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Review

Prefrontal neuronal circuits of contextual fear conditioning

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Over the past years, numerous studies have provided a clear understanding of the neuronal circuits and mechanisms involved in the formation, expression and extinction phases of conditioned cued fear memories. Yet, despite a strong clinical interest, a detailed understanding of these memory phases for contextual fear memories is still missing. Besides the well-known role of the hippocampus in encoding contextual fear behavior, growing evidence indicates that specific regions of the medial prefrontal cortex differentially regulate contextual fear acquisition and storage in both animals and humans that ultimately leads to expression of contextual fear memories. In this review, we provide a detailed description of the recent literature on the role of distinct prefrontal subregions in contextual fear behavior and provide a working model of the neuronal circuits involved in the acquisition, expression and generalization of contextual fear memories.

Keywords: Anxiety disorders, contextual fear conditioning, fear acquisition, fear discrimination, fear expression, fear generalization, freezing, medial prefrontal cortex, neuronal circuits, post-traumatic stress disorder

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Coping with threatening contexts or discrete stimuli is critical for the survival of numerous animal species, yet the underlying neuronal circuits remain largely unknown. In clinical populations, altered emotional regulation is associated with functional changes in neuronal circuits of patients with anxiety or trauma-related psychiatric conditions, such as post-traumatic stress disorder (PTSD), (Indovina *et al.* 2011; Pitman *et al.* 2012; Rougemont-Bucking *et al.* 2011; Shin *et al.* 2005). A hallmark symptom of PTSD is 're-experiencing', which is an emotional response that can occur when an individual is reminded of the traumatic event by exposure to a sensory cue that was present during, or even resembles, the original trauma. In view of the powerful control that trauma-related cues have on re-experiencing, it is believed that during a traumatic event, sensory elements contained in the environment are combined to form a single context and become associated with the emotional experience itself. Additionally, the brain structures that are functionally altered in PTSD patients largely overlap with circuits that are responsible for fear learning and expression (Shin & Handwerger 2009; Vanelzakker *et al.* 2014). As such, how specific neuronal circuits support associative learning during aversive or traumatic events, their role in the generation, and regulation of fear- and anxiety-related behaviors in the presence of trauma-related stimuli, is essential for an understanding of the development of anxiety disorders.

In the laboratory, contextual fear conditioning has been a useful model for studying the neural circuits of associative learning processes. In its most basic form, this Pavlovian learning paradigm consists of the association between a context and a mild electric footshock, referred to as the unconditioned stimulus (US). In contrast to the tone fear conditioning paradigm, where a discrete tone conditioned stimulus (CS) is paired with footshock, contextual fear conditioning is the pairing of footshock with a context that contains several stable sensory stimuli. These stable environmental stimuli, herein referred to as feature components, are bound together to form a conjunctive representation of the context (Mcclelland *et al.* 1995) that becomes associated with the US (Rudy & O'reilly 1999). Importantly, *context* is not necessarily restricted to a multisensory conjunctive representation; interoceptive stimuli, temporal properties and social settings can also be incorporated into context (Maren *et al.* 2013).

Even a single pairing of the context and US produces a range of conditioned fear responses when the animal is re-exposed to the context, including an innate immobilization response in rodents termed 'freezing' (Blanchard & Blanchard 1969). The freezing conditioned response (CR) is a behavioral measure supported by a long-lasting associative memory that depends on activity-dependent plasticity mechanisms (Fanselow & Kim 1994; Maren *et al.* 2003; Sindreu *et al.* 2007). Although other behavioral paradigms, such as fear-potentiated startle (Davis *et al.* 1993), or eyeblink conditioning (Freeman & Steinmetz 2011), have been developed to study the neural circuits underlying emotional memories, several studies on contextual fear conditioning used freezing as a behavioral measure of the context-US association. For this reason, the main focus of this review will be restricted to experimental data using freezing as an index of contextual fear conditioning.

Pavlovian conditioning with aversive USs has been extensively investigated and some of the necessary brain regions for this type of associative learning have been identified. In particular, the basolateral amygdala (BLA) and central nucleus of the amygdala (CeA) are repeatedly implicated in contextual and cued fear acquisition and expression (Ciocchi *et al.* 2010; Ehrlich *et al.* 2009; Gale *et al.* 2004; Maren 2001; Muller *et al.* 1997; Phillips & Ledoux 1992). However, contextual fear conditioning also depends on the hippocampus (HPC).

The HPC is thought to be critical for contextual processing (Anagnostaras *et al.* 2001; Maren *et al.* 2013; Rudy 2009) and appears to be exclusively engaged during contextual, but not tone, fear conditioning (Kim & Fanselow 1992; Phillips & Ledoux 1992; Phillips & Ledoux 1994). In the intact animal the dorsal hippocampus (dHPC) is necessary for the encoding the conjunctive representation of the context, as lesions administered after contextual fear conditioning impair memory retrieval (Biedenkapp & Rudy 2009; Lehmann *et al.* 2009; Maren *et al.* 1997; Wiltgen *et al.* 2006). Specifically the dorsal region containing the dentate gyrus (DG) is necessary for contextual encoding (Kheirbek *et al.* 2013), a process that occurs automatically (Good *et al.* 1998). Interestingly, the dHPC is not the only region encoding contextual information, as dHPC lesions administered before contextual fear conditioning do not greatly affect freezing (Biedenkapp & Rudy 2009; Maren *et al.* 1997; Wiltgen *et al.* 2006). These findings indicate that multiple structures can encode the feature components of contextual representations during conditioning, but under normal circumstances the dHPC plays a dominant role.

Given the dominance of the dHPC in contextual encoding, the ontogeny of contextual fear conditioning has been examined with regard to HPC functional maturation. The expression of contextual fear in mice emerges from postnatal days 13–14 and becomes persistent between postnatal days 16–30 (Akers *et al.* 2012). In rats, contextual fear memories are formed at postnatal day 23–24 and are HPC-dependent (Raineki *et al.* 2010; Rudy 1993). Because shortly following birth the balance of excitatory/inhibitory neurotransmitters, maturation of synaptic plasticity mechanisms, and granule cell neurogenesis in the HPC are still underway, the adolescent HPC may not be able to support contextual fear memories (Raineki *et al.* 2010). However, the ontogeny of contextual fear memory may not exclusively depend on HPC function *per se*, but also on the formation of a context-US association, which requires functional maturity of multiple brain regions, such as the amygdala (Akers *et al.* 2012; Foster & Burman 2010).

Indeed, the HPC and amygdala are functionally connected as measured by local field potentials (LFPs) and within these regions neuroplasticity mechanisms are engaged during both contextual fear conditioning and expression (De Oliveira Coelho *et al.* 2013; Huff & Rudy 2004; Lesting *et al.* 2011; Maren & Hobin 2007; Trifilieff *et al.* 2007). Although the roles of the BLA, CeA and dHPC have been well-documented in contextual fear behavior, they are likely part of a larger neural circuit supporting contextual fear conditioning, as other structures are also necessary for the acquisition and expression of conditioned freezing.

An additional structure that has been investigated in contextual fear conditioning is the medial prefrontal cortex (mPFC). Indeed, a number of studies have attributed a variety of functions to the mPFC that lend this structure well to the control of fear behavior, including emotional regulation (Pitman *et al.* 2012; Roy *et al.* 2012), decision making (Euston *et al.* 2012), threat responsivity (Wood *et al.* 2012) and context encoding (Hyman *et al.* 2012). Experiments using Pavlovian tone fear conditioning have extensively investigated the role of the mPFC in the acquisition and expression of conditioned freezing (Courtin *et al.* 2013; Sotres-Bayon & Quirk 2010). However, the contribution of mPFC neuronal elements and mechanisms to various aspects of contextual fear behavior is still largely unknown. In this review we will first document the relevant anatomy of the mPFC as it relates to contextual fear. Next, we will highlight the functional role of the mPFC in contextual fear conditioning, expression, generalization and extinction. Lastly, we will conclude with a proposal of a general schema of how the mPFC participates in contextual fear.

Functional anatomy of the mPFC related to contextual fear conditioning

Over the past years there has been animated discussion about mPFC homology between animal species, in part due to cytoarchitectonic and hodological differences among species. Nowadays, it is commonly accepted that the rodent mPFC can be divided into four distinct areas which are, descending from the most dorsal region, the medial precentral cortex (PrCm), the anterior cingulate cortex (AC, dorsal and ventral part), the prelimbic cortex (PL) and the infralimbic cortex (IL) (Krettek & Price 1977; Matyas *et al.* 2014; Ray & Price 1992; Van De Werd *et al.* 2010; Van Eden & Uylings 1985). The mPFC can be functionally separated into the PrCm and AC areas, which regulate various motor behavior, whereas PL and IL are implicated in emotional, mnemonic and cognitive processes (Heidbreder & Groenewegen 2003). PL and IL can be separated using cytoarchitectural criterion such as the wideness of layer II, which is characteristic of the IL subregion (Krettek & Price 1977; Matyas *et al.* 2014; Ray & Price 1992; Van De Werd *et al.* 2010; Van Eden & Uylings 1985). Furthermore, based on their differing target structures, the PL has been implied in emotional regulation and cognitive processes whereas the IL is more particularly involved in the regulation of visceral and autonomic functions (Vertes 2006).

Concerning hodological features, tracing experiments have reported that the mediodorsal nucleus of the thalamus (MD) is bidirectionally connected with the mPFC. More precisely, the medial segments of the MD preferentially contact the IL and PL, whereas its lateral segments more often contact the AC and PrCm (Groenewegen 1988; Uylings & Van Eden 1990). These projections are mostly ipsilateral and terminate in layers I/III of the mPFC (Kuroda *et al.* 1993). The mPFC also receives massive inputs emanating from the BLA that terminate in the ventral AC, PL and IL. The mPFC projects back to both the BLA and the CeA (Mcdonald *et al.* 1996). However, the IL and PL display differences in the nuclei they preferentially contact. The IL is highly connected with lateral

capsular, where the intercalated cell mass (ITC) is located, and the lateral amygdaloid nuclei (LA). In contrast the AC and PL project mostly to the basal nucleus of the amygdala (BA) (Hoover & Vertes 2007; Krettek & Price 1977; Mcdonald 1991; Shinonaga *et al.* 1994). Additionally, the mPFC also shares reciprocal connectivity with the ventral tegmental area (Thierry *et al.* 1973), the basal ganglia (Groenewegen *et al.* 1997) and the dorsal and lateral regions of the periaqueductal gray (PAG) matter (Gabbott *et al.* 2005).

The mPFC also projects to other cortical areas such as the paralimbic cortex, which sends reciprocal projections back to the ventral AC, PL and IL and the somatosensory and motor cortices that primarily contact the PrCm and dorsal AC. The mPFC receives ipsilateral inputs from the ventral HPC (vHPC) (CA1 region and subiculum) that terminate in layers I/III of the PL and IL, with sparse inputs from the dHPC (Cenquizca & Swanson 2007; Hoover & Vertes 2007; Jay & Witter 1991). Interestingly, there are no direct projections from the mPFC to HPC. However the mPFC indirectly projects to the HPC via the nucleus reuniens of the thalamus (NR), which serves as a major source of thalamic input to the HPC (Varela *et al.* 2014; Vertes *et al.* 2007). The mPFC can also indirectly influence the HPC via projections to the entorhinal cortex, the MD or the amygdala (Vertes 2006). Finally, the mPFC also contains important internal connections, with the PL projecting to the ventral AC and the IL region projecting preferentially to the PrCm and dorsal AC (Hoover & Vertes 2007; Sesack *et al.* 1989).

Identifying how dedicated neuronal circuits regulate fear behavior has recently gained insight from functional anatomical analyses combining both immediate early gene expression (IEG) and anatomical tracing approaches. The first innovative approach used transgenic rats expressing a PSD-95 Venus fusion protein under the control of a c-Fos promoter that was targeted to dendrites using the PSD-95 and 3′-UTR of *Arc* mRNA. Since PSD-95 is a major component of postsynaptic densities, and *Arc* UTRs contain dendrite-localizing sequences, Venus rats allow for fluorescent tagging of the dendrites and synapses of activated neurons following c-Fos induction. In conjunction with anterograde tracer injections in distinct neuronal regions, this anatomical technique also allows for the examination of discrete inputs onto the cell bodies and dendrites of IEG-activated neurons.

Interestingly, a recent study using these approaches demonstrated that during the retrieval of extinction memory, the dominant inputs to c-Fos activated neurons in the LA were from the IL, whereas recovery of fear memory was associated with c-Fos active neurons receiving inputs from the PL and the vHPC (Knapska *et al.* 2012). These data strongly suggest that fear recovery depends on contextual inputs from the HPC to the mPFC, a hypothesis that has received some experimental support (see the section below *Role of the mPFC in encoding context specificity of fear extinction*).

Another approach to identify how specific neuronal circuits regulate fear behavior came from antidromic and orthodromic electrical stimulation preformed after recording from neuronal populations involved in fear behavior. The motivation of these experiments was to identify the inputs and outputs of previously recorded neurons during fear behavior. This approach, first described in the BLA, demonstrated that BLA neurons activated during high fear behavior (the so-called 'fear neurons') receive inputs from the vHPC and project to the mPFC. In contrast, neurons activated during extinction learning (the so-called 'extinction neurons') receive inputs and project to the dorsal mPFC (Courtin *et al.* 2014, Herry *et al.* 2008). More recently, Courtin *et al.* (2014), used the same approach to demonstrate that mPFC neurons activated during presentations of conditioned tones, preferentially project to the BLA. Furthermore, recent technical advances have allowed the use of optogenetics to identify functional neuronal circuits by generating antidromic spikes following short stimulations of neurons containing light-activated opsins (Jennings *et al.* 2013). Future studies are required to determine if these functional anatomical approaches can be used to evaluate the role of mPFC circuits in contextual fear behavior. Given the connections that we describe above, the mPFC is ideally positioned to receive and modulate spatial and limbic information from structures throughout the brain and implicated in contextual fear behavior.

Involvement of the prefrontal cortex in the acquisition of contextual fear behavior

In addition to the well-established involvement of the HPC in contextual fear conditioning (Biedenkapp & Rudy 2009; Kheirbek *et al.* 2013; Kim & Fanselow 1992, Maren *et al.* 2013, Phillips & Ledoux 1992, Phillips & Ledoux 1994, Rudy 2009), a number of studies in both humans and animals have also identified the mPFC as playing a key role in the acquisition, expression and extinction of contextual fear behavior. These findings are extensively reviewed in the following sections.

The functional role of the mPFC in contextual fear conditioning has long been assumed given its role in emotional regulation (Charney 2004; Kim *et al.* 2011a; Quirk & Beer 2006; Wager *et al.* 2008). However, a complete understanding and schema of the distinct prefrontal regions involved in contextual fear behavior remains to be formulated. Clinical studies using functional magnetic resonance imaging (fMRI) have demonstrated that the prefrontal cortices, primarily the subgenual AC, are engaged during acquisition and expression of contextual fear conditioning (Alvarez *et al.* 2008; Lang *et al.* 2009; Marschner *et al.* 2008; Pohlack *et al.* 2012). Interestingly, Alvarez et al. (2008) showed that during context conditioning, activation of the prefrontal cortex was negatively correlated to amygdalar activity, as has been documented in other clinical imaging studies investigating anxiety- and phobia-related disorders (Hahn *et al.* 2011; Kim *et al.* 2011b; Milad *et al.* 2006).

Converging evidence in clinical populations demonstrates that at the very least the prefrontal cortex and amygdala interact during contextual fear expression. However, to understand the necessary functional connectivity between structures, the specific cell types contained in circuits supporting fear behavior, and the patterns of neuronal activity occurring during fear acquisition, additional approaches are required.

Recent findings in rodents using IEG analyses, lesions or inactivation studies have provided important data on mPFC functions during contextual fear conditioning.

In one study using contextual fear conditioning, c-Fos protein expression following fear acquisition was examined in the AC, but no elevations in c-Fos were observed (Goshen *et al.* 2011). Another study testing the causality of c-Fos expression following fear acquisition found that suppression of c-Fos mRNA before fear acquisition did not alter contextual-based freezing in rats that received tone-footshock conditioning (Morrow *et al.* 1999a). Although this study was not specifically designed to assess context fear, it provides evidence that elevated c-Fos expression in the mPFC following acquisition is not directly related to context-conditioned freezing. Consistent with the mPFC and HPC anatomical connections described above, the mPFC has been implicated in the encoding of contextual information. Recent experiments examining cytoplasmic and nuclear labeling of Arc mRNA, reinforced the idea that the mPFC is involved in the acquisition of contextual fear memories. Indeed, context exposure as well as the context-US association activated the PL, but not IL. These findings provide evidence that the PL may serve as a structure where information from the BLA and dHPC is combined following conditioning (Zelikowsky *et al*. 2014). However, whether the mPFC is necessary for contextual fear acquisition is open to question.

In particular, electrolytic or pharmacological lesions of the mPFC before contextual fear expression had either no effect on conditioned freezing (Antoniadis & Mcdonald 2006; Baran *et al.* 2010; Bissiere *et al.* 2008; Chang & Maren 2010; Fernandez Espejo 2003; Gewirtz *et al.* 1997; Holson 1986; Morgan *et al.* 1993; Morrow *et al.* 1999b; Zelinski *et al.* 2010) or increased freezing (Lacroix *et al.* 2000; Morgan & Ledoux 1995). Although lesions can be performed within the dorsal/ventral axis of the mPFC to produce dissociations in conditioned freezing (Morgan & Ledoux 1995), one limitation of lesioning is that non-neuronal tissue is destroyed and in the case of electrolytic lesions, downstream targets may also be negatively affected due to the supraphysiological stimulation associated with this method. Additionally, following lesioning, structures that typically do not support memory encoding can compensate for loss of function (Biedenkapp & Rudy 2009).

Recently, refined molecular techniques that either completely block synaptic transmission using recombinant adeno-associated virus (AAV)-mediated expression of tetanus-toxin light chain (TetTox), or that disrupt the kinetics of neurotransmitter release using an AAV-mediated knockdown of the Ca^{+2} sensor synaptotagmin-1 (Syt1), were used to investigate the role of the mPFC in contextual fear acquisition. A few weeks before training, mice received infusions of AAV encoding either TetTox or Syt1 locally in the mPFC. The authors demonstrated that neither complete blockade, nor impaired synaptic transmission, in the mPFC during memory acquisition affected conditioned freezing during the test phase (Xu *et al.* 2012). Although these studies suggest that the mPFC is unnecessary for contextual fear conditioning, it is still possible that the long treatment period necessary to reduce synaptic transmission resulted in compensatory mechanisms that might have masked the contribution of the mPFC in contextual fear acquisition. Moreover, the irreversibility of these methods does not allow the dissociation of mPFC function during acquisition and expression phases.

Reversible inactivation of the mPFC during contextual fear acquisition using pharmacological agents has yielded more consistent findings. When considering specific subregions of the mPFC, the AC appears to be necessary for the acquisition of contextual fear. Indeed, injection of the $GABA_A$ receptor agonist muscimol into the AC before conditioning reduced context-conditioned freezing (Tang *et al.* 2005). Additionally, given the role of the *N*-methyl-D-aspartate (NMDA) receptor subtype 2B (NR2B) in long-term memory formation (Plattner *et al.* 2014), the NR2B antagonist Ro25-6981 was administered to determine whether the AC was necessary for contextual fear memory consolidation. Administration of Ro25-6981 immediately before fear conditioning reduced conditioned freezing during the test phase (Einarsson & Nader 2012; Zhao *et al.* 2005). Additionally, virally mediated disruption of learning-induced dendritic spine growth in the AC during memory consolidation reduced context freezing during the test phase (Vetere *et al.* 2011). Together these studies suggest that in addition to a global role in contextual fear memory formation, the AC may be preferentially involved during the consolidation phase.

Manipulations of other mPFC regions have yielded mixed findings. For instance, pharmacological inhibition of the PL with tetrodotoxin during fear conditioning did not alter contextual fear expression the following day (Corcoran & Quirk 2007). However, optogenetic manipulation of the PL and IL during fear acquisition has yielded different findings. Interestingly, extended periods of optogenetic-induced inhibition of CaMKII-positive, but not parvalbumin-positive, neurons with stable step-function opsins immediately before fear conditioning, blocked context-conditioned freezing the following day (Yizhar *et al.* 2011). Taken together, these findings indicate that the AC is likely activated during acquisition of contextual fear memories and is required for memory encoding (Fig. 1a). However, the participation of other mPFC regions during fear acquisition remains to be elucidated.

Involvement of the prefrontal cortex in the expression of contextual fear behavior

Functional anatomy studies have provided an interesting insight into the regional specificity of mPFC activation during fear expression. Following contextual fear conditioning c-Fos immunoreactivities in the PL, IL or AC were significantly elevated in rats trained and tested in the conditioning context (Albrechet-Souza *et al.* 2013; Almada *et al.* 2009; Beck & Fibiger 1995; Fujisaki *et al.* 2004; Lemos *et al.* 2010). Additionally, relatively few context-US pairings also produced significant increases in c-Fos immunoreactivity during the first test of contextual fear conditioning in the ventral PL and IL regions (Do-Monte *et al.* 2010). However, others have reported no significant changes in c-Fos expression among PL, IL and AC subregions one day after conditioning, during fear expression (Tulogdi *et al.* 2012).

Besides c-Fos, expression of other IEGs has been correlated with contextual fear expression. For instance, Zif268,

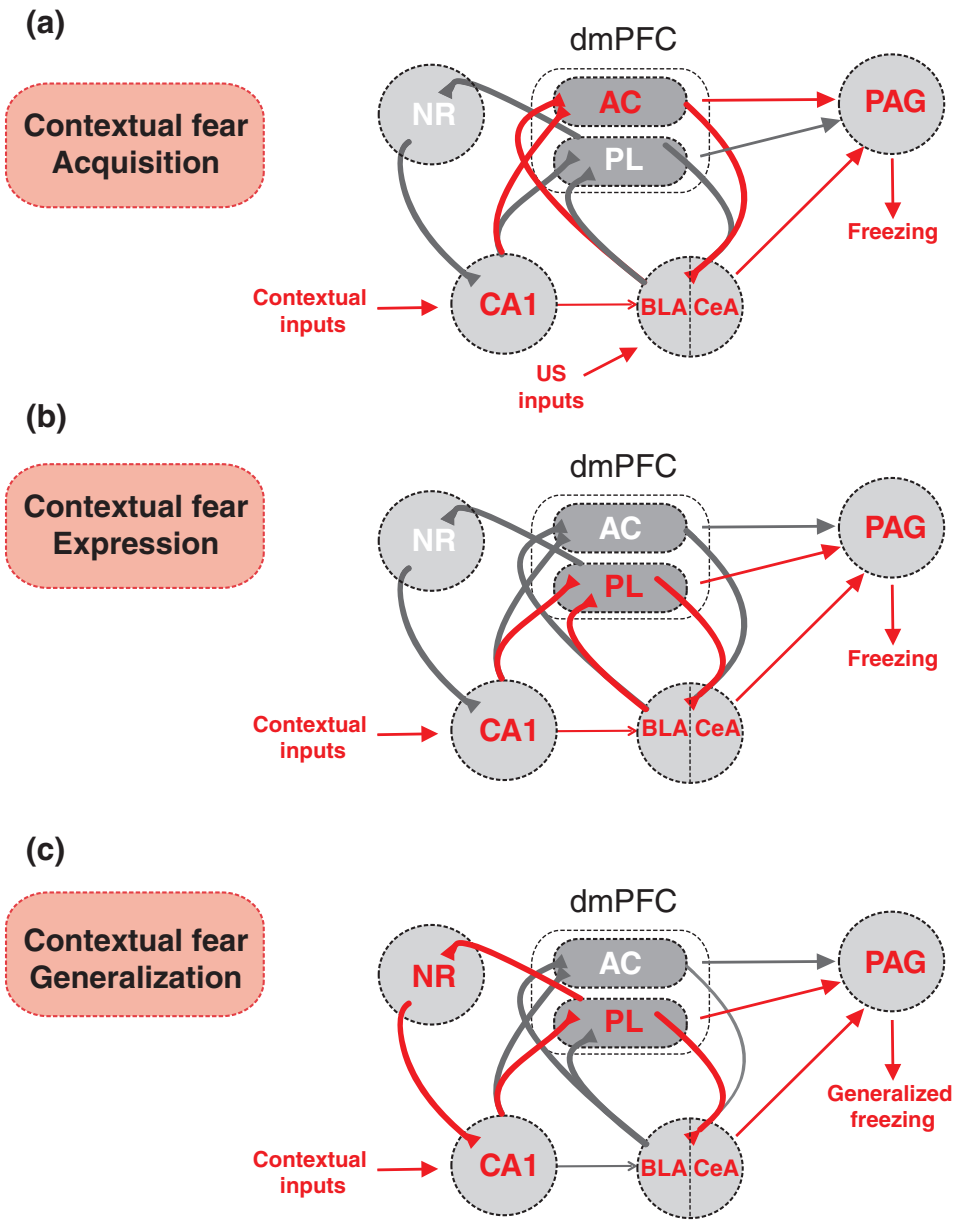

Figure 1: Neuronal circuits mediating contextual fear acquisition, expression and generalization. (a) Contextual fear acquisition requires a representation of the environment within the CA1 region of the ventral hippocampus. This contextual information is then relayed to the basolateral and central nuclei of the amygdala (BLA/CeA), a structure of the medial temporal lobe involved in the formation of context-US associations. Contextual fear acquisition has also been shown to depend on dorsal medial prefrontal (dmPFC) regions, including the AC, which is known to receive inputs from the ventral CA1. Output circuits involved in the genesis of conditioned fear responses include projections from the AC or AMG to the ventrolateral periaqueductal grey (vlPAG). (b) Contextual fear expression has been shown to be dependent on the prelimbic region (PL) of the dmPFC, which receives inputs from both the ventral CA1 and the BLA. Output circuits involved in the genesis of conditioned fear responses include projections from the PL or BLA/CeA to the vlPAG. (c) Contextual fear discrimination has been shown to strongly depend on precise contextual representations provided by a reciprocal circuit between the ventral CA1, the PL, and the NR, which projects back to the hippocampus. Alterations within this circuit leads to fear generalization in non-conditioned contexts. The expression of fear generalization might involve direct projections from the PL to the vlPAG or indirect connections through the BLA/CeA. Red arrows and letters indicate structures and projections involved during specific contextual fear memory phases. Gray arrows and projections indicate hypothesized functional connectivity during specific contextual fear memory phases.

or early growth response gene 1 (Egr-1), mRNA and protein expression was elevated in the AC and PL following context fear retrieval (Stern *et al.* 2014; Thomas *et al.* 2002). However, others have reported no changes in Egr-1 protein expression following context fear expression (Fujisaki *et al.* 2004). In a related paradigm to classic contextual fear conditioning, the context pre-exposure facilitation effect, elevated zif268 mRNA was observed in the IL and PL during fear expression in rats pre-exposed to the conditioning context (Asok *et al.* 2013). Moreover, zif268 knockout mice exhibited decreased levels of context-conditioned freezing (Besnard *et al.* 2013). However, it is not clear whether elevated IEG expression in the mPFC during memory retrieval supports conditioned freezing *per se*, as opposed to memory recall, updating or reconsolidation processes.

Studies using reversible pharmacological inactivation of the mPFC during fear memory recall suggest a regional specificity within the mPFC that is responsible for conditioned freezing. For example, inactivation of the AC and PL with lidocaine immediately before retrieval of context fear memory did not alter behavioral freezing when mice were tested 24 h after conditioning (Frankland *et al.* 2004). Alternatively, inactivation of the PL with tetrodotoxin, or the PL and IL with muscimol, immediately before fear expression greatly reduced context freezing (Corcoran & Quirk 2007; Laurent & Westbrook 2008). Additionally, bilateral injection of the sodium channel inhibitor bupivacaine into the dorsal mPFC during retrieval/expression also reduced contextual freezing (Stevenson 2011). Although the findings reviewed above do not necessarily support each other, they generally support the notion that the PL is likely implicated in the expression of contextual fear memories (Fig. 1b).

The use of more temporally precise techniques of neuronal inhibition, such as light-induced neuronal inhibition following infection of CaMKII-positive cells in the AC with the light-controllable opsin, halorhodopsin (NpHr), has recently contributed to our understanding of how the mPFC might be involved in expression of context-conditioned fear. In this study, contextual freezing was unaltered by light-induced inhibition of the AC when animals were conditioned 24 h earlier (Goshen *et al.* 2011). Expanded use of temporally precise optogenetic manipulations is needed to disentangle the functional pathways of the AC, IL and PL during contextual fear expression. Moreover, cell type specific optogenetic manipulations using transgenic mice will be extremely valuable in understanding how inhibitory and excitatory neurons interact in the mPFC during contextual fear expression.

Studies using *in vivo* electrophysiological recordings in the mPFC of behaving mice during contextual fear expression are less numerous. One reason for the paucity of recordings in behaving animals during contextual fear expression is perhaps the absence of discrete conditioned stimuli to which one can correlate unit firing patterns. Despite this potential limitation, single units were recorded in the PL and IL of rats during presentation of a fear-conditioned context (Baeg *et al.* 2001). The authors found decreased firing rates in approximately half of the units they recorded when the rat was placed in the conditioning context. Interestingly, a separate population of units increased their firing rates during exposure to the fear-conditioned context. These heterogeneous neuronal responses during exposure to the shock-conditioned context suggest a number of interesting possibilities. For example, the mPFC may be composed of two discrete neuronal circuits that independently support the initiation of freezing and the cessation of freezing. Another possibility, which is not mutually exclusive from neural circuits coding for contextual freezing, is an mPFC circuit that processes contextual information in concert with the HPC (Hyman *et al.* 2012; Zelikowsky *et al.* 2014).

Although the above data provide an insight into the prefrontal neuronal circuits mediating contextual fear behavior, further work is required to precisely understand the conditions in which the mPFC becomes activated during contextual fear expression/recall, and the functional connectivity that it shares with other structures such as the dHPC and BLA. Additionally, the development of behavioral paradigms that manipulate the context (Jezek *et al.* 2011) will be critical in advancing our understanding of the neuronal circuits and elements that support contextual fear conditioning.

The mPFC and recent and remote contextual fear expression

As mentioned above, the dHPC is critical for the acquisition and expression of contextual fear memories. However, many of the investigations supporting this claim have tested for contextual fear memory retrieval one day after conditioning. Interestingly, in humans, it has been observed that damage to the hippocampal formation produces temporally graded retrograde amnesia, particularly of context-dependent memories (Squire & Alvarez 1995). These findings suggest that with the passage of time, memory retrieval becomes independent of HPC input, a phenomenon termed systems consolidation. The field of systems consolidation has been discussed previously (Dudai 2012; Frankland & Bontempi 2005; Sutherland *et al.* 2010) and a complete reappraisal falls outside the scope of this review. However, pertinent to this review is the intriguing hypothesis that retrieval of remote contextual fear memories becomes dependent upon the mPFC, rather than the dHPC. For example, retrieval of a remote contextual fear memory elevated expression of c-Fos and zif268 in the mPFC, whereas levels of these IEGs were decreased in CA1 (Frankland *et al.* 2004; Goshen *et al.* 2011). Additionally, contextually conditioned freezing was impaired following microinjections of either lidocaine or the AMPA/kainate receptor antagonist CNQX into the AC, but not PL, at least 18 days after fear conditioning (Einarsson *et al.* 2014; Frankland *et al.* 2004). In agreement, when AC CaMKII-positive cells were infected with NpHr and light-induced neuronal inhibition was performed during contextual fear expression, remote, but not recent, context fear memory was impaired. Interestingly, when NpHr was transduced into CaMKII-positive cells in the dorsal portion of CA1, light-induced inhibition impaired both recent and remote contextual fear memories (Goshen *et al.* 2011). These optogenetic experiments indicate that contextual fear memories continually depend upon the dHPC for retrieval, but with the passage of time, recall of these traces requires assistance from the AC. As mentioned above,

it appears that for recent memories, activation of the AC is less critical, as compared to the PL and IL.

Role of the mPFC in contextual fear generalization

The mPFC receives rich projections from the ventral CA1 and as such, it has been implicated in contextual encoding and/or memory (Hyman *et al.* 2012; Lee & Solivan 2008; Zelikowsky *et al.* 2014). In light of its connectivity with the HPC and amygdala, the mPFC may serve as a structure that regulates emotion depending on contextual information. Additionally, functional imaging studies examining activity in the prefrontal cortex and amygdala in patients with PTSD have led to the proposal that reduced activation of the ventral mPFC (vmPFC) is associated with an inability to regulate fear and anxiety (Kim *et al.* 2011b; Pitman *et al.* 2012; Rougemont-Bucking *et al.* 2011), and thereby could be critical for regulating fear generalization. Generalization has been extensively studied using a variety of behavioral paradigms (Honig & Urcuioli 1981; Mclaren & Mackintosh 2002; Pavlov 1927; Rohrbaugh & Riccio 1968, Shepard 1987) and the results of these studies have provided information about the breadth of functional control that a CS has over a behavior.

Typically, a generalization paradigm consists of conditioning an organism to behaviorally respond to a CS. Then, the organism is presented with several stimuli that more or less physically resemble the CS and a generalization gradient is plotted. Gradients are often defined by their slope and are commonly qualitatively described as either 'flat' or 'steep'. Here, a flat generalization gradient refers to a similar level of behavior/response between the CS and test stimuli. Alternatively, a steep generalization gradient refers to a large reduction in behavior/response to the test stimuli, in comparison to the CS. If the subject minimally responds to a test stimulus during a generalization test, that stimulus can be said to have good 'discriminability' (Honig & Urcuioli 1981).

A number of studies have investigated fear generalization in human populations. Predictably, the mPFC and amygdala have been implicated. For instance, studies of fear generalization gradients have suggested that the mPFC regulates emotional responses via its connectivity to the amygdala. Interestingly, analyses of fMRI BOLD signal during presentation of stimuli that most resemble the original CS revealed low and high activity in mPFC and BLA, respectively. Conversely, during presentation of stimuli that least resemble the CS, mPFC activity was high and amygdala activity was low (Greenberg *et al.* 2013; Lissek 2012). Additionally, cortical thickness and functional connectivity between relevant neural structures have been correlated to fear generalization gradients (Cha *et al.* 2014). Indeed, higher levels of ventral mPFC thickness were associated with steeper generalization gradients. Interestingly, increased functional connectivity between the vmPFC and amygdala was associated with flat generalization gradients. As the authors note, this finding was contrary to another study investigating functional connectivity of these structures in patients with social anxiety disorder (Hahn *et al.* 2011). One limitation of functional connectivity is that it does not provide information about the directionality of communication between structures. Further studies are required to understand how functional connectivity and fMRI BOLD signals are related, and to determine the unique information that each of these methods can provide to our understanding of fear generalization gradients.

Together these human imaging studies demonstrated that the mPFC and amygdala are involved in fear generalization and that altered signaling between these structures contributes to flat generalization gradients. Importantly, participants in these studies were conditioned to a discrete CS, rather than a context. Currently there are no imaging studies that have examined contextual fear generalization gradients with human participants. Future studies are required to determine whether fear generalization to a context and to a discrete CS share the same neural structures.

The relationship between the mPFC and contextual fear generalization has also been studied using rodent models. Rather than investigating contextual fear generalization gradients, the following studies examined fear discrimination. That is, freezing behavior was compared between conditioned and non-conditioned contexts, rather than comparisons following multiple tests to gradations of the conditioned context.

Interestingly, pre-training NMDA-induced lesions of the PL/IL produced similar conditioned freezing between an unconditioned context and the conditioned context (Antoniadis & Mcdonald 2006). Since lesions were made before conditioning, these findings suggest that the mPFC may participate in the acquisition of a precise contextual representation. However, given the irreversibility inherent with lesions, the roles of the mPFC in acquisition and expression of precise contextual fear memory were irresolvable.

Two recent studies using molecular techniques assessed the role of the mPFC in contextual fear discrimination (Xu *et al.* 2012; Xu & Sudhof 2013). As described above, a number of days before training, the authors stereotaxically injected an AAV encoding TetTox or Syt1 into the mPFC to either block or disrupt synaptic transmission, respectively. Interestingly, mice infected with TetTox or Syt1 froze normally to the conditioned context, but generalized freezing to an unconditioned context. In a second study, two weeks before conditioning the authors expressed TetTox in specific mPFC pathways using an innovative double infection strategy. A cre-dependent AAV encoding TetTox was injected in the mPFC, while a trans-synaptically transported AAV encoding cre-recombinase was injected in the projection regions of the mPFC. This double-infection strategy enabled pathway-specific expression of TetTox. Interestingly, complete blockade of synaptic transmission within the mPFC to the NR pathway did not alter freezing to the conditioned context, but produced freezing to an unconditioned context. Notably, mice similarly expressing TetTox displayed normal freezing to a previously conditioned tone-CS, and did not generalize freezing to an altered tone.

However, the irreversibility of these manipulations prevented establishing a specific role of the mPFC or NR during contextual fear acquisition or expression. To determine the role of NR during fear expression, the authors injected a lentivirus encoding TetTox into the NR two days following fear conditioning. Now mice did not generalize freezing to

an unconditioned context. Furthermore, the authors demonstrated a role of the NR in context encoding of a contextual fear memory using optogenetic techniques. Pulses of phasic blue light stimulation at 30 Hz of the NR during contextual fear conditioning produced freezing to an unconditioned context during testing the following day.

These studies illustrate that the encoding of precise context representations during contextual fear conditioning relies on faithful communication between the mPFC and the NR (Fig. 1c). Future studies using pathway-specific methods of inactivation are required to determine the role of the mPFC during expression of contextual fear generalization.

mPFC, hippocampus and context encoding

The mPFC receives rich projections from the hippocampal formation, primarily from ventral CA1 and subiculum (Cenquizca & Swanson 2007; Hoover & Vertes 2007; Jay & Witter 1991). Indeed, recordings of multiple unit activity within the mPFC while exposing rats to different contexts revealed that mPFC units preferentially fire during global contextual changes (Hyman *et al.* 2012). Moreover, the firing of a significant portion (40%) of mPFC neurons is entrained by HPC theta rhythm (Siapas *et al.* 2005). These studies indicate that mPFC neurons receive, and their activity can be modulated by, contextual information originating from the HPC.

A vast majority of HPC principal cells are known to have corresponding activity to specific environmental locations, the so-called 'place cells'. Place cells, which comprise between 30% and70% of hippocampal principal cells (Gothard *et al.* 1996; Jung *et al.* 1994), were discovered in the rat dHPC (O'keefe & Dostrovsky 1971) and have been recorded in many species, including humans (Ekstrom *et al.* 2003). These cells are mostly silent under baseline conditions, but increase their firing rate when the organism enters a small part of the environment known as the place field. Different cells have different place fields that are stable across recording sessions in the same environment. Because they collectively cover the entire environment, these spatially tuned cells and more generally the HPC, were considered the key neuronal substrates for cognitive map development (Tolman 1948).

Now, it is widely accepted that place cell firing is not exclusively sensitive to space, but is also directly modulated by various factors. Indeed, several studies have shown that place cells can also encode behavioral choices (Lenck-Santini *et al.* 2001), conjunction of events (Wood *et al.* 1999) and episodic memory (Pastalkova *et al.* 2008; Smith & Mizumori 2006). Moreover, place cells can undergo global remapping. This remapping has been observed when animals detect a change in the behavioral task and some cells stop firing and other cells switch their responsive fields (Markus *et al.* 1995), or as a more modest 'rate remapping' where each place cell retains its place field but exhibits a different firing rate when the environment is modified (Leutgeb *et al.* 2005). Additionally, place cells can progressively remap when repeatedly exposed to two different environments, reflecting discrimination (Lever *et al.* 2002). Lastly, instability of place cell firing patterns has been observed following lesions of mPFC (Kyd & Bilkey 2003; Kyd & Bilkey 2005), demonstrating that the mPFC modulates place cell patterns and contributes to their firing specificity. Thus, qualitatively different modifications of place cell firing patterns may represent interesting mechanisms for encoding environmental changes, a phenomenon that is likely to occur during context-dependent extinction of fear memory and may require involvement of the mPFC.

Role of the mPFC in encoding context specificity of fear extinction

It has long been known that fear extinction, a phenomenon that occurs when the CS (being a tone, a light, odor) previously paired with a footshock is repeatedly presented without footshock, is a form of inhibitory learning that is contextually dependent and leads to progressive reductions in conditioned fear responses (Fanselow & Poulos 2005; Ledoux 2000). A review of several studies suggests that fear extinction does not result from an erasure of the original CS-US memory, but rather from the learning of a new CS- (No-US) association that will compete with the original CS-US association (Bouton 2004). Interestingly, it has been shown that extinction learning is specific to the context in which it occurs. As a consequence, following extinction learning, presentation of the extinguished CS in a context different from the extinction context, will trigger a full recovery of conditioned fear responses, a phenomenon known as context-dependent fear renewal (Bouton 2002, 2004).

From a clinical standpoint, long-lasting recovery of conditioned fear responses following exposure therapies, an analog of extinction learning in humans, is a major obstacle in the treatment of pathological conditions such as anxiety disorders and PTSD (Yonkers *et al.* 2003). For example, Rodriguez et al. studied this return of fear in a systematic manner on undergraduate students showing spider phobia. They observed a greater recovery of fear when participants were tested outside the exposure context. Hence, although the exposure therapy was successful, extinction of fear strongly depended on the extinction context (Rodriguez *et al.* 1999). The context-dependency of extinction learning has been confirmed by other human studies, which supports the similarity of extinction processes between humans and animals (Alvarez *et al.* 2007; Milad *et al.* 2005). For clinical purposes, the understanding of the neuronal circuits involved in the contextual modulation of fear extinction and renewal processes represents an important challenge to develop efficient therapeutic approaches to fear-related pathological conditions.

Since extinction has been clearly shown to be context-dependent, scientists have tried to identify brain structures responsible for encoding the context-dependency of extinction learning under both normal and pathological conditions. Imaging studies performed in healthy subjects suggest that three key regions are involved in extinction learning: the amygdala, the vmPFC and the HPC. More specifically, decreased amygdala activity was observed during early extinction that correlated with conditioned fear responses, while the magnitude of the vmPFC responses

during late extinction correlated with extinction learning (Phelps *et al.* 2004). On the other hand, Labar et al. observed that context-dependent fear reinstatement in healthy subjects was disturbed in amnesic patients, indicating the importance of HPC in contextual modulation of extinction (Labar & Phelps 2005). In the same line, using fMRI Kalisch et al. observed that HPC, but also vmPFC, were activated when a CS was presented in the extinction context, but not when presented in the conditioning context (Kalisch *et al.* 2006).

Behavioral studies in humans have revealed that PTSD patients submitted to a fear conditioning paradigm displayed altered extinction learning (Blechert *et al.* 2007; Peri *et al.* 2000), supporting the hypothesis that symptoms associated with PTSD results from a deficit in the extinction of the trauma-related associations. Interestingly, and consistent with this view, PTSD patients display altered amygdala, vmPFC and HPC activities (Rauch *et al.* 2006). Indeed, imaging studies revealed hyper-responsivity of the amygdala when the traumatic event was recalled (Liberzon *et al.* 1999) but also a hypoactivation of the vmPFC and a decreased blood flow in the HPC (Bremner *et al.* 1999; Rougemont-Bucking *et al.* 2011). Interestingly, Rougement-Bucking and colleagues compared PTSD patients with trauma-exposed subjects that did not develop PTSD, in a contextual extinction-learning task. They observed that in the conditioning context, which signaled danger, PTSD patients showed (1) a hyperactivation of the dorsal AC and (2) a hypoactivation of the vmPFC. Importantly, this pattern of activity was also present in the extinction context, or safe context, after extinction learning had occurred. These results strongly suggest that PTSD patients display altered neuronal responses to a safe context and supports the view that PTSD results from altered contextual processing (Liberzon & Sripada 2008). Overall these results suggest that hyperactivation of the amygdala would result from an inadequate regulation by the mPFC as well as decreased HPC function that may prevent the identification of safe contexts (Rauch *et al.* 2006).

Studies performed in rodents have also identified the amygdala, the HPC and the mPFC, as keys structures involved in extinction learning (Maren & Quirk 2004). *Extinction* neurons have been recorded in the BLA and have been shown to display bidirectional connections with the mPFC (Herry *et al.* 2008). Because lesion studies demonstrated that the HPC is necessary to acquire (Phillips & Ledoux 1992) and retrieve contextual representations (Kim & Fanselow 1992), one would expect that the HPC is also involved in encoding context-dependent fear extinction learning. Indeed, rodents displayed fear renewal regardless of testing context when the HPC was inactivated during extinction learning (Corcoran *et al.* 2005; Sierra-Mercado *et al.* 2011). Importantly, the mPFC receives direct projections from the vHPC (CA1, subiculum) (Mcdonald 1998) and single unit recordings in the LA revealed that firing patterns of LA neurons were contextually modulated (Hobin *et al.* 2003), a property that was abolished by HPC inactivation (Maren & Hobin 2007). These observations led to the conclusion that the HPC encodes context specificity of extinction learning.

A number of studies have also revealed a strong contribution of the mPFC during fear extinction. The first evidence that the mPFC processes fear extinction was given by Ledoux and colleagues who showed that while this region was not necessary for the acquisition of fear conditioning, it is required for extinction learning (Morgan & Ledoux 1995; Morgan *et al.* 1993). In this study, the authors reported heterogeneous results depending on the mPFC subregion that was lesioned. Indeed, whereas dorsal mPFC lesions increased freezing responses to both CS and context, during both acquisition and extinction, vmPFC lesions (including ventral PL, medial orbital cortex and IL) specifically altered extinction learning. This result was later confirmed by other studies (Lebron *et al.* 2004; Morrow *et al.* 1999b) although some reports did not find any effect of the lesion (Gewirtz *et al.* 1997; Morgan *et al.* 2003). Hence, these data suggested sub-regional heterogeneity within mPFC, a hypothesis that was later confirmed.

Indeed, in a seminal study, Quirk *et al.* (2000), showed that lesions restricted to the IL, were sufficient to block the recall of extinction memory 24 h after training while sparing fear acquisition. Interestingly, IL lesions did not alter *within session*, but disrupted *between sessions*, extinction learning (Quirk *et al.* 2000), indicating the IL is specifically involved in extinction consolidation. In addition, in a series of studies, the same group observed that (1) IL neurons increased their activity following extinction learning (Milad & Quirk 2002), (2) micro-stimulation of this area mimicked extinction learning (Vidal-Gonzalez *et al.* 2006), and (3) the molecular cascade involved in long-term memory consolidation is altered in the IL after extinction learning (Hugues *et al.* 2004; Santini *et al.* 2004). These results strongly indicate that the vmPFC is involved in the consolidation of extinction memory.

Interestingly, a recent study from the same group indicated that BDNF release in the IL, which originates from the HPC, mimics extinction learning (Peters *et al.* 2010). This study strongly indicates that the HPC-IL pathway is critical for extinction learning and further suggests a key role of this pathway in mediating context-dependent modulation of fear extinction. For instance, in addition to its role during extinction consolidation, the IL could also be involved in the recall of the context-CS association. In agreement with this hypothesis, Morgan et al., observed both altered retention of contextual fear conditioning and disrupted extinction learning in vmPFC lesioned rodents (Morgan *et al.* 2003). Hence, in line with the Peters et al., results mentioned above, this observation supports the involvement of the vmPFC in contextual processing. In direct support of this view, it has also been observed that the facilitation of neuronal activity in the mPFC using the AMPA receptor potentiator PEPA, or the $GABA_A$ receptor antagonist picrotoxin, promotes contextual fear extinction (Thompson *et al.* 2010; Zushida *et al.* 2007). Therefore, the IL may also contain a circuit responsible for recognizing the extinction context as a safe context. However the functional interactions between the IL and PL are not fully understood and require further investigation to determine how contextual information is integrated into these fear and extinction circuits within the mPFC.

A possible interaction between mPFC areas during the contextual modulation of fear extinction has been suggested by

the work of Knapska *et al.*, who compared c-Fos expression in the mPFC between a group of animals that underwent extinction and a second group that was placed in a new context for fear renewal (Knapska & Maren 2009). The authors observed opposite patterns of c-Fos protein within the mPFC, namely, increased c-Fos expression during high fear recovery in the PL of rats that were presented an extinguished CS in a new context, and increased c-Fos levels during low fear recovery in the IL of rats that were presented the extinguished CS in the extinction context.

However, if both the mPFC and HPC regulate the context specificity of extinction learning as described above, it remains to be understood how the mPFC interacts with HPC. As mentioned earlier, a specific class of HPC neurons called place cells have been extensively studied for their spatial properties, but very few experiments have evaluated how their activity is modulated during extinction learning. For instance, very few studies have investigated how the firing rate of HPC place cells correlates with fear behavior. Kim *et al.* (2007) observed a rate remapping, but no alteration of place field stability, when rats were submitted to audiogenic stress. Interestingly, following auditory fear conditioning, Moita *et al.* (2003) observed increased activity of HPC place cells to the CS when the animal was in a place field. In a second study, the authors confirmed the effect of contextual fear conditioning on place cells and observed a partial remapping of place cells after learning (Moita *et al.* 2004). From these results, the authors concluded that place cells also encode the emotional and motivational aspects of the environment. Despite the well-known role of the HPC and place cells in encoding the contextual aspects of fear memory, to our knowledge there is not a single study addressing the role of HPC place cells in the modulation of context-dependent fear extinction.

It is interesting to note that in the above mentioned studies, place cells were recorded from the dorsal CA1 subfield of HPC, yet both the amygdala and mPFC are connected to ventral CA1 (Mcdonald 1998). Jung *et al.*, observed lower proportions and less selective place cells in ventral CA1, compared to place fields in dorsal CA1 (Jung *et al.* 1994; Maurer *et al.* 2005). Altogether, these results lead us to hypothesize that (1) given its anatomical connections, the ventral CA1 could be even more sensitive to the emotional value of the environment and (2) ventral CA1 place cells might encode contextual information conveyed to the BLA and mPFC during fear extinction learning.

Altogether, these results from human and animal literatures indicate that extinction learning, which is context-dependent learning, relies on the interactions of three main regions: amygdala, HPC and mPFC. Future investigations on place cell activity during extinction, as well as their influence on the mPFC, are necessary to understand how PL and IL interact to encode contextual modulation of extinction learning.

Conclusions and perspectives

The mPFC is responsible for many functions. In the domain of emotional regulation, the accumulating evidence reviewed above suggests that the mPFC plays functional roles in both acquisition and expression of contextual fear memories. The known and hypothesized pathways involved in contextual fear acquisition, expression and generalization are illustrated in Fig. 1. Encoding of precise contextual fear memories requires faithful neural communication within a circuit containing the mPFC and the NR. Although the hippocampus is necessary for encoding context representations, how place cells contribute to contextual encoding of aversive memories within this prefrontal-based circuit remains to be delineated. Additionally, further studies directed at understanding how the mPFC interacts with the BLA to form fear memories are required.

The mPFC is also recruited during the expression of context-conditioned freezing responses. However, future studies examining the precise pathways responsible for the expression of contextual fear behavior will likely provide a more detailed view of the downstream targets of the mPFC, such as the CeA and the PAG, which are responsible for the generation of fear behavior within this circuit. Once more, the contribution of CA1 to the mPFC during contextual fear expression requires further studies; most interestingly, identifying whether abnormalities in contextual processing occur in this circuit during periods of fear generalization. Additionally, the relationship between the AC and PL during fear expression poses an interesting question as it is unknown how the circuits required for acquisition interact with the circuits responsible for fear expression. Related to context fear expression, the neural circuits responsible for contextual fear extinction remain to be investigated as well. Interestingly, whether contextual fear extinction recruits a separate hippocampal-mPFC ensemble that competes with the original fear conditioning memory trace or if place cells in the hippocampus undergo remapping remains to be investigated. Lastly, studies investigating how the PL and IL interact, and integrate contextual information from the HPC during fear extinction, are required. Future studies using temporally precise techniques such as multisite unit recordings in behaving animals and optogenetic manipulations will contribute to our understanding of the specific circuits engaged and their interactions during context fear acquisition, expression and extinction. Additionally, the use of pathway-specific manipulations, such as optogenetics, is necessary to determine the interactions between the mPFC, amygdala and HPC during formation and expression of aversive memories.

Finally, it is likely that the understanding of the detailed functional role of dedicated neuronal circuits and elements within the mPFC, BLA and HPC involved in the regulation of contextual fear behavior will open new therapeutic avenues for the treatment of anxiety disorders, including PTSD and other related psychiatric conditions. For instance, recent non-invasive transcranial magnetic stimulation (TMS) approaches targeting prefrontal regions have been shown to modulate cortical inhibition in rodents likely through an action on different classes of interneurons (Funke & Benali 2011; Hoppenrath & Funke 2013; Volz *et al.* 2013) and to reduce core symptoms of PTSD (Bluhm *et al.* 2009; Grisaru *et al.* 1998). Although additional studies are required to fully understand how TMS might reduce PTSD core symptoms within

mPFC-BLA-HPC circuits, these approaches are very promising for the treatment of pathological anxiety.

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