in the amygdala might represent a novel strategy to treat patients with psychiatric conditions.

Material and methods

Animals

Male C57BL6/J mice (3 months old; RCC Ltd) were individually housed for 7 days before all experiments, under a 12 h light/dark cycle, and provided with food and water ad libitum. All animal procedures were performed in accordance with institutional guidelines and were approved by the Veterinary Department of the Canton of Basel-Stadt.

Behaviour

Fear conditioning and extinction took place in two different contexts (context A and B). The conditioning and extinction boxes and the floor were cleaned with 70% ethanol or 1% acetic acid before and after each session, respectively. To score freezing behaviour, an automatic infrared beam detection system placed on the bottom of the experimental chambers (Coulbourn Instruments) was used. The animals were considered to be freezing if no movement was detected for 2 s. On day 1, mice were submitted to a habituation session in context A, in which they received 4 presentations of the CS+ and the CS– (total CS duration of 30 s, consisting of 50-ms pips repeated at 0.9 Hz, 2-ms rise and fall; pip frequency: 7.5 kHz or white-noise, 80 dB sound pressure level). Discriminative fear conditioning was performed on the same day by pairing the CS+ with a US (1-s foot shock, 0.6 mA, 5 CS+/US pairings; inter-trial interval: 20–180 s). The onset of the US coincided with the offset of the CS+. The CS– was presented after each CS+/US association but was never reinforced (5 CS– presentations, inter-trial interval: 20–180 s). The frequencies used for CS+ and CS– were counterbalanced across animals. On day 2 and day 3, conditioned mice were submitted to extinction training in context B, during which they received 4 and 12 presentations of the CS– and the CS+, respectively. Recall of extinction and context-dependent fear renewal were tested 7 days later in context B and A, respectively, with 4 presentations of the CS– and the CS+. Pharmacological experiments were performed using the same conditioning and extinction protocol except for one group of mice that was not submitted to extinction training but tested for conditioned fear with 4 CS– and 4 CS+ presentations on day 2 in context B. Seven days later, mice were submitted to 2 sessions of extinction recall 5 h apart in context B (4 presentations of each CS for each session).

Finally, 10 min after the second recall session, mice were submitted to 4 CS– and 4 CS+ presentations in context A for context-dependent fear renewal.

For discriminative extinction, mice were habituated on day 1 to 4 presentations of two different CS in context A (total CS duration of 30 s, consisting of 50-ms pips repeated at 0.9 Hz, 2 ms rise and fall; pip frequency: 7.5 kHz or white-noise, 80 dB sound pressure level). Both CS were subsequently paired with a US (1-s foot shock, 0.6 mA, 5 CS/US pairings for each CS; inter-trial interval: 20–180 s). The onset of the US coincided with the offset of the CS. On days 3 and 4, only one of the two CS was extinguished by 16 and 12 presentations in context B, respectively. At the end of the second extinction session, mice were exposed to 4 presentations of the non-extinguished CS in context B.

US-induced flinching behavior and vocalizations were compared in the presence and absence of muscimol in freely moving mice. Delivered footshocks ranged from 0.1-1 Ma.

Surgery and recordings

Mice were anesthetized with isoflurane (induction 5%, maintenance 2.5%) in O2. Body temperature was maintained with a heating pad (CMA/150, CMA/Microdialysis). Mice were secured in a stereotaxic frame and unilaterally implanted in the amygdala with a multi-wire electrode aimed at the following coordinates: 1.7 mm posterior to bregma; ±3.1 mm lateral to midline; and 4 mm to 4.3 mm deep from the cortical surface. For CEA implantations, we used the following coordinates: 1.3 mm posterior to bregma; ±2.9 mm lateral to midline; and 3.9 mm to 4.3 mm deep from the cortical surface. The electrodes consisted of 8 to 16 individually insulated nichrome wires (13 -µm inner diameter, impedance 0.05–3 MΩ; California Fine Wire) contained in a 26 gauge stainless steel guide canula. The wires were attached to a 10 pin to 18 pin connector (Omnetics). The implant was secured using cyanoacrylate adhesive gel. After surgery mice were allowed to recover for 7 days. Analgesia was applied before, and during the 3 days after, surgery (Metacam, Boehringer). Electrodes were connected to a headstage (Plexon) containing eight to sixteen unity-gain operational amplifiers. The headstage was connected to a 16-channel computer-controlled preamplifier (gain 100x, bandpass filter from 150 Hz to 9 kHz, Plexon). Neuronal activity was digitized at 40 kHz and bandpass filtered from 250 Hz to

8 kHz, and was isolated by time–amplitude window discrimination and template matching using a Multichannel Acquisition Processor system (Plexon). At the conclusion of the experiment, recording sites were marked with electrolytic lesions before perfusion, and electrode locations were reconstructed with standard histological techniques.

Single-unit spike sorting and analysis

Single-unit spike sorting was performed using Off-Line Spike Sorter (OFSS, Plexon) as described (Supplementary Methods Fig.1, Supplementary Methods Fig.2). Principal component scores were calculated for unsorted waveforms and plotted on three-dimensional principal component spaces, and clusters containing similar valid waveforms were manually defined. A group of waveforms was considered to be generated from a single neuron if it defined a discrete cluster in principal component space that was distinct from clusters for other units and if it displayed a clear refractory period (>1 ms) in the auto-correlogram histograms. In addition, two parameters were used to quantify the overall separation between identified clusters in a particular channel. These parameters include the J3 statistic, which corresponds to the ratio of between-cluster to within-cluster scatter, and the Davies–Bouldin validity index (DB), which reflects the ratio of the sum of within-cluster scatter to between-cluster separation. High values for the J3 and low values for the DB are indicative of good single-unit isolation (Supplementary Methods Fig.1, Supplementary Methods Fig.2). Control values for this statistics were obtained by artificially defining two clusters from the centred cloud of points in the principal component space from channels in which no units could be detected. Template waveforms were then calculated for well-separated clusters and stored for further analysis. Clusters of identified neurons were analysed offline for each recording session using principal component analysis and a template-matching algorithm. Only stable clusters of single units recorded over the time course of the entire behavioural training were considered. Long-term single-unit stability isolation was first evaluated using Wavetracker (Plexon) in which principal component space-cylinders were calculated from a 5 min segment of data spontaneously recorded before any training session. Straight cylinders suggest that the same set of single units was recorded during the entire training session (Supplementary Methods Fig.1, Supplementary Methods Fig.2).

Second, we quantitatively evaluated the similarity of waveform shape by calculating linear correlation (r) values between average waveforms obtained over training days49 (Supplementary Methods Fig.1, Supplementary Methods Fig.2). As a control, we computed the r values from average waveforms of different neurons.
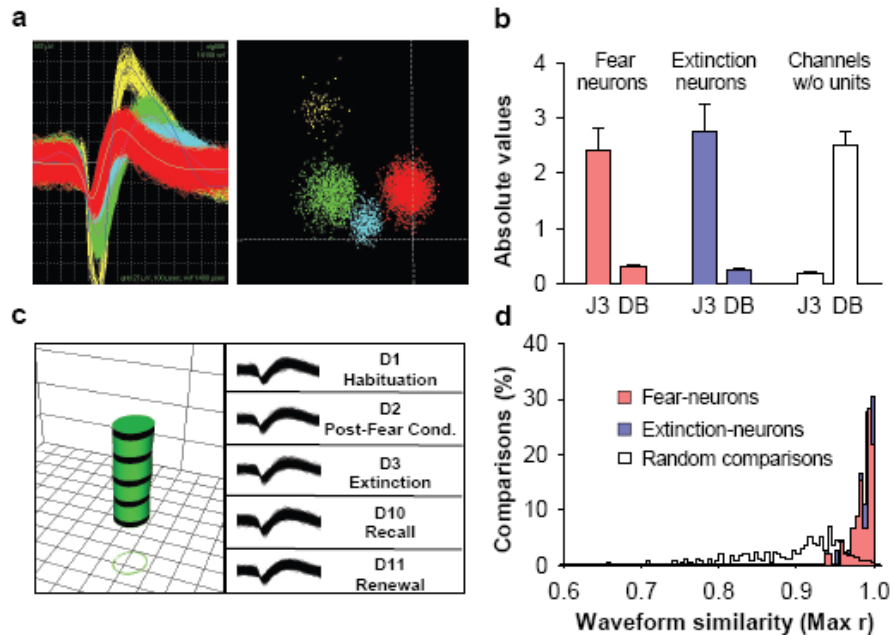
Supplementary Methods Figure 1. Stability of chronic single unit recordings from mouse basal amygdala. a, Top left: Superimposed waveforms recorded from four different units. Top right: Spikes originating from individual units were sorted using 3D-principal component analysis. **b,** Quantitative J3 and Davies Bouldin validity index (DB) statistics calculated for fear and extinction neurons. Controls values were obtained using two clusters defined from the centered cloud of points from channels in which no units could be detected. High values for the J3 and low values for the DB are indicative of good single unit isolation. **c,** Left: Stability of clustered waveforms during long-term recordings was assessed by calculating principal component (PC) space cylinders. Straight cylinders suggest that the same set of single units was recorded during the entire training session. Right: Superimposed waveforms used to calculate the PC space cylinder recorded before habituation, extinction, recall and renewal sessions. **d,** In addition, to quantitatively evaluate similarity of different spike shapes recorded on different days, linear correlation values between time-shifted average waveforms were calculated for fear and extinction neurons. As a control we computed the r values from average waveforms of different neurons.The maximum r value across time shifts was used to quantify similarity (r = 1 would indicates identical spike shapes). These calculations revealed that 94.4% of extinction neurons and 95.65 % of fear neurons had an r value above 0.95, compared with only 17.9% of similarity scores calculated between waveforms of different cells.

a

b

c
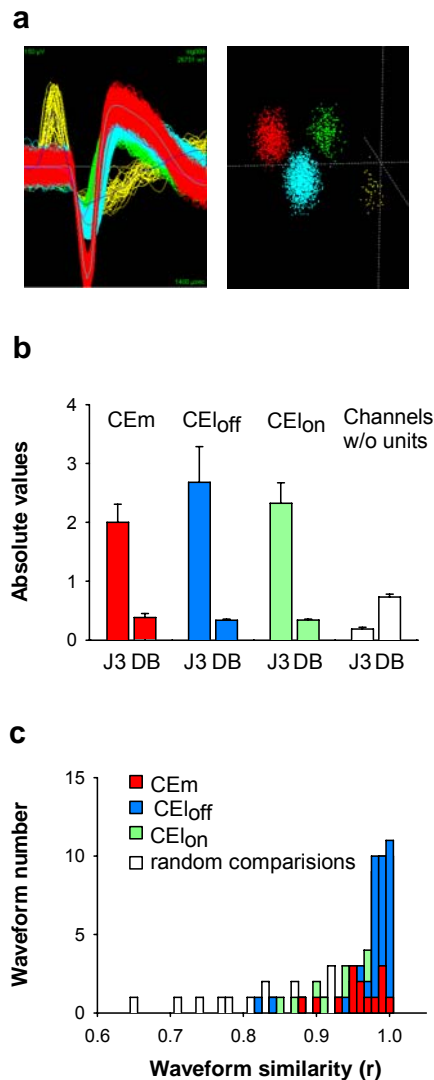
Supplementary Methods Figure 2 . Isolation and stability of unit recordings from central amygdala. a, Left: Superimposed waveforms recorded from four different units. Right: Spikes originating from individual units were sorted using 3D-principal component analysis. **b,** Quantitative J3 and Davies Bouldin validity index (DB) statistics calculated for CEm, CEl_{off} and CEl_{on} neurons. Controls values were obtained using two clusters defined from the centered cloud of points from channels in which no units could be detected. High values for the J3 and low values for the DB are indicative of good single unit isolation. **c,** In addition, to quantitatively evaluate similarity of different spike shapes recorded on different days, linear correlation values between time-shifted average waveforms were calculated for CEm, CEl_{off} and CEl_{on} neurons. As a control we computed the r values from average waveforms of different neurons. The r value across time shifts was used to quantify similarity ($r = 1$ would indicates identical spike shapes). These calculations revealed that 77% of CEm, 93% of CEl_{off} and 73% of CEl_{on} neurons had an *r* value above 0.95, compared with only 18% of similarity scores calculated between waveforms of randomly selected neurons.

Third, for each unit we used correlation analysis to quantitatively compare the similarity of waveform shape during CS+-stimulation and during a 60 s period of spontaneous activity recorded before each behavioural session (Supplementary Methods Fig.3). To avoid analysis of the same neuron recorded on different channels, we computed cross-correlation histograms. If a target neuron

presented a peak of activity at a time that the reference neuron fires, only one of the two neurons was considered for further analysis. CS-induced neural activity was calculated by comparing the firing rate after stimulus onset with the firing rate recorded during the 500 ms before stimulus onset (bin size, 20 ms; averaged over blocks of 4 CS presentations consisting of 108 individual sound pips in total) using a z-score transformation. z-score values were calculated by subtracting the average baseline firing rate established over the 500 ms preceding stimulus onset from individual raw values and by dividing the difference by the baseline standard deviation. Only CS-excited neurons were considered for analysis. Classification of units was performed by comparing the largest significant z-score values within 100 ms (within 200ms for CEA neuronal responses) after CS-onset during post-fear conditioning and extinction sessions according to the freezing levels. For high-fear states, the entire post-fear conditioning session was analysed, whereas, for low-fear states, analysis was restricted to the block of 4 CS presentations during which the fear level was the lowest. A unit was classified as a fear neuron if it exhibited a significant z-score value after fear conditioning (when freezing levels were high), but no significant z-score value after extinction (when freezing levels were low), and vice versa for extinction neurons. Finally, units were classified as extinction-resistant neurons if they displayed a significant z-score value during both post-fear conditioning and extinction sessions, independently of freezing levels. For statistical analysis, z-score comparisons were performed using the average z-score value calculated during the 40 ms after CS-onset (for CEA, we used the averaged z-score value over 100ms after CS-onset). In cases in which shorter or longer CS-evoked activity was observed, the average z-score was calculated during the 20 ms and 80 ms after CS-onset, respectively. To identify the trial in which individual neurons changed their CS-evoked responses during fear conditioning and extinction, we applied a change point analysis algorithm. Change point analysis identifies the trial(s) exhibiting a significant change in neuronal activity or freezing behaviour relative to the preceding trials. Change points are graphically represented by a change in the slope of a plot showing the cumulative sums of the averaged and normalized z-score and freezing values. Statistical analyses were performed using paired Student's t-tests post hoc comparisons at the $P < 0.05$ level of significance unless indicated otherwise. Results are presented as mean ± s.e.m.
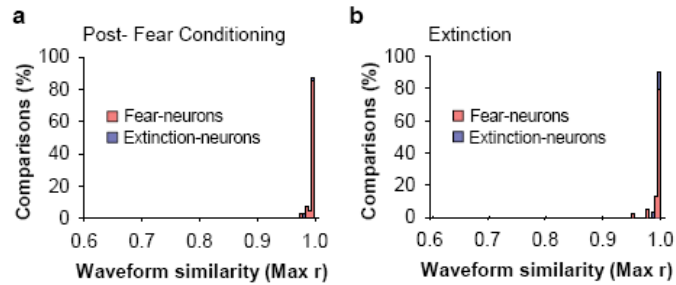
Supplementary Methods Figure 3. Quantitative comparisons of waveforms accross periods of spontaneous activity and sensory stimulation. a, For each identified fear- and extinction-neuron we calculated linear correlation values between time-shifted average waveforms obtained during a 60 s period of spontaneous activity recorded prior to each behavioral session and during CS stimulation. The maximum r value across time shifts was used to quantify similarity ($r = 1$ would indicates identical spike shapes). These calculations revealed r values above 0.95 for 100% of all units. **b,** Same plot for all units recoded before and during the extinction session.

To address CS-evoked latencies of the three CEA neuronal populations, normalized peri-stimulus time histograms (PSTH) were computed for each single neuron of each category using 5 ms bins. Populations PSTHs obtained established by averaging single neuron PSTHs. CS-evoked onset latencies were calculated for the population PSTH based on the first significant bin (at least 2.5 SD of baseline activity)

Extracellular stimulation

To determine the connectivity of recorded neurons, we used extracellular stimulation of the mPFC and the vHip in a subset of animals. At the end of the training procedure, animals were anesthetized using urethane (1.4 g kg-1), and concentric stimulating electrodes (FHC) were lowered in the mPFC (2 mm anterior to bregma; ±0.3 mm lateral to midline; and 1.6 mm to 2 mm deep from the cortical surface) and the ventral hippocampus (3.6 mm posterior to bregma; ±3.1 mm lateral to midline; and 4 mm to 4.2 mm deep from the cortical surface). For stimulation of CEA neurons, we used the following coordinates: for mesencephalic axon bundle (3.9 mm posterior to bregma; ±1.1 mm lateral to midline; and 2.1 mm to 3.9 mm deep from the cortical surface) and the parabrachial (5.0 mm posterior to bregma; ±1.3 mm lateral to midline; and 1.6 mm to 4.2 mm deep from the cortical surface). During the experiments, the stimulation electrodes were advanced in steps of 5 µm by a motorized micromanipulator (David Kopf Instruments), and BA-evoked responses were recorded. Stimulation-induced and spontaneous spikes were sorted using principal component analysis and template matching. The similarity of

stimulation-induced spike waveforms was quantitatively compared to the waveforms of units previously identified in the awake animal and recorded on the same wire using correlation analysis (Supplementary Methods Fig. 4). To be classified as antidromic, evoked responses had to meet at least two out of three criteria: (1) stable latency (<0.3 ms jitter), (2) collision with spontaneously occurring spikes, and (3) the ability to follow high-frequency stimulation (200 Hz). At the end of the experiments, stimulating sites were marked with electrolytic lesions before perfusion, and electrode locations were reconstructed with standard histological techniques. For each stimulation site, orthodromic and antidromic response probabilities of fear and extinction neurons were analysed using binomial statistics, with $P < 0.05$ indicating non-random connectivity.

To determine the responsiveness of CEl neurons to nociceptive inputs, a footshock of 0.6-1.3 mA (3 ms) was delivered to the hindlimb of anesthetized mice.
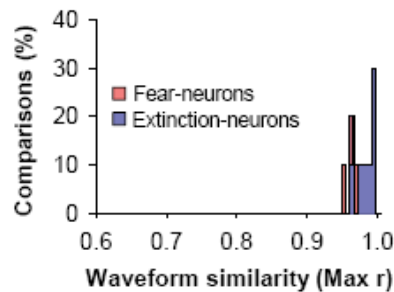
Supplementary Methods Figure 4. Identification of units activated by extracellular stimulation. Similarity of stimulation-induced spike waveforms was quantitatively compared to the waveforms of fear- and extinction-neurons previously identified in the awake animal and recorded on the same wire using correlation analysis. For each unit we calculated linear correlation values between time-shifted average waveforms obtained during the extinction session and during extracellular stimulation in the anaesthetized animal. The maximum r value across time shifts was used to quantify similarity ($r = 1$ would indicates identical spike shapes). These calculations revealed r values above 0.95 for 100% of all units.

Muscimol iontophoresis

Muscimol micro-iontophoresis injection was performed in chronically implanted animals. Single-barrel micropipettes with a tip diameter of 10 to 15 µm were cut

at 1 cm length and filled with a solution containing muscimol covalently coupled to a fluorophore (Muscimol-Bodipy-TMR conjugated, Invitrogen; 5 mM in phosphate buffered saline (PBS) 0.1 M, DMSO 40%) or with bodipy alone (Invitrogen; 5 mM in PBS 0.1 M, DMSO 40%). Mice were bilaterally implanted at the following coordinates: 1.7 mm posterior to bregma; 3.1 mm lateral to midline; and 4 mm to 4.3 mm deep from the cortical surface. For CEA experiments, we used the following coordinates: 1.3 mm posterior to bregma; 2.9 mm lateral to midline; and 3.9 mm to 4.3 mm deep from the cortical surface. Chlorided silver wires were inserted in each micropipette and attached to a connector. A third silver wire screwed onto the skull and attached to the connector served as a reference electrode. The entire miniature was secured using cyanoacrylate adhesive gel. After surgery, mice were allowed to recover for 2 days. On the injection day, iontophoretic applications were performed by means of cationic current (+12 µA to +15 µA) for 15 min per side using a precision current source device (Stoelting). Mice were submitted to the behavioural procedure 5 min after the end of iontophoretic injections and were immediately perfused at the end of the experiments. Brains were collected for further histological analysis. Serial slices containing the amygdala were imaged at X5 using an epifluorescence stereo microscope (Leica), and the location and the extent of the injections were controlled. Mice were included in the analysis only if they presented a bilateral injection targeting exclusively the BA and if the targeted injections cover at least 25% of the BA. Statistical analyses were performed using paired and unpaired Student's t-tests post hoc comparisons at the $P < 0.05$ level of significance. Results are presented as mean ± s.e.m.

Immunohistochemistry

Mice were transcardially perfused with ice-cold 4% paraformaldehyde in 0.1 M PBS 120 min after the onset of the training session[26]. Brains were prepared for immunohistochemistry using primary polyclonal rabbit anti-c-Fos antibody (Calbiochem; anti-c-Fos, Ab-5, 4-17, rabbit pAb, PC38; 1:20,000 dilution). A fluorescent-dye-coupled goat anti-rabbit antibody (Invitrogen; Alexa-Fluor 633; 1:1,000 in PBS) was used as secondary antibody. Stained slices were imaged at 40 using an LSM 510 Meta confocal microscope (Carl Zeiss Inc.). Quantitative analysis of c-Fos-positive nuclei was performed using a computerized image analysis system (Imaris 4.2, Bitplane). Structures were defined according to ref.

50. Immunoreactive neurons were counted bilaterally using a minimum of three sections per hemisphere per animal. Statistical analyses were performed using unpaired Student's t-tests at the $P < 0.05$ level of significance. Results are presented as mean ± s.e.m.

Intracellular recordings and morphological reconstructions

Intracellular recordings sessions were done in mice under chloral hydrate anesthesia (400 mg/kg), and ended the same day with the animal being transcardially perfused and the brain kept for morphological reconstruction of the neurobiotin-filled recorded neurons using standard methods (for example: Lang and Paré, 1997). During the experiment, the animal's head was held firmly by a holding bar cemented on the cranium. The absence of earbars allowed the use of earphones (ER-2 earphones, Etymotic Research, Elk Grove Village, Illinois, Bok et al. 2003) for auditory stimulation. Auditory responses of CEl neurons were determined by the presentation of tones of different frequencies (1 to 30 kHz) and intensities (using a RP2.1 processor and a HB7 headphone driver from TDT, Alachua, FL).

Intracellular electrodes were pulled from borosilicate glass tubing (1.5 mm outer diameter, 0.84 mm inner diameter; World Precision Instruments, Sarasota, FL) using a Flaming-Brown micropipette puller (model P-97; Sutter Instruments, Novato, CA). Electrodes were filled with 1.5% neurobiotin (Vector Laboratories Inc., Burlingame, CA) in 1 M potassium acetate. Impedances were measured in situ and ranged from 65 to 120 MΩ. Electrodes were slowly lowered in the brain via a micromanipulator (LN mini/ combi, Luigs & Neumann, Ratingen, Germany). Recordings were acquired and analyzed with: ClampEx9.0 and ClampFit9.0 (Axon Instruments(AI), Union City, CA, USA) through an intracellular recording amplifier (Axoclamp-2B; AI) and a data digitizer (Digidata 1322A, AI). Positive DC pulses (0.1-1.0 nA, 500 msec, 1Hz) were used to eject neurobiotin into the neurons. Mice were then perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and stored in the perfusion fixative. They were later sliced on a microtome into 80-μm-thick sections and labeled for neurobiotin using the Vectastain Elite avidin-biotin complex peroxidase kit (Vector Laboratories Inc.). Neurons were reconstructed with the Neurolucida software (Microbrightfield, Berlin, Germany).

Virus injections

For retrograde tracing of projections, replication defective Herpes Simplex Virus (HSV-1)(Neurovex, Oxon, UK) expressing eGFP was injected into either CEl or CEm. Deeply anesthetized animals were fixed in a stereotactic frame (Kopf Instruments, Tujunga, USA) and the skin above the skull was cut. A small hole was drilled at coordinates -1.2 mm posterior and -2.9 mm lateral to bregma. Glass pipettes (tip diameter 10-20 µm), connected to a Picospritzer III (Parker Hannifin Corporation, Fairfiled, USA), were lowered by a Micropositioner (Kopf Instruments, Tujunga, USA) to depths of 4.0 to 4.4 mm. About 300 nl HSV-1 Neurovex-EF1α-eGFP (Neurovex, Oxon, UK) were pressure injected to CEl or CEm. For identification of the injection site, the virus solution was mixed at 1:1000 with blue fluorescing polymer microspheres (Duke Scientific Corp., Palo Alto, USA). The skin was sutured and the wound disinfected. Before and after the surgery, systemic (Metacam, Boehringer Ingelheim, Germany) and local analgesic (Naropin, AstraZeneca AG, Zug, Switzerland) were administered. After 1 week of expression, animals were transcardially perfused with 4% PFA. The brain was removed and cut into 80 µm coronal slices. To improve the fluorescent signal, an immunostaining was performed. Slices were kept in blocking solution (3% BSA, 0.2% Triton in 0.1M PBS) for 1 h at room temperature, before application of the primary antibody (Goat anti-GFP, Abcam plc, Cambridge, UK; 1:500 in blocking solution) and incubated at 4°C over night. After washing, slices were incubated with secondary antibody (Alexa Fluo 488, donkey anti goat, Invitrogen, Basel, Switzerland; 1:1000 in PBS) at 4°C over night. After a final wash, slices were mounted on cover slips and imaged.

Acknowlegements

Finally, I am very thankful to my parents for giving me the opportunity to study biology and recognize in that its personal and interpersonal benefits.

Thanks to Raphaël for your exceptional understanding of science even as a political scientist!

Last, but not least, thanks to Yannick for your scientific curiosity and friendship.

CURRICULUM VITAE

Personal information

Particulars

Name:	Stephane Ciocchi
Date of birth:	20.06.1979
Nationalities:	Swiss and Italian
Origin:	Bärschwil, SO
Marital Status:	Married to Muriel Ciocchi
E-mail:	stephane.ciocchi@fmi.ch
Address:	Rue Pré-Guillaume 13, 2800 Delémont
Telephone:	+41 79 480 96 52 (mobile),
	+41 32 422 36 79 (home)

Education

2004-2009	Ph.D thesis with Prof. Dr Andreas Lüthi *"Fear conditioning-induced neuronal plasticity in the mouse amygdala"* Friedrich Miescher Institute, Basel
2004	Research associate at Novartis Pharma, Basel Biology teacher at the Lycée cantonal, Porrentruy
1998-2003	Diploma in biologist, University of Lausanne
2002-2003	Diploma thesis with Prof. Dr Andreas Trumpp, Swiss Institute of Cancer Research *"Role of c-Myc and P19ARF during adipocyte differentiation"*

Publications
Shaban H., Humeau Y., Herry C., Cassasus G., Shigemoto R., **Ciocchi S.**, Barbieri S., van der Putten H., Kaupmann K., Bettler B., Lüthi A.. (2006)
Generalization of amygdala LTP and conditioned fear in the absence of presynaptic inhibition. Nature Neuroscience 9:1028-35.

Herry C.*, **Ciocchi S.***, Senn V., Demmou L., Müller C., Lüthi A.. (2008)
*equal contribution
Switching on and off fear by distinct neuronal circuits. Nature 454, 600-606

Stephane Ciocchi*, Cyril Herry*, Francois Grenier, Steffen B.E. Wolff, Michael Stadler, Christian Müller and Andreas Lüthi.
In preparation. * Equal contribution.
Dissociable roles for tonic and phasic inhibitory network activity in fear conditioning

Scientific communications

Talks
4th Meeting on "*Cellular and Molecular Neurobiology of Mental Disease*", Giessbach, 2008
Plasticity of dis-inhibitory circuits in the central amygdala gates expression of conditioned fear

Annual Meeting FMI, 2008
Plasticity of dis-inhibitory circuits in the central amygdala gates expression of conditioned fear

Poster presentation
Swiss Society of Neuroscience, Annual Meeting, Basel, 2006, *Fear conditioning enhances auditory responses of single neurons in the lateral and basal amygdale of freely moving mice.*

Swiss Society of Neuroscience, Annual Meeting, Bern, 2007, *Encoding of fear extinction in the basal amygdala*

FENS Forum, Geneva, 2008, *Fear conditioning- and extinction–induced neuronal plasticity in the central amygdala*

EMBO conference "The assembly and function of neuronal circuits", 2007, Ascona

Honors
Volker-Henn Best Poster Award**,** Swiss Society of Neuroscience, Annual Meeting, Bern, 2007, *Encoding of fear extinction in the basal amygdala.*

References

Adolphs, R., D. Tranel, et al. (1994). "Impaired recognition of emotion in facial expressions following bilateral damage to the human amygdala." Nature 372(6507): 669-72.

Amaral, D. G. and R. Insausti (1992). "Retrograde transport of D-[3H]-aspartate injected into the monkey amygdaloid complex." Exp Brain Res 88(2): 375-88.

Amorapanth, P., J. E. LeDoux, et al. (2000). "Different lateral amygdala outputs mediate reactions and actions elicited by a fear-arousing stimulus." Nat Neurosci **3**(1): 74-9.

Anglada-Figueroa, D. and G. J. Quirk (2005). "Lesions of the basal amygdala block expression of conditioned fear but not extinction." J Neurosci **25**(42): 9680-5.

Asan, E. (1998). "The catecholaminergic innervation of the rat amygdala." Adv Anat Embryol Cell Biol 142: 1-118.

Bale, T. L., A. M. Davis, et al. (2001). "CNS region-specific oxytocin receptor expression: importance in regulation of anxiety and sex behavior." J Neurosci 21(7): 2546-52.

Balleine, B. W. and S. Killcross (2006). "Parallel incentive processing: an integrated view of amygdala function." Trends Neurosci 29(5): 272-9.

Bartos, M., I. Vida, et al. (2007). "Synaptic mechanisms of synchronized gamma oscillations in inhibitory interneuron networks." Nat Rev Neurosci 8(1): 45-56.

Bargmann, W. (1951). "[The midbrain and neurohypophysis; a new concept of the functional significance of the posterior lobe.]." Med Monatsschr **5**(7): 466-70.

Berretta, S., H. Pantazopoulos, et al. (2005). "Infralimbic cortex activation increases c-Fos expression in intercalated neurons of the amygdala." Neuroscience 132(4): 943-53.

Bouret, S., A. Duvel, et al. (2003). "Phasic activation of locus ceruleus neurons by the central nucleus of the amygdala." J Neurosci 23(8): 3491-7.

Bouton, M. E. and D. A. King (1983). "Contextual control of the extinction of conditioned fear: tests for the associative value of the context." J Exp Psychol Anim Behav Process 9(3): 248-65.

Bouton, M. E., R. F. Westbrook, et al. (2006). "Contextual and temporal modulation of extinction: behavioral and biological mechanisms." Biol Psychiatry 60(4): 352-60.

Buzsaki, G. (2002). "Theta oscillations in the hippocampus." Neuron 33(3): 325-40.

Buzsaki, G., J. Csicsvari, et al. (2002). "Homeostatic maintenance of neuronal excitability by burst discharges in vivo." Cereb Cortex 12(9): 893-9.

Canteras, N. S., R. B. Simerly, et al. (1992). "Connections of the posterior nucleus of the amygdala." J Comp Neurol 324(2): 143-79.

Canteras, N. S. and L. W. Swanson (1992). "Projections of the ventral subiculum to the amygdala, septum, and hypothalamus: a PHAL anterograde tract-tracing study in the rat." J Comp Neurol 324(2): 180-94.

Carlsen, J. and L. Heimer (1988). "The basolateral amygdaloid complex as a cortical-like structure." Brain Res 441(1-2): 377-80.

Cassell, M. D., L. J. Freedman, et al. (1999). "The intrinsic organization of the central extended amygdala." Ann N Y Acad Sci 877: 217-41.

Chieng, B. C., M. J. Christie, et al. (2006). "Characterization of neurons in the rat central nucleus of the amygdala: cellular physiology, morphology, and opioid sensitivity." J Comp Neurol 497(6): 910-27.

Ciriello, J., M. P. Rosas-Arellano, et al. (2003). "Identification of neurons containing orexin-B (hypocretin-2) immunoreactivity in limbic structures." Brain Res 967(1-2): 123-31.

Collins, D. R. and D. Pare (2000). "Differential fear conditioning induces reciprocal changes in the sensory responses of lateral amygdala neurons to the CS(+) and CS(-)." Learn Mem 7(2): 97-103.

Corcoran, K. A., T. J. Desmond, et al. (2005). "Hippocampal inactivation disrupts the acquisition and contextual encoding of fear extinction." J Neurosci 25(39): 8978-87.

Corcoran, K. A. and S. Maren (2001). "Hippocampal inactivation disrupts contextual retrieval of fear memory after extinction." J Neurosci 21(5): 1720-6.

Cousens, G. and T. Otto (1998). "Both pre- and posttraining excitotoxic lesions of the basolateral amygdala abolish the expression of olfactory and contextual fear conditioning." Behav Neurosci 112(5): 1092-103.

Davis, M. (1992). "The role of the amygdala in fear and anxiety." Annu Rev Neurosci 15: 353-75.

Delaney, A. J., J. W. Crane, et al. (2007). "Noradrenaline modulates transmission at a central synapse by a presynaptic mechanism." Neuron 56(5): 880-92.

Dumont, E. C., M. Martina, et al. (2002). "Physiological properties of central amygdala neurons: species differences." Eur J Neurosci 15(3): 545-52.

Faber, E. S., R. J. Callister, et al. (2001). "Morphological and electrophysiological properties of principal neurons in the rat lateral amygdala in vitro." J Neurophysiol 85(2): 714-23.

Falls, W. A., M. J. Miserendino, et al. (1992). "Extinction of fear-potentiated startle: blockade by infusion of an NMDA antagonist into the amygdala." J Neurosci 12(3): 854-63.

Fu, Y. and P. Shinnick-Gallagher (2005). "Two intra-amygdaloid pathways to the central amygdala exhibit different mechanisms of long-term potentiation." J Neurophysiol 93(5): 3012-5.

Gerren, R. A. and N. M. Weinberger (1983). "Long term potentiation in the magnocellular medial geniculate nucleus of the anesthetized cat." Brain Res 265(1): 138-42.

Gewirtz, J. C. and M. Davis (1997). "Second-order fear conditioning prevented by blocking NMDA receptors in amygdala." Nature 388(6641): 471-4.

Goosens, K. A., J. A. Hobin, et al. (2003). "Auditory-evoked spike firing in the lateral amygdala and Pavlovian fear conditioning: mnemonic code or fear bias?" Neuron 40(5): 1013-22.

Goosens, K. A. and S. Maren (2004). "NMDA receptors are essential for the acquisition, but not expression, of conditional fear and associative spike firing in the lateral amygdala." Eur J Neurosci 20(2): 537-48.

Gray, T. S., M. D. Cassell, et al. (1984). "Distribution of pro-opiomelanocortin-derived peptides and enkephalins in the rat central nucleus of the amygdala." Brain Res 306(1-2): 354-8.

Henke, P. G., R. M. Sullivan, et al. (1988). "Interactions of thyrotropin-releasing hormone (TRH) with neurotensin and dopamine in the central nucleus of the amygdala during stress ulcer formation in rats." Neurosci Lett 91(1): 95-100.

Herry, C., S. Ciocchi, et al. (2008). "Switching on and off fear by distinct neuronal circuits." Nature 454(7204): 600-6.

Herry, C. and N. Mons (2004). "Resistance to extinction is associated with impaired immediate early gene induction in medial prefrontal cortex and amygdala." Eur J Neurosci 20(3): 781-90.

Herry, C., P. Trifilieff, et al. (2006). "Extinction of auditory fear conditioning requires MAPK/ERK activation in the basolateral amygdala." Eur J Neurosci 24(1): 261-9.

Honkaniemi, J., M. Pelto-Huikko, et al. (1992). "Colocalization of peptide and glucocorticoid receptor immunoreactivities in rat central amygdaloid nucleus." Neuroendocrinology 55(4): 451-9.

Huber, D., P. Veinante, et al. (2005). "Vasopressin and oxytocin excite distinct neuronal populations in the central amygdala." Science 308(5719): 245-8.

Jolkkonen, E. and A. Pitkanen (1998). "Intrinsic connections of the rat amygdaloid complex: projections originating in the central nucleus." J Comp Neurol 395(1): 53-72.

Kemppainen, S. and A. Pitkanen (2000). "Distribution of parvalbumin, calretinin, and calbindin-D(28k) immunoreactivity in the rat amygdaloid complex and colocalization with gamma-aminobutyric acid." J Comp Neurol 426(3): 441-67.

Kolber, B. J., M. S. Roberts, et al. (2008). "Central amygdala glucocorticoid receptor action promotes fear-associated CRH activation and conditioning." Proc Natl Acad Sci U S A 105(33): 12004-9.

Koob, G. F. (2003). "Neuroadaptive mechanisms of addiction: studies on the extended amygdala." Eur Neuropsychopharmacol **13**(6): 442-52

Lang, E. J. and D. Pare (1998). "Synaptic responsiveness of interneurons of the cat lateral amygdaloid nucleus." Neuroscience 83(3): 877-89.

LeDoux, J. E. (2000). "Emotion circuits in the brain." Annu Rev Neurosci 23: 155-84.

LeDoux, J. E., C. Farb, et al. (1990). "Topographic organization of neurons in the acoustic thalamus that project to the amygdala." J Neurosci 10(4): 1043-54.

LeDoux, J. E., C. R. Farb, et al. (1991). "Overlapping projections to the amygdala and striatum from auditory processing areas of the thalamus and cortex." Neurosci Lett 134(1): 139-44.

LeDoux, J. E., J. Iwata, et al. (1988). "Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear." J Neurosci 8(7): 2517-29.

LeDoux, J. E., D. A. Ruggiero, et al. (1985). "Projections to the subcortical forebrain from anatomically defined regions of the medial geniculate body in the rat." J Comp Neurol 242(2): 182-213.

Likhtik, E., J. G. Pelletier, et al. (2006). "Identification of basolateral amygdala projection cells and interneurons using extracellular recordings." J Neurophysiol **96**(6): 3257-65.

Lopez de Armentia, M. and P. Sah (2003). "Development and subunit composition of synaptic NMDA receptors in the amygdala: NR2B synapses in the adult central amygdala." J Neurosci 23(17): 6876-83.

Lopez de Armentia, M. and P. Sah (2007). "Bidirectional synaptic plasticity at nociceptive afferents in the rat central amygdala." J Physiol 581(Pt 3): 961-70.

Luskin, M. B. and J. L. Price (1983). "The laminar distribution of intracortical fibers originating in the olfactory cortex of the rat." J Comp Neurol 216(3): 292-302.

Marchant, N. J., V. S. Densmore, et al. (2007). "Coexpression of prodynorphin and corticotrophin-releasing hormone in the rat central amygdala: evidence of two distinct endogenous opioid systems in the lateral division." J Comp Neurol 504(6): 702-15.

Maren, S. (1998). "Overtraining does not mitigate contextual fear conditioning deficits produced by neurotoxic lesions of the basolateral amygdala." J Neurosci 18(8): 3088-97.

Maren, S. and G. J. Quirk (2004). "Neuronal signalling of fear memory." Nat Rev Neurosci 5(11): 844-52.

Maren, S., S. A. Yap, et al. (2001). "The amygdala is essential for the development of neuronal plasticity in the medial geniculate nucleus during auditory fear conditioning in rats." J Neurosci 21(6): RC135.

Martina, M., S. Royer, et al. (1999). "Physiological properties of central medial and central lateral amygdala neurons." J Neurophysiol 82(4): 1843-54.

Mascagni, F. and A. J. McDonald (2003). "Immunohistochemical characterization of cholecystokinin containing neurons in the rat basolateral amygdala." Brain Res 976(2): 171-84.

McDonald, A. J. (1982). "Cytoarchitecture of the central amygdaloid nucleus of the rat." J Comp Neurol 208(4): 401-18.

McDonald, A. J. (1982). "Neurons of the lateral and basolateral amygdaloid nuclei: a Golgi study in the rat." J Comp Neurol 212(3): 293-312.

McDonald, A. J. (1998). "Cortical pathways to the mammalian amygdala." Prog Neurobiol 55(3): 257-332.

McDonald, A. J. and J. R. Augustine (1993). "Localization of GABA-like immunoreactivity in the monkey amygdala." Neuroscience 52(2): 281-94.

McDonald, A. J. and R. L. Betette (2001). "Parvalbumin-containing neurons in the rat basolateral amygdala: morphology and co-localization of Calbindin-D(28k)." Neuroscience 102(2): 413-25.

McDonald, A. J. and F. Mascagni (2001). "Colocalization of calcium-binding proteins and GABA in neurons of the rat basolateral amygdala." Neuroscience 105(3): 681-93.

McDonald, A. J., F. Mascagni, et al. (1996). "Projections of the medial and lateral prefrontal cortices to the amygdala: a Phaseolus vulgaris leucoagglutinin study in the rat." Neuroscience 71(1): 55-75.

McEchron, M. D., H. Bouwmeester, et al. (1998). "Hippocampectomy disrupts auditory trace fear conditioning and contextual fear conditioning in the rat." Hippocampus 8(6): 638-46.

McGaugh, J. L. (2004). "The amygdala modulates the consolidation of memories of emotionally arousing experiences." Annu Rev Neurosci 27: 1-28.

McKernan, M. G. and P. Shinnick-Gallagher (1997). "Fear conditioning induces a lasting potentiation of synaptic currents in vitro." Nature 390(6660): 607-11.

Milad, M. R. and G. J. Quirk (2002). "Neurons in medial prefrontal cortex signal memory for fear extinction." Nature 420(6911): 70-4.

Millhouse, O. E. and J. DeOlmos (1983). "Neuronal configurations in lateral and basolateral amygdala." Neuroscience 10(4): 1269-300.

Miserendino, M. J., C. B. Sananes, et al. (1990). "Blocking of acquisition but not expression of conditioned fear-potentiated startle by NMDA antagonists in the amygdala." Nature 345(6277): 716-8.

Moreno, N. and A. Gonzalez (2007). "Evolution of the amygdaloid complex in vertebrates, with special reference to the anamnio-amniotic transition." J Anat 211(2): 151-63.

Muller, J. F., F. Mascagni, et al. (2006). "Pyramidal cells of the rat basolateral amygdala: synaptology and innervation by parvalbumin-immunoreactive interneurons." J Comp Neurol 494(4): 635-50.

Muller, J. F., F. Mascagni, et al. (2007). "Postsynaptic targets of somatostatin-containing interneurons in the rat basolateral amygdala." J Comp Neurol 500(3): 513-29.

Myers, K. M. and M. Davis (2007). "Mechanisms of fear extinction." Mol Psychiatry 12(2): 120-50.

Neugebauer, V., W. Li, et al. (2004). "The amygdala and persistent pain." Neuroscientist 10(3): 221-34.

Nie, Z., P. Schweitzer, et al. (2004). "Ethanol augments GABAergic transmission in the central amygdala via CRF1 receptors." Science 303(5663): 1512-4.

Ottersen, O. P. and Y. Ben-Ari (1979). "Afferent connections to the amygdaloid complex of the rat and cat. I. Projections from the thalamus." J Comp Neurol 187(2): 401-24.

Pare, D. and H. Gaudreau (1996). "Projection cells and interneurons of the lateral and basolateral amygdala: distinct firing patterns and differential relation to theta and delta rhythms in conscious cats." J Neurosci 16(10): 3334-50.

Pare, D., G. J. Quirk, et al. (2004). "New vistas on amygdala networks in conditioned fear." J Neurophysiol 92(1): 1-9.

Pare, D., Y. Smith, et al. (1995). "Intra-amygdaloid projections of the basolateral and basomedial nuclei in the cat: Phaseolus vulgaris-leucoagglutinin anterograde tracing at the light and electron microscopic level." Neuroscience 69(2): 567-83.

Pascoe, J. P. and B. S. Kapp (1985). "Electrophysiological characteristics of amygdaloid central nucleus neurons during Pavlovian fear conditioning in the rabbit." Behav Brain Res 16(2-3): 117-33.

Paton, J. J., M. A. Belova, et al. (2006). "The primate amygdala represents the positive and negative value of visual stimuli during learning." Nature 439(7078): 865-70.

Paz, R., J. G. Pelletier, et al. (2006). "Emotional enhancement of memory via amygdala-driven facilitation of rhinal interactions." Nat Neurosci 9(10): 1321-9.

Phelps, E. A., M. R. Delgado, et al. (2004). "Extinction learning in humans: role of the amygdala and vmPFC." Neuron 43(6): 897-905.

Pitkanen, A., M. Pikkarainen, et al. (2000). "Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review." Ann N Y Acad Sci 911: 369-91.

Pitkanen, A., V. Savander, et al. (1997). "Organization of intra-amygdaloid circuitries in the rat: an emerging framework for understanding functions of the amygdala." Trends Neurosci 20(11): 517-23.

Pitkanen, A., L. Stefanacci, et al. (1995). "Intrinsic connections of the rat amygdaloid complex: projections originating in the lateral nucleus." J Comp Neurol 356(2): 288-310.

Quirk, G. J. (2002). "Memory for extinction of conditioned fear is long-lasting and persists following spontaneous recovery." Learn Mem 9(6): 402-7.

Quirk, G. J., J. L. Armony, et al. (1997). "Fear conditioning enhances different temporal components of tone-evoked spike trains in auditory cortex and lateral amygdala." Neuron 19(3): 613-24.

Quirk, G. J., C. Repa, et al. (1995). "Fear conditioning enhances short-latency auditory responses of lateral amygdala neurons: parallel recordings in the freely behaving rat." Neuron 15(5): 1029-39.

Rescorla, R. A. (2001). "Retraining of extinguished Pavlovian stimuli." J Exp Psychol Anim Behav Process 27(2): 115-24.

Rescorla, R. A. and C. D. Heth (1975). "Reinstatement of fear to an extinguished conditioned stimulus." J Exp Psychol Anim Behav Process 1(1): 88-96.

Roberts, G. W., P. L. Woodhams, et al. (1982). "Distribution of neuropeptides in the limbic system of the rat: the amygdaloid complex." Neuroscience 7(1): 99-131.

Rodrigues, S. M., G. E. Schafe, et al. (2001). "Intra-amygdala blockade of the NR2B subunit of the NMDA receptor disrupts the acquisition but not the expression of fear conditioning." J Neurosci 21(17): 6889-96.

Rodriguez, B. I., M. G. Craske, et al. (1999). "Context-specificity of relapse: effects of therapist and environmental context on return of fear." Behav Res Ther **37**(9): 845-62.

Roozendaal, B., G. H. Schoorlemmer, et al. (1992). "Opposite effects of central amygdaloid vasopressin and oxytocin on the regulation of conditioned stress responses in male rats." Ann N Y Acad Sci **652**: 460-1.

Rosenkranz, J. A. and A. A. Grace (2002). "Cellular mechanisms of infralimbic and prelimbic prefrontal cortical inhibition and dopaminergic modulation of basolateral amygdala neurons in vivo." J Neurosci **22**(1): 324-37.

Rogan, M. T. and J. E. LeDoux (1995). "LTP is accompanied by commensurate enhancement of auditory-evoked responses in a fear conditioning circuit." Neuron 15(1): 127-36.

Rogan, M. T., U. V. Staubli, et al. (1997). "Fear conditioning induces associative long-term potentiation in the amygdala." Nature 390(6660): 604-7.

Rumpel, S., J. LeDoux, et al. (2005). "Postsynaptic receptor trafficking underlying a form of associative learning." Science 308(5718): 83-8.

Samson, R. D., S. Duvarci, et al. (2005). "Synaptic plasticity in the central nucleus of the amygdala." Rev Neurosci 16(4): 287-302.

Samson, R. D. and D. Pare (2005). "Activity-dependent synaptic plasticity in the central nucleus of the amygdala." J Neurosci 25(7): 1847-55.

Samson, R. D. and D. Paré (2005). "Activity-dependent synaptic plasticity in the central nucleus of the amygdala." J Neurosci 25(7): 1847-55.

Savander, V., C. G. Go, et al. (1996). "Intrinsic connections of the rat amygdaloid complex: projections originating in the accessory basal nucleus." J Comp Neurol 374(2): 291-313.

Schiess, M. C., P. M. Callahan, et al. (1999). "Characterization of the electrophysiological and morphological properties of rat central amygdala neurons in vitro." J Neurosci Res 58(5): 663-73.

Schulkin, J., M. A. Morgan, et al. (2005). "A neuroendocrine mechanism for sustaining fear." Trends Neurosci 28(12): 629-35.

Seidenbecher, T., T. R. Laxmi, et al. (2003). "Amygdalar and hippocampal theta rhythm synchronization during fear memory retrieval." Science 301(5634): 846-50.

Shaban, H., Y. Humeau, et al. (2006). "Generalization of amygdala LTP and conditioned fear in the absence of presynaptic inhibition." Nat Neurosci 9(8): 1028-35.

Shi, C. and M. Davis (2001). "Visual pathways involved in fear conditioning measured with fear-potentiated startle: behavioral and anatomic studies." J Neurosci 21(24): 9844-55.

Shi, C. J. and M. D. Cassell (1998). "Cascade projections from somatosensory cortex to the rat basolateral amygdala via the parietal insular cortex." J Comp Neurol 399(4): 469-91.

Shi, C. J. and M. D. Cassell (1998). "Cortical, thalamic, and amygdaloid connections of the anterior and posterior insular cortices." J Comp Neurol 399(4): 440-68.

Shimada, S., S. Inagaki, et al. (1992). "Synaptic contacts between CGRP-immunoreactive terminals and enkephalin-immunoreactive neurons in the central amygdaloid nucleus of the rat." Neurosci Lett 134(2): 243-6.

Smith, Y. and D. Pare (1994). "Intra-amygdaloid projections of the lateral nucleus in the cat: PHA-L anterograde labeling combined with postembedding GABA and glutamate immunocytochemistry." J Comp Neurol 342(2): 232-48.

Smith, Y., J. F. Pare, et al. (1998). "Cat intraamygdaloid inhibitory network: ultrastructural organization of parvalbumin-immunoreactive elements." J Comp Neurol 391(2): 164-79.

Siggins, G. R., G. Martin, et al. (2003). "Glutamatergic transmission in opiate and alcohol dependence." Ann N Y Acad Sci **1003**: 196-211.

Sotres-Bayon, F., D. E. Bush, et al. (2004). "Emotional perseveration: an update on prefrontal-amygdala interactions in fear extinction." Learn Mem **11**(5): 525-35.

Sotres-Bayon, F., D. E. Bush, et al. (2004). "Emotional perseveration: an update on prefrontal-amygdala interactions in fear extinction." Learn Mem 11(5): 525-35.

Sotres-Bayon, F., D. E. Bush, et al. (2007). "Acquisition of fear extinction requires activation of NR2B-containing NMDA receptors in the lateral amygdala." Neuropsychopharmacology 32(9): 1929-40.

Stalnaker, T. A., M. R. Roesch, et al. (2007). "Cocaine-induced decision-making deficits are mediated by miscoding in basolateral amygdala." Nat Neurosci 10(8): 949-51.

Sun, N. and M. D. Cassell (1993). "Intrinsic GABAergic neurons in the rat central extended amygdala." J Comp Neurol 330(3): 381-404.

Sun, N., L. Roberts, et al. (1991). "Rat central amygdaloid nucleus projections to the bed nucleus of the stria terminalis." Brain Res Bull 27(5): 651-62.

Sun, N., H. Yi, et al. (1994). "Evidence for a GABAergic interface between cortical afferents and brainstem projection neurons in the rat central extended amygdala." J Comp Neurol 340(1): 43-64.

Tsvetkov, E., W. A. Carlezon, et al. (2002). "Fear conditioning occludes LTP-induced presynaptic enhancement of synaptic transmission in the cortical pathway to the lateral amygdala." Neuron 34(2): 289-300.

Turner, B. H. and M. Herkenham (1991). "Thalamoamygdaloid projections in the rat: a test of the amygdala's role in sensory processing." J Comp Neurol 313(2): 295-325.

Uvnas-Moberg, K., S. Ahlenius, et al. (1994). "High doses of oxytocin cause sedation and low doses cause an anxiolytic-like effect in male rats." Pharmacol Biochem Behav 49(1): 101-6.

Veening, J. G., L. W. Swanson, et al. (1984). "The organization of projections from the central nucleus of the amygdala to brainstem sites involved in central autonomic regulation: a combined retrograde transport-immunohistochemical study." Brain Res 303(2): 337-57.

Veinante, P. and M. J. Freund-Mercier (1995). "Histoautoradiographic detection of oxytocin- and vasopressin-binding sites in the amygdala of the rat." Adv Exp Med Biol 395: 347-8.

Veinante, P. and M. J. Freund-Mercier (1997). "Distribution of oxytocin- and vasopressin-binding sites in the rat extended amygdala: a histoautoradiographic study." J Comp Neurol 383(3): 305-25.

Veinante, P. and M. J. Freund-Mercier (1998). "Intrinsic and extrinsic connections of the rat central extended amygdala: an in vivo electrophysiological study of the central amygdaloid nucleus." Brain Res 794(2): 188-98.

Walker, D. L., K. J. Ressler, et al. (2002). "Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats." J Neurosci 22(6): 2343-51.

Waters, S. M. and J. E. Krause (2000). "Distribution of galanin-1, -2 and -3 receptor messenger RNAs in central and peripheral rat tissues." Neuroscience 95(1): 265-71.

Wilensky, A. E., G. E. Schafe, et al. (2006). "Rethinking the fear circuit: the central nucleus of the amygdala is required for the acquisition, consolidation, and expression of Pavlovian fear conditioning." J Neurosci 26(48): 12387-96.

Wilensky, A. E., G. E. Schafe, et al. (1999). "Functional inactivation of the amygdala before but not after auditory fear conditioning prevents memory formation." J Neurosci 19(24): RC48.

Woodruff, A. R., H. Monyer, et al. (2006). "GABAergic excitation in the basolateral amygdala." J Neurosci 26(46): 11881-7.

Yu, B. and P. Shinnick-Gallagher (1998). "Corticotropin-releasing factor increases dihydropyridine- and neurotoxin-resistant calcium currents in neurons of the central amygdala." J Pharmacol Exp Ther 284(1): 170-9.

Zardetto-Smith, A. M., M. M. Moga, et al. (1988). "Lateral hypothalamic dynorphinergic efferents to the amygdala and brainstem in the rat." Peptides 9(5): 1121-7.

Ich erkläre, dass ich die Dissertation, **Fear conditioning- and extinction-induced neuronal plasticity in the mouse amygdala,** nur mit der darin angegeben Hilfe verfasst und bei kein anderen Fakultät eingereicht habe.