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Ha-Rang Kim

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POUR OBTENIR LE GRADE DE

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L'UNIVERSITÉ DE BORDEAUX**

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ
SPÉCIALITÉ NEUROSCIENCES

Par Ha-Rang KIM

**Neuronal mechanisms underlying contextual
fear memory discrimination**

Sous la direction de : Dr. Yann HUMEAU

Soutenue le 29 Novembre 2022

Membres du jury :

M. FOSSAT Pascal, Dr. (PR)
Mme. POPA Daniela, Dr. (DR)
Mme. GIRARDEAU Gabrielle, Dr. (CR)
M. ROZESKE Robert, Dr. (Asst. Prof)
M. BENCHENANE Karim, Dr. (DR)
M. HERRY Cyril, Dr. (DR)
M. HUMEAU Yann, Dr. (DR)

Université de Bordeaux, Bordeaux
IBENS, Paris
IFM, Paris
University of Toronto, Canada
ESPCI-ParisTech, Paris
NCM, Bordeaux
IINS-Centre Broca, Bordeaux

Président
Rapporteure
Rapporteure
Examineur
Examineur
Membre invité
Directeur de thèse

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Titre : Mécanismes neuronaux de discrimination de la mémoire de peur contextuelle.

RESUME :

Les interactions de tous les jours nécessitent de prendre en compte le contexte afin de réagir de manière appropriée. Par exemple, la rencontre d'un lion dans un environnement ouvert ou bien derrière la vitre d'un zoo doit amener à des réactions différentes. Cette évaluation permanente de l'environnement est donc essentielle par bien des aspects. Un cerveau sain a donc la capacité permanente de sélectionner et garder distincts de nombreux stimuli sensoriels constitutifs de notre environnement et de les rendre résistants au temps et à la confusion. L'altération de cette capacité amène à des désordres psychologiques et est au cœur d'un nombre important de pathologies telles que l'anxiété et les désordres post-traumatiques. Étant donné le rôle important de l'évaluation contextuelle dans les émotions et la cognition, la compréhension des mécanismes cérébraux qui la sous-tendent et qui permettent sa restitution est fondamentale, ainsi que leur modulation par les émotions associées aux contextes.

Au sein des laboratoires, la mise en place d'une aversion contextuelle est aisée et est classiquement utilisée pour étudier les circuits neuronaux impliqués. Ce protocole, appelé conditionnement contextuel à la peur, consiste à placer un animal dans une enceinte de conditionnement dans laquelle il va recevoir un stimulus aversif. Comme ce contexte va devenir prédictif de l'arrivée de ce stimulus, l'animal conditionné va adopter un comportement de crainte lorsqu'il sera repositionné dans ce contexte quelques jours plus tard. Cette réponse comportementale sera spécifique à ce contexte, n'étant pas observée dans un autre contexte même proche. Ce phénomène est appelé discrimination contextuelle.

Mon but est de visualiser les phénomènes neurophysiologiques qui sont en jeu à ce moment crucial où les contextes sont ambigus et doivent être évalués afin de déterminer la réponse à adopter. La difficulté est d'abord méthodologique : il est difficile de modifier le niveau d'ambiguïté d'un contexte dans une seule modalité sensorielle qui doit également être perçue. Le début de mon travail de thèse a donc été de déterminer expérimentalement la meilleure modalité à utiliser, qui s'est avérée être la forme de l'enceinte de conditionnement.

Ensuite, j'ai mis en place des enregistrements extracellulaires multisites ciblant trois zones impliquées dans la peur contextuelle : le cortex préfrontal médian (mPFC), l'amygdale basolatérale (BLA) et l'hippocampe ventral (vHPC). Les enregistrements ont été menés sur plusieurs jours, incluant les phases de tests ainsi que les phases de repos, qui sont essentiels pour la consolidation de la mémoire. Une

attention particulière a été portée aux phases de transition entre les contextes que notre appareillage permet de mener de façon progressive. Nous avons voulu répondre à plusieurs questions ambitieuses :

1. Quels sont les mécanismes fonctionnels instruisant le cortex préfrontal lors de l'expressions de la peur contextuelle ?
2. Quels sont les mécanismes fonctionnels ayant lieu au sein du circuit mPFC-BLA-vHPC qui permettent l'ajustement de la réponse comportementale a un changement de la valence contextuelle ?
3. Au niveau cellulaire, quels sont les déterminants entraînant la sélection d'un neurone dans l'encodage de la peur ou la discrimination contextuelle ?

De façon générale, nous pensons qu'une meilleure compréhension du processus par lequel le cerveau décode le contexte environnant est essentielle afin de comprendre la flexibilité comportementale que nous montrons quotidiennement, et dont l'importance est démontrée par les pathologie associée à son dysfonctionnement. Le propos de ce travail était d'apporter une petite brique supplémentaire à cet édifice.

Mots-clés : Peur Contextuelle, Discrimination contextuelle, Consolidation de la Mémoire, Electrophysiologie, Etude Comportementale

Title: Neuronal mechanisms underlying contextual fear memory consolidation.

ABSTRACT

Encountering a particular stimulus may require radically different responses in different situations. Imagine yourself facing a lion, an animal that is generally integrated in our consciousness as threatening. This lion would express a different meaning when it is encountered in the wild or when it is seen behind glass in a zoo. This observation emphasizes the context processing allowing to elicit the most appropriate response. Normal brains keep biologically significant events distinct and resistant to confusion. If not, it may lead to psychological dysfunction because of inaccurate context processing. This is one of the core symptoms observed in patients suffering from anxiety disorders and posttraumatic stress disorder (PTSD). Given the essential role of the context in emotion and cognition, a major scientific challenge is to understand how the brain processes and restitute contextual information between neutral and aversive emotional valence.

In the laboratory condition, the classical contextual fear conditioning (CFC) is a useful model for studying neural circuits of associative learning processes. In its most basic form, it consists of placing the animal in a conditioning chamber in which is delivered an aversive stimulus. In rodent studies the environmental context itself act as an “occasion setter” to predict the arrival of the US, thus replacing the animal back in this context leads to the expression of conditioned responses (CR) usually freezing behavior. The latter observation is highly context specific, such that when placed in another context animals do not exhibit any fear behavior, a phenomenon called contextual fear discrimination.

My goal is to visualize how brain deals with moments of context ambiguity! But how to catch them! It is first a methodological problem: it is difficult to manipulate the level of ambiguity in the surrounding context along a single sensory dimension. During my thesis, I tested and validated smooth area shape transitions as unique context changes to elicit contextual fear discrimination. Among areas potentially involved in the contextual fear processing, three brain regions - the medial prefrontal cortex (mPFC), the basolateral amygdala (BLA), and the hippocampus (HPC) - are particularly important, and share functional connectivity. We therefore performed extracellular recording simultaneously in the three aforementioned areas, along the contextual fear learning process, i.e., fear acquisition, retrieval and discrimination, with a particular focus on transition periods to let emerge the “partition of the symphony”. We wanted to answer a few specific questions:

1. What are the functional mechanisms instructing the PFC during contextual fear expression?

2. What are the functional mechanisms within the interconnected tripartite circuit that mediate appropriate behavior according to the contextual valence?

3. At cellular level, what will determine if a neuron is implicated or not in contextual discrimination?

In general, we believe that a more comprehensive view of the brain circuits that mediate contextual processing and modulation will greatly enrich the future understanding of flexible, adaptive responses to environmental stimuli, and pathophysiological processes that interfere with this flexibility. The purpose of this thesis is to bring another brick in this wall.

Keywords: Contextual Fear, Fear Memory Discrimination, Memory Consolidation, Electrophysiology, Behavioral study.

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LIST OF ABBREVIATIONS

AMG – amygdala
AC – autocorrelogram
ACC – anterior cingulate cortex
BA – basal amygdala
BLA – basolateral nucleus of amygdala
BMA/BM – baso-medial amygdala
BNST – bed nucleus of the stria terminalis
BOLD – Blood oxygen level dependent
CEA – central amygdala
CEl – lateral section of the central amygdala
CEm – medial section of the central amygdala
CFC – contextual fear conditioning
CFD – contextual fear discrimination
CI – confidence interval
CR – conditioned response
CS – conditioned stimulus
Ctx – contextual
DG – dentate gyrus
dHPC – dorsal hippocampus
dmPFC – dorso-medial prefrontal cortex
EEG – electroencephalogram
EPM – elevated plus maze
FC – fear conditioning
fMRI – functional magnetic resonance imaging
FZ – freezing
GAD – generalized anxiety disorder
GC – granule cells
HPC – hippocampus
IL – infralimbic cortex
IN – interneuron
ITC – intercalated cells
KS – Kolmo-smirnov
LA – lateral amygdala
LFP – local field potential
LIA – large irregular activity
LTP – long term potentiation
MB – mobility
mPFC – medial prefrontal cortex
NAc – nucleus accumbens
NR – nucleus reuniens
Non-Ctx – non-contextual
OF – open field
PAG – periaqueductal gray
PKC – protein kinase C
PL – prelimbic cortex

PN – principal neuron
PTSD – posttraumatic stress disorder
REM – rapid eye movement
Si – sensory insula
SNr – substantia nigra pars reticulata
SOM – somatostatin
SWR/SPW-R – sharp wave ripple
SWS – slow wave sleep
US – unconditioned stimulus
vHPC – ventral hippocampus
vmPFC – ventro-medial prefrontal cortex
vIPAG – ventro-lateral PAG
VTA – ventral tegmental area
WF – waveform
WT – wild-type

PREFACE

Encountering a particular stimulus may require radically different responses in different situations. Imagine yourself facing a lion, an animal that is generally integrated in our consciousness as threatening. This lion would express a different meaning when it is encountered in the wild, where it could signal “danger”, and elicit the well-known “fight or flight” body response. On the contrary when it is seen behind glass in a zoo, it could mean “interesting” and trigger curiosity and excitement. This observation emphasizes the important function of a context processing to elicit the most appropriate response (Bouton 1993). Context by definition refers to the general cognitive, semantic or emotional background that provides critical information for response selection (Kim & Fanselow 1992). Context is routinely encoded without awareness (Barrett & Kensinger 2010), and includes the perception of time, so that it frames the memory of an experience, and shapes for future expectations (Maren 2013). Finally, context also plays a central role in resolving ambiguity, through flexible representation and retrieval of information (Maren 2013). As a result, it allows discrimination between different stimuli, a fundamental cognitive ability that bases on the recognition and identification of the similarity between present situation and previously learned meaningful experience.

Normal brain function is built to keep biologically significant events (in our case fear memories) distinct and resistant to confusion. Indeed, a misbalanced contextualization may lead to psychological dysfunction (Dunsmoor and Paz 2015), mainly characterized by inaccurate context processing (Liberzon & Abelson, 2016), resulting in an inappropriate response even in context that are not trauma-associated (Lissek 2012). This is a one of the core symptoms observed in patients suffering from anxiety disorders and posttraumatic stress disorder (PTSD). Given the essential role of the context in emotion and cognition, a major scientific challenge is to understand how the brain processes and reconstitute contextual information between context that is associated with neutral and aversive emotion.

In the laboratory condition, the classical contextual fear conditioning (CFC) is a useful model for studying neural circuits of associative learning processes. In its most basic form, it consists of placing the animal in a conditioning chamber in which is delivered an aversive stimulus, usually a mild electric foot shock, referred as the unconditional stimulus (US). In rodent studies the environmental context itself serves as a conditioned stimulus (CS) and acts as an “occasion setter” (Bouton & Nelson 1998) to predict the delivery of the US. The term “context” here has been used in a broader sense including multisensory variables, defined as a mixture of unspecific cues within the environment (Kim & Fanselow 1992). Once

the context has been associated with the US, replacing the animal back in this context leads to the expression of conditioned responses (CR). The latter observation is highly context specific, such that when placed in another context animals do not exhibit any fear behavior, a phenomenon called contextual fear discrimination. Two important information needs to be taken into consideration:

1. **What is learned in a certain environment is best remembered in the same environment.** For instance, following CFC, re-exposure to the same environment induces fear memory retrieval. On the same line, extinction of fear response to a cue will be expressed only in the context in which extinction occurred, as fear expression will be renewed when extinguished cue is presented in any other context. How and where such processes take place?
2. **And a hallmark of successful learning is the ability to produce appropriate behavioral action that is associated to specific external stimulus.** This means that souvenir retrieval allows appropriate behavioral action after souvenir processing. How and where such processes take place?

In my thesis project, I aim to visualize these processes by implementing a novel behavioral paradigm. Previously, some methodological problems have been reported. For instance, in many of the typical contextual discrimination tasks used, several caveats were identified (Kim 2012). First, contexts used are not representative of the complex natural environment. Second, it is difficult to manipulate the level of ambiguity in the surrounding context along a single sensory dimension. Finally, it is difficult to switch between different context, one specific cue at a time while maintaining all other environmental cues intact. During my thesis I first worked on these questions by testing a novel behavioral apparatus that enables automated and progressive transitions between multisensorial contexts (see Figure 4). My observations drive me to believe that in order to establish solid functional relationship between a neural cognitive process and underlying behavior, it would be sufficient/essential to use well-defined stimuli to know exactly what types of inputs within context are integrated. Therefore, for the rest of my thesis, I used arena shape transitions as unique contextual change to elicit contextual fear discrimination (see Figure 2 and Figure 7).

Discrimination between sensory stimuli is achieved by sensory cortices, however the attribution of emotional value or saliency to a given context is related to experience, and must be treated in high order cortical areas and other regions. Among areas potentially involved in the contextual fear processing, three brain regions are particularly important, and share functional connectivity: First, the **medial prefrontal cortex (mPFC)** draws particular attention since it is well known structure for a top-down

regulation of fear responses (Courtin, 2014, Dejean 2016). Its level of activity is also associated with fear discrimination, and signals safety/danger through communication with other subcortical areas (Likhtik, 2014). It also plays a role in fear memory formation (Vetere, 2011; Einarsson & Nader, 2012) and allows appropriate fear response by gradual updating and rebalancing of memory representation. Second, the **amygdala (AMG)** is often qualified as a vital “hub” that allows both integration of cognitive and limbic information (Ledoux, 2007), but also crucial to execute appropriate and adapted behavior according to the final decision made upstream (Dunsmoor & Paz, 2015). It is therefore an essential structure for danger detection, and coordinate threat-related behavior showing differential activation according to the emotional valence (Herry 2008, Grosso 2018). Finally, the **hippocampus (HPC)** is a central structure for encoding and storing precise contextual representations (Maren & Quirk, 2004), exclusively engaged during CFC as compared to cued FC (Perusini & Fanselow, 2015). Importantly, the ventral portion of the HPC (vHPC) related to stress, emotion and affect, shares direct connectivity with both the mPFC and the AMG (Stujenske, 2014), and is preferentially engaged to provide contextual information about emotional content (Fanselow & Dong, 2010).

As of now, past studies have revealed general mechanism by which the brain processes contextual information. **However, mechanisms through which discrimination between a previously threatening context from a neutral context still remain to be more deeply investigated, with a particular focus on the tight interaction between the three brain structures above-mentioned.** As demonstrated previously, cued FC is fairly easy to integrate and thus allows rapid acquisition, with the US onset that is clearly time-locked to the CS. As opposed to the cued FC, CFC learning can be proposed as a more complex variant that critically depends on the mPFC function (Gilmartin 2014). What is not clear is how the mPFC integrates this information and facilitates associative plasticity and memory storage in other subcortical structures. **We believe that the mPFC is a good candidate well positioned to allow both threat assessment and gate fear responses appropriate to the situation at hand, through dynamic interaction with other two subcortical areas.**

The questions that motivate us are: 1. What are the functional mechanisms instructing the PFC during contextual fear expression? 2. What are the functional mechanisms within the interconnected tripartite circuit that mediate appropriate behavior according to the context? We therefore performed extracellular recording simultaneously in the three aforementioned areas, along the contextual fear learning process, i.e., fear acquisition, retrieval and discrimination.

Fascinating enough is the persistence/maintenance of these fearful memories, especially given the fact that they can eventually result from single experiences (Maren, 2013). Do these “super memories” rely on exacerbated memory consolidation mechanisms? It has been suggested a key role of the HPC in mediating consolidation of episodic and spatial memory (Ramirez, 2013; Penn, 2017). Also, fear learning and expression is associated with synchronized oscillatory activity between HPC and the dmPFC (Adhikari, 2011). Mechanistically, one hippocampal oscillatory event, the sharp-wave ripple complex (SWR), that occurs during slow wave sleep (SWS) is specifically involved in memory reactivation and consolidation (Girardeau 2009; Benchenane, 2010). SWR are transient oscillatory events around which consolidation-related cortical reactivations allow memory replay (Buzsaki, 2015), crucial for the transfer and storage of task-relevant information from HPC to PFC (Peyrache, 2009). However very little is known in the frame of fear memory, as well as the possible influence of this hippocampal inputs onto the AMG. By recordings performed along CFC during sleeping phases, we tested how SWR coordinate the reactivation and redistribution of hippocampus-dependent memories to the mPFC and the AMG, and correlated their level of reactivation with post-learning fear expression and the degree of context discrimination.

At cellular levels, what will determine if a neuron is implicated or not in contextual discrimination (physiological determinants)? Does becoming a “CFC fear-modulated” neuron rely on preexisting local and/or long-range connectivity shaping its activity in a stereotyped manner during awake or sleeping states? Our longitudinal experimental design will aim at answering this question. It will also profit from the diversity of behavioral responses displayed by animals: Indeed, our behavioral protocol allows generation of diverse groups of behavioral performance, ranging from animals that do not learn the fearful context, to animals that show discrimination or others generalizing the two contexts. We take advantage of this very interesting aspect to exploit differential neuronal activities between groups, and potentially attribute a signature activity that leads to fear memory discrimination.

In conclusion, we believe that a more comprehensive view of the brain circuits that mediate contextual processing and modulation will greatly enrich the future understanding of flexible, adaptive responses to environmental stimuli, and pathophysiological processes that interfere with this flexibility.

INTRODUCTION

I. Emotion and fear

1. Definition of fear and anxiety

The term “fear” is used to describe the feeling, emotion that emerges when we experience an impending threat, and has powerful effects on triggering physiological and behavioral changes that are thought to promote threat avoidance and survival. Fear has been of great interest in the scientific community for decades, however it is at the heart of an intense debate when it comes to its definition, whether it concerns the feeling of fear or its underlying physiology (Gross & Canteras, 2012). Indeed, in animal studies, the term “fear” or “fear response” refers to the combined behavioral and physiological responses observed when presented threat-related signals. However, to what extent correlation can be made between the term used in this way for animal and the actual human feeling of fear is largely debatable (Gross & Canteras, 2012). Nevertheless, there is a growing agreement that understanding the neural circuits that support the behavioral and physiological response to threats will bring a better understanding of fear, de facto, the underlying psychological pathologies.

According to J. LeDoux, fear is a conscious experience of feeling afraid, meaning that it is a subjective state of inner awareness. (LeDoux, 2000; 2012; 2014). Supporting his concept, distinction between circuits underlying two classes of responses elicited by threats is suggested, the “two-system framework” (LeDoux & Pine, 2016): one circuitry for the behavioral responses and associated physiological changes in the brain and body, and another circuitry for the conscious feeling states reflecting fear and anxiety. Briefly, the first system mostly involves subcortical areas and innate circuitry inherited from ancestors. It therefore operates unconsciously, and when activated leads to behavioral and physiological responses. On the other hand, the second system relies on cortical areas and is essentially involved in generating conscious and subjective feelings. This means that such complex and personal experience can only be specific to humans, so that it is hardly translatable to other animals.

This definition however was challenged by M. Fanselow, who defines fear as a functional neural-behavior system that generates defensive behaviors that have evolved to provide protection against environmental threats (Bolles & Fanselow, 1980; Fanselow, 2019). Opposing to LeDoux, he argues that the various physiological, behavioral and cognitive-emotional responses to danger emerge from a central neuronal circuit, best conceptualized one single system, namely “central fear generator” (Fanselow, 2018).

He also proposes the concept of threat-imminence continuum (Fanselow 2018; 2019), where defensive reactions are organized along a continuum that corresponds to the proximity to fatal contact with the predator. The physical distance to the predator is one prominent factor that allow for the prey assessment of where it stands on this continuum, and adapt its feeding and drinking demands accordingly. In addition to the distance with predator, its size, identity, effectiveness, probability of occurrence, and timing until predator gets closer are all elements of predatory imminence. There are four phases of defense: 1 - **preferred phase**, when animals are in a safe location, such as a nest, demonstrating non defensive behaviors; 2 - **pre-encounter phase**, where there is no immediate danger, but the risk is present due to the animal activity like foraging. It relates to anxiety and mostly engages cortical circuits; 3 - **post-encounter phase**, when the threat is detected, but the interaction prey-predator is not yet established. It relates to fear and responses need to be immediate, therefore engages mainly the amygdala and brainstem structures. 4 - **circa-strike phase**, during which the prey is detected and attacked. It triggers panic reaction and mainly brainstem and autonomic arousal are engaged. Altogether, this indicates that the distance to threat may have an influence on the selection of defensive response triggered.

Before going on further, difference should be made between fear and anxiety. Whereas fear is an emotional response to a known or definite threat, resulting in brief defensive behaviors. Fear causes to act and deal with the threat but if no action is taken, the body remains in a state of readiness. On the other hand, anxiety can be operationalized as a more diffuse feeling of worry and increased vigilance associated with the anticipation of vague and potential threats, that is either perceived or not, therefore is longer lasting (Gross & Canteras, 2012; Tovote & Fadok, 2015). Under normal conditions, anxiety states promote adaptive avoidance behaviors, through rapid recognition of threatening stimuli and information processing within structures that can modulate defensive behavior (Jiminez, 2018). However, when maladaptive, defensive responses are excessive and inappropriate, leading to enhanced avoidance (Jiminez, 2018), and general state of distress lasting for long time (Sadock, 2009). In addition, it has been proposed that the state of anxiety can be elicited by cues associated with threat (i.e., environment in which threat-related stimuli occurred), and expression of lengthy and resistant contextual fear can be suggested to model human anxiety (Davis, 2009).

2. Classical fear conditioning – associative learning

First of all, distinction should be made between innate and learned behaviors. Innate behaviors, such as reflexes, are shared by all members of a species as part of their heritage and requires minimal

learning. On the contrary learned behaviors are innate responses that are under the control of novel stimuli through associative learning, namely Pavlovian conditioning (Pavlov, 1927; Gross & Canteras, 2012). Although we agree on the fact that at some point innate fear has been learned, transmitted to the offspring then further developed within organism through observational learning, or epigenetic factors, we can deduce that during innate fear, there is no need to establish an association between a stimulus and a negative outcome, to later consider the stimulus as a threat.

Pavlovian fear conditioning has been broadly used for decades, and enabled extensive understanding of emotional defensive behaviors in mammals. In the classical cued fear conditioning, an emotionally neutral conditioned stimulus (CS) (typically a tone or a light), is paired with an aversive unconditioned stimulus (US), usually mild electrical footshocks. The subjects thereby learn the association of the CS with the US, and by their close temporal relationship, the CS acquires aversive properties. Upon reexposure to the CS, animals display conditioned responses (CR) that are specifically elicited by the CS (Phillips & LeDoux, 1992; Ressler & Maren 2019). Importantly, “fear” or defensive responses to the US was first considered as an innate response, but it turns out not to be the case. CRs are not elicited by the CS before the pairing with the US and occur only specific to the CS. Thereby, they can be referred to as learned CR, where the US does not innately elicit fear, rather it conditions fear.

These defensive responses can be triggered automatically and rapidly and are characterized by a variety of body responses. In animal models, **freezing** is one of the most obvious readouts of fear behaviors. It can be characterized as the complete body immobilization, excepting movement related to breathing, and are typically accompanied by a crouching posture (Blanchard & Blanchard, 1969). The freezing CR is a behavioral measure supported by a long-lasting associative memory that depends on activity-dependent plasticity mechanisms (Fanselow & Kim, 1994; Maren, 2003). In addition to freezing behaviors, other body response can be observed in rodents: externally, vocalization (screaming), jumping, darting, but also defecation, piloerection. Internally expressed behaviors are stereotyped increases in blood pressure and heart rate, and the release of adrenal stress hormones (corticosterone) into the systemic circulation (Blanchard & Blanchard, 1969; Bolles & Fanselow, 1980).

Nevertheless, as mentioned above, the selection of body reaction to threat depends on the environment, i.e., the threat imminence (Fanselow, 2019). Notably, during pre-encounter state, due to the past experiences with threats, the prospection of an eventual threat is characterized by risk assessment behaviors like stretch-approach behaviors. During post-encounter state, animals enter in passive strategies, during which they disengage from environment to avoid detection from the threat and

exhibit freezing and thigmotaxis (tendency to remain close to the walls) as typical coping strategy. Finally, during circa-strike phase animals engage active strategies characterized by increased motor activity, encompassing fight and flight behavior, such as vocalization, vigorous escape attempts such as jumping and darting. However, when threat imminence increases to the point of inescapability, it is more likely to observe passive coping strategies. In humans, research on fear conditioning focuses predominantly on sympathetic autonomic arousal, as measured by increases in the skin conductance response, or potentiation of the startle eyeblink response (Dunsmoor & Paz, 2015).

Lastly, common measure of anxiety is often performed using Elevated plus maze (EPM) and Open field (OF). However, their unreliability has been contested and Headley & Pare (2019) provided supporting arguments, among them: low test-retest reliability, inconsistent results according to the duration of the tests, lack of correlation between the 2 tests, inconsistent sensitivity to different types of anxiolytic drugs, or contradictory results in optogenetic studies (opposite behavior was observed when manipulating same neuronal population). Therefore, they suggested to consider other variables as a readout of anxiety level, such as the stretch-attend behavior, defecation, stimulus generalization of fear response, that wouldn't be influenced by the methodological bias from behavioral setups.

II. Contextual Fear Discrimination

For decades, extensive studies in both humans and animals have explored how context are encoded in the brain, in both healthy subjects and patients with psychiatric disorders. Although past findings have provided a foundation to better understand the neuroanatomy of context processing, a detailed understanding of mechanisms involved in the memory phases for contextual fear memories is still less explored (compared to cued fear memories). There is a general consensus that context facilitates memory retrieval, and we will suffer from memory lapses when people, places or things are experienced “out of context” (Maren & Holt, 2000). Likewise, memory retrieval is facilitated when the context in which memory retrieval occurs is similar to the context where learning occurred, the so-called contextual effect on memory retrieval (Tulving & Thomson, 1973). To illustrate these words, we will for instance more easily remember the face/name of someone we met in a memorable place if encountered again on the same spot, as opposed to in the street.

Memory retrieval of a context operates on many forms of learning, and we will particularly put the emphasis on the learning and memory of aversive context. We know that brains are built to detect

biologically significant events (or stimulus) in the environment and signals for the presence (or absence) of an eventual threat. Importantly, when the presence of a threat is ambiguous, this ambiguity is resolved at least in part, by information provided by the context (Bouton, 1994). All things considered, we would like to suggest that context is a central element involved in the learning process up until fear memory retrieval, and takes an active part in modulation of engaging an appropriate behavior.

1. Definition of context

Before going into details on the physiological mechanisms of context fear conditioning (CFC) and discrimination (CFD), the concept of context requires to be clearly defined. Already, considerable number of literatures propose a nice overview about its definition and provide detailed information in support of its importance. We thus kindly invite the reader to refer to the following reviews: Maren, 2013; Fanselow, 2000; Fanselow & Dong, 2010; Philips & Ledoux, 1992.

Stephen Maren brings a rather “easy to integrate”, yet exhaustive definition of a context, and defines it as a set of circumstances around an event, that are typically multisensory, diffuse and continuously present in time. Importantly, he added that it is not only restricted to a general notion of a space, but many other settings can also be incorporated into a context, such as the temporality, hormonal and physiological states, or even social and cultural backgrounds of an experience. Functionally, the concept of context has been referred as a general cognitive and “emotional” background that allows us to acquire the meaning of the environment. Context is routinely encoded without awareness (Barrett & Kensinger, 2010). Most of all, context includes the perception of time, which shapes the memory of an experience and defines future expectations of similar experiences. As a result, it assigns contingencies, enables flexible representation and retrieval of information by recognition of familiar event (salient cues), while the situation is ongoing. All things considered, context thus has a central role in resolving ambiguity (Maren, 2013), and allows matching of behavioral response adapted to a given situation (Liberzon & Abelson 2016).

Bouton and Nelson, 1998, have attributed the term “**occasion setter**” to context. Simply speaking, environmental stimuli not only elicit learned behavioral response but also sets the condition under which certain associative contingencies are valid. Occasion setters themselves do not elicit conditioned behavior, rather, they serve as modulators that inform when another stimulus will result in a particular outcome. In a context discrimination task for example, one context sets the occasion for when and where the

environment predicts an aversive outcome, that favors retrieval of “fearful” CU-US memory, and the “safe” CS- no-US memory in another context (Harris, 2000).

Finally, in rodents studies the term “context” has been defined as any stimuli in the animal background that provide critical conditional information for response selection (Kim & Fanselow, 1992). Likewise, it also includes spatial, visual, temporal and internal variables of the animal (Hirsh, 1974). In a typical context fear conditioning task, context is defined as a mixture of unspecified cues within the room, concrete examples can be ambient odors, light, or fan sound (Kim & Fanselow, 1992).

2. Mechanisms of contextual fear conditioning

Contextual fear conditioning (CFC) is a useful model for studying neural circuits of associative learning processes, and has been widely used for a global understanding of the interaction occurring between anatomically defined structures. A typical CFC takes place, by placing an animal in a chamber that is at first emotionally neutral, in which an aversive US, (often time a mild footshock) is experienced. In this situation, animals form a representation of the context by combining sensory elements contained in the environment, a process called “**context encoding**” (Maren, 2013). Therefore, during CFC, the environment itself becomes the CS. This contextual representation is learned incidentally (Barrett & Kensinger, 2010), and are acquired very rapidly as a “gestalt” (Maren, 2013). Only after encoding, this representation comes to be associated with the emotional experience itself, the US (e.g., arrival of footshocks), a process referred as “**context conditioning**”. Of note, prior period of exploration and prospection of the context is crucial in order to encode context-US association and further consolidate the emotional memory. Indeed, it was demonstrated that animals that were shocked immediately after placement in the context did not show context conditioning (Fanselow, 1990, 1986).

After learning, re-exposure to the shock-associated context produces a range of conditioned fear responses, even with a single pairing of the context and the US (Blanchard & Blanchard, 1969), among them, CFC often uses freezing as a behavioral measure of fear responses (Fanselow & Kim 1994; Hobin, 2003). The expression of conditioned fear response is highly context dependent, so that when placed in a sufficiently different context, fear response is dampened. Following those lines, the importance of context is emphasized during fear extinction procedure. For instance, in a cued fear conditioning the diminished fear response to the CS will be only expressed in the context in which extinction occurred, which means that fear response will renew if the extinguished CS is presented outside the extinction context. This strongly suggests that extinction is context dependent (Maren & Quirk, 2004) and also presents a special

clinical relevance. Indeed, a major challenge in psychotherapy targeting post-traumatic stress disorder (PTSD) patients is to achieve a reduction in fear-related behavior that is generalized beyond the therapeutic settings (i.e., outside the extinction context where the therapy usually takes place) (Bouton, 1988; 2002).

Although the emotional responses elicited by contextual and cued CSs are identical, the two forms of conditioning each has parallel, yet separate information processing systems (Phillips & LeDoux, 1992). First, in contextual conditioning the CS is not restricted to a single sensory modality. Second, unlike an explicit CS, contextual CSs are continuously present and are thus not delivered to the animal in a precise, time-dependent manner in relation to the US. Third, contextual CSs are predictive of the general situation in which the US is likely to occur but are not predictive of the onset of any particular US. These observations suggest that different neural pathways may mediate the analysis of the stimulus properties of explicit and contextual stimuli but that common pathways may be involved in the expression of the conditioned emotional responses elicited by either kind of CS. Likewise, the context-processing system is essential for understanding the meaning of cues in a particular context.

3. Overview on fear discrimination

Under stressful condition, we are often faced with the challenge of quickly interpreting ambiguous information in uncertain and threatening environments. In such situation, efficient evaluation of threat thereby requires disambiguation of contextual information associated with either safety or threat, and detection of the probability (certainty) with which a cue predicts the threat (Besnard & Sahay, 2016; Maren, 2013). It is likely to interpret potentially dangerous stimuli as threatening until contextual information proves otherwise. Such cognitive ability is referred as generalization and allows organisms to deal with the complexity of real-world situations. It bases on detecting the similarities between the current situation and previous experience and enables judiciously applying that information from the to the new situation (Onat & Büchel, 2015). It makes sense to have wider generalization and respond to stimuli that are not necessarily similar to the original memory. This is because miss-correctly identifying dangerous stimulus as safe is more costly than incorrectly identifying a safe stimulus as a conditioned one (Mineka & Sutton, 1992; Dunsmoor & Paz, 2015).

Fear generalization can thus be defined as a mechanism through which the conditioned response, initially elicited by the CS (context), is also elicited by other stimuli that were not predicted by the US before. In this way, generalization acts as a factor that influences associative learning process of the

original memory, and therefore should be distinguished from the possible non-associative effects, like sensitization or habituation of the CS (Dunsmoor & Paz, 2015; Lissek, 2014).

Generalization, however, must be actively controlled, and requires that behaviors should rely on similarities between events to the extent that is useful for the organisms. Indeed, uncontrolled generalization or overgeneralization, induces perceiving the situation more dangerous than they really are, and may lead to exaggerated physiological and behavioral arousal. On the other hand, if the learning of a context is too specific, it results in unnecessary exploration of the new environment which could have been otherwise deduced from past experience (Onat & Büchel, 2015). This thereby emphasizes the relative importance of prior experience on the current information, so they can be balanced optimally.

Nevertheless, exaggerated generalization can be maladaptive, such as overreacting to supposedly harmless stimuli that for instance vaguely resemble a learned threat can be a burden on our daily life. Notably, overgeneralization behavior has been suggested as a pathogenic marker that cuts across different anxiety-related disorders (Lissek, 2010; 2014) by contributing to avoidance of stimuli/situations that are indirectly associated with a feared outcome, thus preventing from the opportunity to experience safety and disconfirm negative expectations. As opposed to generalization, discrimination consists of comparing information about the context with stored knowledge, and when new stimuli are perceptually similar to those associated with danger in the past, organisms respond with defensive responses, such as avoidance or immobility, whereas greater dissimilarity yields to curiosity and exploratory behavior (Grosso, 2018).

In the face of constantly changing environment, the adaptive flexibility to engage the most appropriate behavior presents therefore an evident clinical significance. However, to date, the precise neural mechanisms underlying such process are not thoroughly understood.

Additionally, it is also important to bring out the notion of **ambiguity**. One of the questions we put the focus on are neural mechanisms by which context modulates memory retrieval induced by ambiguous cues. Ambiguity can be defined as missing, yet potentially knowable information regarding the probability of a certain outcome (Camerer, 1995). Ambiguity generates uncertainty and most likely uncertainty to a potential threat creates aversion, a state of emotional arousal, which later has effects on cognitive and affective functions, and promotes a state of heightened vigilance (Bouton, 1994; Dunsmoor & Paz, 2015). For survival when confronted to ambiguous situation (where the presence of a threat is uncertain) animals must be temporally efficient in evaluating possible consequence of a new situation while it is ongoing and

engage the most appropriate behavior. This situation is well illustrated at the beginning of this manuscript (i.e., lion encountered in different environment), where same stimulus can elicit opposite behavioral response depending on the context. From our point of view, in addition to ambiguity related to the surrounding environment, it can also be seen as a cognitive state that rise from a poor evaluation of the sensory stimuli within the environment, for instance, due to a lack of attention and focus. Finally, the use of an ambiguous situation might be more relevant and better suited to study individual differences in fear conditioning, because it increases the variability of interindividual responses and will make the proposed maladaptive responses of vulnerable individuals more apparent (Lissek, 2006).

4. Psychiatric disorders – pathophysiology

Considering the prominent role of context in retrieving the meaning of a stimulus, thereby resolving ambiguity, deficits in contextual processing often leads to inappropriate behavioral responses. The resulting failure of adaptive regulation of fear is a core symptom of many anxiety, stress and trauma-related disorders (Liberzon & Abelson, 2016). Moreover, it is highly adaptive for humans and animals to adjust their defensive reaction to the updated reality, and the reduced discriminatory fear learning is evidenced among patients suffering from anxiety disorders, such as generalized anxiety disorders (GAD), panic disorders or post-traumatic stress disorders (PTSD) (Lissek, 2005). Dunsmoor and Paz (2015) have proposed in their review nice examples of how overgeneralization is characterized in anxiety disorders. A person suffering from phobia of spiders may react excessively to all crawling bugs, if suffering from panic disorder, experiencing panic attack in an elevator may increase the chance of having a panic attack in other enclosed spaces, for a patient of GAD the reminder of death may cause excessive worrying about one's own health and safety, and PTSD patient may experience intense physiological response when presented myriad cues related to a trauma.

Among these disorders, PTSD may be the most representative of context processing pathology. One of the core symptoms of PTSD is an excessive generalization of fear that is characterized not only by a strong response to a previously learned fearful cue, but also a failure to suppress these responses even in the presence of cues that signal safety (Lissek, 2005; Ghosh, 2015). This is due to an altered contextual processing, leading to focused behavioral reactivity that is less flexible, therefore more likely to be situation inappropriate (Liberzon & Abelson, 2016). It suggests that patients with PTSD may not be able to use contexts to limit fear responses to a CS that no longer signal aversive outcome or, not able to learn new relationships that define when dangerous cues are safe (Liberzon, 2007). From a computational

standpoint, anxiety and PTSD are disorders of prediction in which the estimation of future threats is deficient (Levy & Schiller, 2020), resulting in heightened emotional reactivity, aberrant learning, impaired inhibition, hyper-avoidance, maladaptive memories, biased decision making.

All things considered, a comprehensive view of the fundamental neural circuitry involved in context processing and modulation appears to be necessary in identifying abnormalities associated with various psychiatric disorders, and thereby presents a real clinical relevance. This will greatly enrich the future understanding of mechanisms governing adaptive responses during periods of contextual uncertainty.

5. Important notions about the contextual fear discrimination

As mentioned above, disambiguation of contextual information and prediction of the probability of threat are required strategies for efficient appraisal of threat. Essential to this process is the need to decrease interference between the ongoing new information and previously stored similar traumatic memories promoted by a mechanism named **pattern separation** (Besnard & Sahay, 2016). In terms of cognitive processes, it can be conceptualized as resolution of interference between similar memories that are ambiguous or uncertain, and the previously encoded traumatic memories. In result, it allow efficient discernment of threat on the given situation, while maintaining the precision of stored traumatic memories, necessary mechanisms for context discrimination.

Pattern separation, originally defined as a computational process, pattern separation represents segregating overlapping neural inputs onto more separated neural ensembles (O'Reilly & McClelland, 1994). It occurs automatically along the learning, and defects in this ability may be prone to anxiety disorders (Kheirbek & Hen, 2014). The mechanism is supported by the granule cells (GC) within the dentate gyrus (DG) of the dorsal hippocampus (dHPC), mainly because DG has more neurons and a lower firing rate than its input structure, the entorhinal cortex (Sangha, 2020), and because this mechanism has been attributed mostly the spatial working memory tasks (Hainmueller & Bartos, 2020). GC activity is exceptionally sparse and fewer than 5% of rodent's GCs are active when animals explore a given environment (Hainmueller & Bartos, 2018).

Consistent with this idea, GC activity seems to be more sensitive to subtle change in the environment, and typically fire in a single environment, as compared to other hippocampal cells (e.g., place cells in CA1 and CA3, later detailed) (GoodSmith, 2017). There is strong evidence that DG pattern separation highly participates in discrimination of contextual fear and other hippocampus-dependent

memories. Indeed, DG lesions (Baker, 2016), or perturbation of DG function by knocking out the NMDA receptors (McHugh, 2007) impaired specificity of contextual fear memory, and impaired the ability to learn a contextual fear discrimination. Silencing DG during re-exposure to the conditioned context, when animals were previously exposed to similar shock-paired and safe context, impaired freezing because of an impaired ability to distinguish between the conditioned and safe context (Bernier, 2017). These findings suggest that impairments of DG do not necessarily leads to excessive fear, rather its dysfunction will instead depend on the history of the animals, i.e., on the composition of stored memories.

In addition, pattern separation is mainly contributed by mature GCs and Raineke et al. (2010) provide an interesting explanation on the expression of contextual fear in mice being persistent only at a later time point post birth. That's because shortly after birth, maturation of synaptic plasticity and GC neurogenesis are still underway, and may not support contextual fear memories yet. For a more comprehensive and detailed view on this concept of pattern separation, we kindly invite the reader on the following reviews: Kheirbek & Hen, 2013; 2014; Besnard & Sahay 2016; Hainmueller & Bartos, 2020.

Another way to allow fear discrimination is through learning that one context is safer than the other context, namely **safety learning**. The effect of learned safety has rewarding properties and facilitating effects on the behavioral performance during memory retrieval, indicating that it may be associated with positive affective brain state (Kong, 2014). In humans, impaired safety signal learning has been proposed as a biomarker for anxiety disorder and PTSD (Pollak, 2008).

A common paradigm used for studying safety learning is to train animals to associate one stimulus (CS+) with the US, while another stimulus (CS-) that is perceptually different with the other CS, is unpaired from the US. Fear response to the CS+ and CS- is tested the next day, and generalization is inferred when responding to both CSs in a similar manner, while discrimination is said when fear response for CS+ is greater than for the CS-. By contrast to learned fear, safety learning results from a negative correlation of a CS- with the aversive US, where CS- is a safety cue and animals develop the ability to predict that CS- is a protection from the US (Lissek, 2006). Because of the threat un-ambiguity where the CS+ clearly signals danger, and the CS- signals safety, this type of learning can be expected to be relatively simple, and behavioral response to be uniform across individuals (Lissek, 2006). Taken together, it is suggested that learned safety and fear are opposite associative processes, where learned safety (CS-) inhibits the physiological responses evoked by learned fear (CS+), a behavioral phenomenon termed conditioned

inhibition (Pollak, 2008). Therefore, while learned safety and learned fear are both important for survival, they can be expected to be served by related, but independent neural circuitries.

Clinical studies using functional neuroimaging revealed the involvement of the AMG and striatum in learned safety, where safety exposure led to reduced blood oxygenation level-dependent activity in AMG and increased activity in striatum (Pollak, 2010). Based on rodent studies, it has been suggested that the basolateral amygdala (BLA) works in concert with the sensory insula (Si), such that the sensory input of the safety signal is received by the thalamus and sensory cortices, which project to the Si. The Si then projects directly to the BLA and the signals are conveyed towards the striatum. At the same time, inputs from the Si inhibit other possible output pathways of the AMG that mediate behavioral effects of learned fear (Kong, 2014).

In addition, differential contribution of anterior and posterior vmPFC has been described, which respectively induces, safety and threat computation (Ongur, 2003). Safety signals activate anterior vmPFC which then contact the insula, BLA, and the striatum, as described above. Whereas the posterior vmPFC that involved in threat signal, should activate another population of the BLA that contact other output structures, namely, the central amygdala (CEA), hypothalamus and periaqueductal gray (PAG) (Piantadosi, 2020). In conclusion, AMG most likely constitutes the prime site for both acquisition and consolidation of learned safety, with a specific involvement of the BLA, and the anterior vmPFC serves as a main guide to which other subcortical structure depend on (Tashjian, 2021; Kong, 2014).

III. Neurobiology of contextual fear conditioning and contextual fear discrimination

Before going into a detailed review on the neuroanatomy, we will first present simplified picture of the neural mechanisms involved in contextual fear conditioning. In order for context learning to occur, animals must first form a representation of the context. As context are typically multisensory, sensory information defining the contextual environment is first received within different thalamic nuclei which then relay sensory information directly to the AMG or indirectly via sensory cortices. Importantly, FC to a context typically recruits plasticity within HCP, where multisensory stimuli are detected and encoded, which then assembles a cognitive map of the environment. This process is called **context encoding**. The processed information are then relayed to the AMG for the context and US association, called **context conditioning**. Then the efferent projections either from the AMG, or direct long-range projection from the

mPFC towards midbrain structures like the PAG induces expression of conditioned fear responses. Most importantly, as already mentioned above, the reciprocal connection between mPFC, HCP, AMG regulate contextual fear expression, which will be detailed below.

In line with this idea, PTSD patients display altered function in AMG, mPFC and HPC (Rauch, 2006), with hyper-responsivity of AMG during recall (Liberzon, 1999), hypoactivation of vmPFC and decreased blood flow in the HPC (Rougement-Bucking 2011). This hyperactivation of AMG may result from decreased HPC functions due to inadequate identification of safe context and decreased mPFC regulation (Rauch, 2006). This is consistent with symptoms of deficit in the extinction of the trauma-related associations.

1. HIPPOCAMPUS

Like most forms of memory recollections, emotional memories also undergo three basic phases that determine their durability and vividness: acquisition, consolidation and retrieval. The contextual information encoded during CFC is highly supported by the hippocampus (HPC) (Holland & Bouton, 1999), because of its role in the context representation, as well as for the emotional content of the task. Indeed, HPC has been described to be exclusively engaged during CFC but not cued FC, since pharmacological and lesion manipulations of the HPC all produced a deficit in context fear memory, and suggested that this selective deficit of context fear was caused by a failure in context processing, and rather than by a general emotional deficit (Kim & Fanselow 1992; Philips & LeDoux 1992; 1994).

HPC function can be especially highlighted during the encoding of context representation, with hippocampal damage marked by a deficit in context fear memory related to a deficit in forming and storing context representation, whereas context-US association is not disrupted (Wilson & Bouton, 1995; Frohardt, 2000). Similarly, the blocking of contextual fear memories by NMDA antagonists and protein synthesis inhibitors directed at the HPC prior to context exposure deteriorated contextual fear memories, but not when given prior to conditioning (Barrientos, 2002; Stote & Fanselow, 2004). These findings all suggest hippocampal implication for the cognitive integration of the context and not the emotion-based association of the context with the US.

When it comes to the affective component of the CFC, HPC has been intimately tied to emotion and stress response regulation on the basis of more dense connectivity with the amygdala (Canteras & Swanson, 1992) and regulatory control of endocrine stress response through the hypothalamic-pituitary-adrenal axis (Dedovic, 2009). In humans, decreased hippocampal function and hippocampal volume are

associated with disrupted emotional control, which are major symptoms of psychological disorders, such as anxiety and PTSD (Maren, 2013).

This functional segregation of the HPC can be, according to Fanselow and Dong (2010), visualized as a set of separate structures with a dorsal portion that serves the “cold” cognitive function (particularly spatial memory) and a ventral portion that serves the “hot” and affective function, involved in emotional responses. This functional distinction is supported by studies from Moser et al., (1993) in which they controlled the size and locus of lesions, for a cleaner attribution of their function. This indicated that the input and output connections of dorsal hippocampus (dHPC) and ventral hippocampus (vHPC) are distinct, and display topographically arranged, non-overlapping neuronal connectivity patterns.

a. dHPC

We first briefly present anatomical connectivity of the dHPC. The most prominent projection from dHPC (dCA1) are to the retrosplenial cortex and ACC, involved in memory processing and spatial navigation. Through massive projections to mammillary nuclei and thalamic nuclei complex, they register a cognitive map for the navigation that allows to orient and execute behavior. dHPC also enables locomotion and movement orientation through projection towards the ventral tegmental area (VTA) and Substantia nigra pars reticulata (SNr) (Furhmann, 2015).

The cognitive and spatial representation function of the dHPC is greatly contributed by a considerable density of place cells as compared to vHPC. They comprise about 30-70% of hippocampal PNs and exhibit specific pattern of activation that respond to a unique location of an environment, termed place fields, characterized by O’Keefe & Dostrovsky (1971). Place cell firing is mostly silent under baseline condition, but present spatially tuned activity for a given environment, and stable across time. It is widely accepted that place cells are not exclusively sensitive to space, but can also encode behavioral choices, conjunction of events, and episodic memory (Lisman, 2017). They undergo remapping as animals explore new space, or detect a change in the behavioral task and this process is thought to play a role in the memory function of the HPC, along with context-specific location discrimination (Anderson & Jeffery 2003; Hayman, 2003; Hainmuller & Bartos, 2018). This suggest that this HPC population coding of the spatial properties serves as index of the strength of a context representation (De Voodgh, 2020).

Added to the place cells, as we introduced in the previous section, DG granule cells contained in the dHPC support the mechanism of pattern separation which play a pivotal role in resolving interference between ambiguous or uncertain environments (or threats), thereby in maintaining the precision of

contextual memories (Besnard & Sahay, 2016; Kheirbek, 2013). The sparsity of DG granule cells activation (1-2%) supports a unique neural code for each context and prevents from interference of firing, thus limiting context generalization. Indeed, affecting DG function, thus pattern separation (or decreased global remapping in the DG) resulted in loss of memory precision accompanied with overgeneralization of memory (Frankland, 1998; Maren, 1997). This observation is supported by the finding of reduction in DG volumes in individuals with PTSD and was reflective of increased interference in the perception of threat or safe stimulus (Wang, 2010).

b. vHPC

The **vHPC** is able to control neuroendocrine activities via strong projections to lateral septum and BNST and both structures project to neuroendocrine neurons within the hypothalamus. BNST is therefore a critical relay for the hippocampal regulation of the hypothalamus-pituitary-adrenal response to psychiatric stress and important in understanding neuroendocrine dysfunction associated with psychiatric disorders. Along with inputs from the LA and BLA, vCA1 send direct projection to CEA which contribute to the fear learning, and provide a foundation for investigating the role of HPC in expression of anxiety. vHPC also has a role in reward processing and motivation through direct projection to NAc, as well as controlling for the circadian rhythm through inputs to the hypothalamic nuclei. Most importantly, the intimate bi-directional connectivity between HPC-AMG-PFC allows for the vHPC to support retrieval of fear memory by shaping context-dependent neuronal responses in the AMG: direct (HPC-AMG) and indirect (HPC-PFC-AMG) route support contextual retrieval of fear memory, and enables the AMG to adapt fear behavior according to the context.

Contribution of the **vHPC** is particularly important when it comes to retrieving the emotional content of a memory. Lesion in vHPC is consistent with a reduction in anxiety, as it increases entry into the open arm during EPM task, or augments the time spent in brightly lit chamber, with decreased anxiety-related behaviors and less increased corticosterone level (Kjelstrup, 2002). These lesion data for emotional response showed an anatomically marked ventral-dorsal distinction, with hippocampal response in emotional response segregated only to a ventral portion.

Finally, as in dHPC, NMDA antagonists infused into the vHPC blocked the acquisition of contextual fear but not fear to a tone that signaled the shock (Zhang, 2001). This role of the vHPC in fear expression is consistent with the anatomic connectivity of the vHPC which shares massive bidirectional connectivity with the AMG (Fanselow & Dong, 2010).

HPC and AMG are functionally connected and neuroplasticity mechanisms within these regions are engaged during both CFC learning and expression. Notably the AMG receives direct hippocampal input only via the vHPC (Maren & Fanselow, 1995), and it has been shown that vHPC manipulation alters fear conditioning by depriving the AMG of both dorsal and ventral hippocampal information (Anagnostaras, 2002; Zhang, 2015). Human fMRI studies demonstrated that context information is encoded by the HPC and converges towards the AMG with information about the US (Fanselow & Maren 1995; Fanselow & Kim 1994), suggesting that AMG has a general role in aversive conditioning, and that context conditioning additionally recruits the HPC.

In the other hand, vHPC also send monosynaptic inputs towards the **mPFC** network, and has been proposed to play a critical role in regulating context-dependent fear memory retrieval after extinction (Jin & Maren 2015a). The disconnection of the vHPC from the mPFC also showed to impair spike firing in the PL and deteriorate fear renewal (Orsini, 2011). Importantly, inputs from the vHPC neurons that project to both the mPFC and the AMG are associated with contextual memory (Jin & Maren 2015a). They provide contextual information about emotional content of an experience (Stujenske, 2004), synchronize activity in PFC-AMG networks for the contextual control of defensive behavior (Kim & Cho, 2017), and they are preferentially engaged during fear renewal (Jin & Maren 2015b). These observations support the idea to bring the vHPC into focus, as it modulates fear memory related to a context by coupling its activity with the mPFC and the AMG, whereby the transmission of contextual information from the HPC to the mPFC generates context-appropriate behavioral response by interacting with the amygdala (Jin & Maren, 2015b).

Finally, if we were to compare the two portions of the HPC, though dHPC is more efficient at spatial processing, vHPC might get involved in context representation in a broader sense. Such that, the dHPC represents specific spatial location in the context, while the vHPC may be necessary for general recognition linked to specific association of cues with emotional value of the context, and thereby suggested as a necessary mechanism to allow discrimination between safe and aversive context (McDonald, 2018). On the same line, study using a visual context associated with an unsignaled US allowed to temporally segregate hippocampal activation along its dorso-ventral axis. For instance, the dHPC was active during early phase of acquisition, reflecting context fear encoding, whereas the vHPC was active during later phase of acquisition, reflecting emotional expression of that fear (Fanselow & Dong, 2010).

2. Medial prefrontal cortex

Considering the reciprocal connectivity with multiple subcortical areas, the mPFC is ideally positioned to receive and modulate both spatial and limbic information from structures implicated in contextual fear behavior. Variety of functions have been attributed to the mPFC, such as emotional regulation (Pitman, 2012), decision making (Euston, 2012), contextual valence evaluation (Coley, 2021), among many. In addition to the well-established role of the vHPC in CFC, consequent numbers of studies in both humans and animals have also identified the mPFC as playing a key role in the acquisition, expression, memory consolidation, up until the extinction of contextual fear behavior. However, its role in fear learning has been mainly investigated using cued FC (Sotres-Bayon & Quirk, 2010; Courtin, 2013), and its contribution to various aspects of contextual fear behavior is still largely understudied.

It is commonly accepted that the mPFC can be divided into 4 distinct areas, following from the most dorsal to ventral region, it comprises the medial precentral cortex (PrCm), the anterior cingulate cortex (ACC), the prelimbic cortex (PL) and the infralimbic cortex (IL) (Krettek & Price, 1977, Van Eden & Uylings 1985; Ray & Price, 1992). The mPFC can be functionally separated into the PrCm and ACC areas, which regulate mainly various motor behavior, whereas PL and IL are implicated in emotional, mnemonic and cognitive processes (Heidbreder & Groenewegen 2003). According to the target areas, 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).

In the following lines, we will briefly review the main prefrontal connectivity. mPFC receive BLA inputs in the ventral ACC, PL and IL, and projects back, though with differential target in the AMG (McDonald, 1996): PL preferentially project to the BLA, while IL projects to the LA and the lateral capsular where the ITC is located. In addition, mPFC also shares reciprocal connectivity with the thalamus which provides sensory information (Groenewegen, 1988), as well as with the dorsal and ventral regions of the periaqueductal gray (PAG) (Gabott, 2005), critically involved in the avoidance and freezing behavior, respectively.

mPFC receive direct and dense projections from vHPC that terminate in PL and IL, whereas inputs from the dHPC are sparser (Cenquizca & Swanson, 2007; Jay & Witter 1991). However, there is no direct projection from the mPFC to HPC, and indirectly connects to HPC through the nucleus reuniens (NR) of the thalamus, which serves as a main provider of thalamic inputs (Vertes, 2007). Moreover, mPFC can influence the HPC indirectly, via the entorhinal cortex or the AMG (Vertes, 2006). Given the connections

described, we may suggest that the mPFC is ideally positioned to receive spatial and limbic information, and may regulate emotional response accordingly through dynamic interaction with other structures.

a. mPFC in the acquisition of contextual fear behavior

Consistent with the anatomical connections between the mPFC, HPC, and AMG, it appears evident that during fear conditioning to a context, the acquisition of CS-US association requires involvement of both the AMG and vHPC which provides emotional and contextual information, respectively to the mPFC. However, given the time-limited role of the HPC in maintaining context memories, it has been suggested that cortical areas with which HPC is reciprocally connected may be essential for the long-term memory storage of context representation (Maren, 2013). Indeed, hippocampal lesion a day after context fear conditioning produced deficit in freezing behavior during retrieval, whereas minimal effect was observed when lesions were made at later time point (30-100 days later) (Kim & Fanselow, 1992; Anagnostaras, 1999). On the same line, inactivation of cortical areas such as the ACC (Frankland, 2004) or the dmPFC (Quinn, 2008) also impaired expression of remote contextual memory.

Past studies in rodent using pharmacological agents have demonstrated mPFC subregion specific contribution during contextual fear acquisition. Above all, the ACC appears to be necessary, since muscimol (GABA_A-receptor agonist) infusion in ACC before conditioning (Tang, 2005), or administration of NMDAR antagonist at the same time point (Einarsson & Nadar, 2012) reduced context-conditioned fear response during retrieval. Clinical studies have confirmed the latter findings as well (Alvarez, 2008), with converging evidence that the mPFC and AMG later interact during contextual fear expression. Moreover, the role of the ACC for contextual fear memory consolidation has been tested. When virally disrupting learning-induced dendritic spine growth in the ACC during memory consolidation, conditioned freezing behaviors were impaired on the following days (Vetere, 2011), suggesting that in addition to a global role in contextual fear memory formation ACC may be involved during consolidation phase (Rozeske, 2015). However, less conclusive findings from other mPFC subregions were obtained. In one hand, context exposure and conditioning activated the PL, but not the IL (Zelikowsky, 2014), on the other hand, while one study have shown that the pharmacological inhibition of the PL before fear conditioning did not alter fear retrieval (Corcoran & Quirk, 2007), another study showed that light-induced inhibition of PL induced complete blockade of conditioned freezing (Yizhar, 2011). Taken together, while ACC involvement is likely activated during acquisition of contextual fear memories and required for memory consolidation (Bissière

2003; 2008), a complete understanding of the distinct prefrontal regions involved in contextual fear behavior modulation remains to be formulated.

b. PFC in the expression of context fear

Appropriate response to conditioned fear is driven by gradual updating and rebalancing PFC memory representation. Studies using pharmacological inactivation of the mPFC during fear memory recall suggest a regional specificity within the mPFC in the expression of conditioned response, and this in a manner that is specific to the phase of learning. For instance, Corcoran & Quirk (2007) performed PL inactivation with TTX infusion at different time point. As shown above, the same study showed that inactivation of PL before conditioning (during context encoding) did not prevent the formation of contextual fear memories, however, PL inactivation immediately before retrieval greatly reduced context freezing. They suggest that PL integrates information from contextual inputs and regulates expression of fear memories via projections to the BLA. Alternatively, neither the pharmacological inactivation of the ACC (Frankland, 2004), nor its opto-inhibition (Goshen, 2011) before retrieval did alter contextual freezing. This finding along with the others, indicate the specific PL involvement during the expression of contextual fear memories.

In the study from Baeg et al. (2001), they have recorded single units in animals' dmPFC (PL and IL) during exposure in the conditioned context. Interestingly, one population of units exhibit increased firing rate, while the other population decreased. This observation is in support with the possibility of two distinct neuronal population that independently support initiation and cessation of freezing. One possible explanation is through subdivisions of mPFC that have opposite role, and have differential target site within the AMG (Hoover & Vertes, 2007, McDonald, 1991). Notably, neurons in PL showed conditioning induced plasticity (Baeg, 2001), and send robust projections to the BLA (McDonald, 1996) and enables fear expression, while IL mostly projects to the ITC cells, which in turn inhibit CEA output (McDonald, 1991; Shinonaga, 1994), and enables fear dampening. Another possibility is that the mPFC circuit may be differentially contacted by hippocampal inputs according to the context, which further allows behavioral adaptation (Maren, 2013). (Hyman et al. 2012; Zelikowsky et al. 2014). Nevertheless, further work is required to precisely understand the conditions in which the mPFC becomes activated during contextual fear expression and recall, especially considering the functional connectivity that it shares with other structures such as the HPC and BLA.

With the passage of time, memory retrieval becomes independent of the HPC input, a phenomenon called system consolidation. Retrieval of remote contextual fear memories becomes dependent upon the mPFC rather than the HPC. For ex, retrieval of a remote contextual fear memory, induced elevated expression of c-fos in the mPFC, whereas level was decreased in the CA1 of HPC (Frankland, 2004; Goshen, 2011).

Additionally, pharmacological manipulation of ACC (but not PL) after at least 18 days from the FC, impaired conditioned freezing expression, (Frankland, 2004), same observation with opto-inhibition of PN in ACC at remote time point. However, inhibition of dCA1 impaired both recent and remote contextual fear memory (Goshen, 2011). This indicates that contextual fear memories continually depend upon the dHPC retrieval, but with the passage of time, memory recall requires ACC. However, for recent memories, activation of PL and IL seems to be more critical.

c. mPFC in context fear generalization

In light of its connectivity with the HPC and AMG, the mPFC may regulate emotion depending on the contextual information. Multiple evidence is in favor of mPFC involvement in the regulation of fear generalization. First, mPFC unit activity while exposing the animals to different contexts revealed a preferential firing during contextual changes (Hyman, 2012). Second, mPFC signals to direct attention to threat-predictive cues in ambiguous environment (Bukalo & Holmes, 2018). Third, functional imaging studies in patients with PTSD showed reduced activation of the mPFC with an inability to regulate fear and anxiety (Kim, 2011; Pitman, 2012). Lastly, in mice submitted to context fear discrimination task, mPFC (PL/IL) was lesioned prior to conditioning which resulted in context generalization, with comparable freezing behavior between the 2 contexts (Antoniadis & McDonald, 2006). All things considered; past studies are in favor to suggest a regulatory ability of the mPFC in fear generalization.

One mechanism the mPFC uses for long-range communication with its subcortical targets is the theta range (4-12Hz) oscillation (Likhtik, 2014). For instance, Siapas et al., (2005) showed that a significant portion of mPFC neurons (40%) is entrained by HPC theta rhythm which allows contextual regulation of the mPFC activity from the HPC. Consistent with this idea, it has been observed that mPFC is entrained to the vHPC theta during anxiety, which however requires tight modulation. While moderate activity of vHPC inputs allows mPFC to construct spatial map, when too excessive, mPFC function is deteriorated, leading to generalization (Adhikari, 2011). Additionally, Popa et al., (2010) suggested that the post-conditioning increase of theta coherence between the AMG and mPFC has functional relevance to optimal fear memory

consolidation. The mPFC, via its widely distributed connections, is therefore in a unique position for both the threat assessment and top-down control of fear discrimination.

As already mentioned above, dorsal hippocampal place cells undergo progressive remapping as animals are exposed to two different environments and switch their firing patterns accordingly, reflecting the ability to discriminate. Notably, instability of place cell firing patterns has been observed following lesions of the mPFC (Kyd & Bilkey, 2003), and demonstrates that the mPFC modulates place cells patterns and contributes to their firing specificity related to the context. Another mechanism that has been suggested is that while the HPC separates overlapping memories, mPFC induces feedforward inhibition of the HPC to prevent from the unwanted co-activation of inappropriate representation, thereby improving memory specificity and generalizability (Koolschijn, 2019). However, mPFC activity in relation to the vHPC during context discrimination still remains to be better explored.

In addition to the hippocampal contribution, mPFC also regulates emotional responses via its connectivity to the AMG. During presentation of 2 different CSs to human subjects, a high fMRI BOLD signal in mPFC with low signal in BLA were observed, whereas presentation of 2 similar CSs, inversed activity was observed (Lissek, 2012). In an anatomical point of view, a thicker vmPFC is associated with a better discrimination ability (Cha, 2014), while increased functional connectivity between the vmPFC and AMG is associated with generalization, although this kind of approach does not inform us about the directionality of communication between structures (Hahn, 2011). Altogether, they demonstrate that the mPFC-AMG network is involved in fear generalization, and an altered signaling between the two contributes to overgeneralization.

Another interesting study, has blocked the synaptic transmission from the mPFC by injecting an AAC encoding TetTox, and showed that mice exhibited normal fear response in conditioned context, but generalized freezing to an unconditioned context (Xu & Sudhof, 2013). Among all projection regions of the mPFC, blockade of synaptic transmission within mPFC-nucleus reuniens (NR) pathway produced generalization of freezing behavior in unconditioned context. Finally, light stimulation of the NR during CFC produced freezing to an unconditioned context during retrieval. Altogether, they indicate that the encoding of precise context representation during CFC relies on faithful communication between the mPFC and the NR. In particular, the dmPFC projections to the PAG (Floyd, 2000; Vertes, 2004) has been described to directly guide defensive responses expression. Notably, recent study from Rozeske et al., (2018) nicely demonstrated that vPAG-projecting dmPFC neurons exhibited dynamic representation of the non-aversive context, and was both necessary and sufficient in mediating contextual fear

discrimination. However, the mechanisms leading to activation of this pathway during fear discrimination are still unclear, and the authors suggest a possible contribution of hippocampal pattern separation mechanism (Kheirbek, 2013; Yassa & Stark, 2011), and excitatory inputs from the thalamic NR (Xu & Südhof, 2013).

When compared to control, PTSD patients in the conditioning context showed hyperactivation of dorsal ACC along with a hypoactivation of vmPFC. This strongly suggests 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).

3. Amygdala

In the face of a danger, it is now globally accepted that the AMG serves as a rapid detector of aversive environmental stimuli, coordinator of threat-response and subsequently produces adaptive responses that is adapted to the behavioral states (LeDoux, 2007; Maddox, 2019). Studies in human and other species have consistently revealed that its structures and functions are highly conserved across species, and is activated by both aversive and fear-conditioned stimuli (Mobbs 2010; Janak & Tye, 2015). Accumulating evidence from clinical neuroimaging studies suggests AMG dysfunction as a shared neural feature of mental disorders associated with altered emotional processing, such as autism, social anxiety, or PTSD (Baron-Cohen 2000, Etkin and Wager 2007). Following these observations, AMG lesions in both rodents and humans revealed an impairment in the recognition of fearful stimuli. For instance, AMG electrolytic lesions performed in rats, impaired contextual fear conditioning learning, with decreased conditioned freezing (J. J. Kim et al., 1993). On the other hand, patients with focal bilateral AMG lesions showed impairment in their ability to recognize fearful faces, and exhibited misjudged social threat signals (Anderson & Adolphs, 2014). All things considered it suggests the role of a brain region important for connecting stimuli with their emotional meaning.

Anatomically, the AMG is nested deep in the temporal lobe, and is comprised of multiple interconnected nuclei. The main nuclei we put the focus on are the basolateral amygdala (BLA), (subdivided in lateral (LA), basal (BA) and baso-medial (BM) amygdala), and the central nucleus of the amygdala (CEA), (subdivided into the lateral (CeL) and medial (CeM) subdivision), which have been extensively investigated during fear-related behaviors. The BLA consists of glutamatergic PN (about 80%) and inhibitory interneurons (about 20%), considered as a cortical-like structure, whereas, the CEA neurons are primarily GABAergic and considered as a striatum-like structure. Finally, an interconnected sheath of

GABAergic neurons, termed the intercalated cells (ITC), is interposed between the BLA and CEA, with a principal role of providing inhibition (Ehrlich, 2009).

a. CS-US association and expression of fear behavior

We first present a highly simplified view of AMG connectivity in the following lines. The AMG receives information about external environment from the sensory cortices and thalamus, which terminate in the LA (Sah, 2013), center for acquisition of conditioned freezing since lesion of the LA blocked the acquisition of conditioned freezing (Nader, 2001). The BLA is reciprocally connected with cortical regions, especially the mPFC and the HPC, thereby transmits information about the emotional content widely throughout cortical regions. The inter-subregion communication is through LA projecting to the BA and BM, as well as to the CEA. Interestingly, the substantial number of regions that communicate with the BLA, may explain its relatively large volume compared with the CEA (Janak & Tye, 2015). The predominant unidirectional output of the BLA include the striatum, the NAc and the BNST and the CEA. Finally, the CEA has connections with hypothalamus and midbrain periaqueductal gray (PAG) among others, controlling the motor expression of emotional responses. Finally, CEA also receive sensory inputs and can contribute to behavioral response independently from the BLA (Corbit, 2005). Altogether, the AMG is more than a simple correlate of the mPFC, such that information about the context is represented in both structures in the similar extent, and it takes active part in making final decision to engage adapted behavior (Cohen and Paz, 2015).

Afferent projections carrying sensory information about the CS and projections carrying US information are conveyed onto the PN in the LA that maintain enhanced strength of excitatory response following Hebbian plasticity mechanism (Quirk 1995; 1997). This experience-dependent potentiation could be induced artificially, for instance, photo-activation of the LA could be used as a substitute for the US (Johansen, 2010). Similarly, high frequency stimulation of auditory stimulus conveying axon terminals could be accounted as a substitute for a tone CS (Rogan & LeDoux, 1995), resulting for both experiments in expression of conditioned freezing. In addition, the pharmacological and molecular blockage of LTP in the amygdala blocks the acquisition and expression of fear conditioning (Bauer, 2002; Maren, 1996; Pape & Pare, 2010). Together, these data nicely demonstrate that BLA is required to process fear-related associative learning (acquisition).

Considering that the expression of conditioned fear is prevented by lesions of the CEA (Kapp, 1979) the projection from the LA to the CEA may be involved in guiding such process (Fanselow & LeDoux, 1999;

Maren, Aharonov, & Fanselow, 1996). The CEA is composed of highly organized inhibitory microcircuit, and information flow from the BLA is first transferred to the CeL, which then set long-range projections towards other structures like the PAG to trigger fear behavior either directly or indirectly via the CeM (Pare & Duvarci, 2012; Tovote, 2015). Importantly, two subpopulations of neurons within the CeL with opposing functions and genetic markers have been identified: neurons that are excited by CS following FC, termed **CeL_{ON}** neurons, on the other hand neurons that are inhibited in response to CS are called **CeL_{OFF}** neurons (Ciocchi, 2010). From the same study, it was shown that pharmacological inactivation of CeM decreased conditioned fear, while CeL inactivation showed promoted fear response. Such observation is directly linked to the inter-population connectivity within the CEA. CeL_{ON} and CeL_{OFF} both project to CeM, and they inhibit one another. Following a fearful CS, CeL_{ON} neurons respond at a shorter latency than CeL_{OFF} (Ciocchi, 2010), and selectively inhibit the CeL_{OFF} neurons that project to the CeM, resulting in disinhibition of CeM output neurons. On the other hand, CeL_{OFF} neurons project to the CeM and tonically inhibit CeM neurons (Haubensak, 2010). In conclusion, neuronal activity in CeL is required for fear acquisition, while conditioned and unconditioned freezing behavior is driven by CeM output neurons which are under tonic inhibitory control originating from CeL (Ciocchi et al., 2010).

The advantage of genetic targeting in mice allowed to identify BLA connectivity with the CEA, especially according to the valence of the CS. First of all, responses to a fear cue in the BLA activate excitatory synapses on somatostatin-positive (SOM+) cells in the CeL, that are the CeL_{ON} cells, with the strength of potentiation greatly reflecting acquisition of fear conditioning (Li, 2013). This results in the initiation of freezing. By contrast, excitatory responses in the BLA following FC also activate protein kinase C expressing (PKC δ +) neurons, that are the CeL_{OFF} cells, which promote anxiolysis. Each subtype of CeL neurons is targeted by distinct functional population of BLA neurons, nicely presented in the study from Herry et al., 2008, namely “fear” and “extinction” neurons, that contact SOM+ CeL_{ON} and PKC δ + CeL_{OFF} neurons, respectively. In conclusion, the presence of experience-dependent potentiation within the CEA points towards a structure that is not only serving as a relay of information from the LA, but is also important in fear conditioning learning (Janak & Tye, 2015).

Long-range inhibitory projections from the CeM output neurons to the ventral lateral PAG (vIPAG) have been described to mediate conditioned freezing behavior (Oka, 2008; Tovote, 2016), since lesions or chemical inactivation of the vIPAG impairs conditioned freezing (J. J. Kim et al., 1993). It is also to be noted that learning activated SOM+ cells project to the PAG as well, bypassing the CeM (Penzo, 2014), and could be suggested as an alternative pathway to modulate fear behavior in addition to the above described

CeM-PAG pathway. This further confirms the implication of the CEA in fear modulation and it is now largely accepted that CEA output to the PAG is instrumental to freezing expression (Herry & Johansen, 2014; Tovote et al., 2016). Although these circuit analyses reveal dynamic interaction between the BLA and CEA in both acquisition and expression of conditioned freezing, multiple neural projections that surround the AMG may regulate behavioral response, to a fearful environment in particular.

b. regulation of fear– integration of contextual and cognitive information

In the following section, we will briefly introduce the modulation of AMG-dependent fear expression, focusing in particular on the dynamic interaction between the AMG, mPFC and HPC. There is a general agreement that these three areas are important for fear expression and modulation, and each of these structures is composed of specific subregions performing different roles within its own local microcircuits, making this inter-structural interaction even more versatile.

For example, as indicated above, the dmPFC is merged with opposing function, with the PL activity necessary for fear retrieval, whereas the IL seems critical for fear extinction learning (Burgos-Robles, 2007; Sierra-Mercado, 2011). Consistent with this idea, a study combining retrograde tracing with optogenetic techniques focused on subpopulation of BA neurons based on their projection target in the mPFC (Senn, 2014). The authors demonstrated that “fear” neurons project exclusively to the PL, whereas “extinction” neurons project to the IL. Moreover, they found that the balance of activity between the BA–PL and BA–IL projection pathways determines the relative expression of fear and extinction memories upon extinction retrieval. This suggests that extinction pathways could directly inhibit fear pathway locally within the AMG, or through competing interaction within subdivision of the mPFC (Tovote, 2015). Of note, extinction training induces NMDAR-dependent plasticity in IL neurons, which in turn stabilizes fear extinction memory (Burgos-Robles, 2007). Notably, IL neurons exhibit CS-evoked responses during retrieval after extinction, and further induces inhibition of the CeM output neurons (Quirk, 2003), through inhibitory ITC cells, and provide a direct pathway to dampen fear responses (Royer, 1999).

The importance of the AMG for the generalization of fear response has been put forward, whereby dissimilarity between stimuli decrease AMG overall activity and prevents inappropriate fear reaction to harmless stimuli (Dunsmoor & Paz, 2015; Rajbhandari, 2016). However, the mechanism by which the AMG interacts with the mPFC and HPC to regulate contextual modulation of AMG activity is currently not well understood. Although conducted on an auditory FC, immunolabeling of BLA and PL showed higher activation in discriminating mice, compared to generalizers. During discrimination a subset of PNs was

engaged during aversive CS, while INs reacted to new stimuli, and inhibited PNs to decrease fear expression (Grosso, 2018).

Notably, Likhtik & Paz (2015), (still on cued FC) nicely reviewed the mPFC-BLA interaction in discriminative learning: they indicate that at the beginning, BLA to mPFC directionality predominates, to later in training when mPFC to BLA directionality is more prominent, that relies on theta and gamma oscillations of mPFC-BLA circuit to transmit information about safety. For example, during safety signal, increased synchrony of mPFC-BLA in the theta range (Likhtik, 2014), or enhanced BLA gamma power along with a strong coupling of BLA gamma to mPFC theta (Stujenske, 2014) were both indicative about discriminative ability. The reciprocal interactions between the BLA and the mPFC may thereby underlie successful discrimination (Likhtik 2014), and the rhythmic entrainment of BLA cells by the mPFC indicate that BLA can code for both safety and danger (Likhtik & Paz, 2015).

Likewise, theta-rhythm entrainment during fear expression also occurs between the LA and the HPC (Seidenbecher, 2003), such that synchrony of the rhythmic activity between both structures correlates with fear behavior. Additionally, it has been shown that opto-activation of a monosynaptic glutamatergic projection from the BLA to the vHPC mediates anxiety-like behavior, while its inhibition induced opposite effect (Felix-Ortiz, 2013). Following those lines, BLA projection to the entorhinal cortex, which in turn provides major input to the hippocampal formation highly contributes to CFC (Sparta, 2014). Finally, pharmacological inactivation of the vHPC not only prevents context-dependent fear renewal (Hobin, 2006), also interferes with context-dependent changes in CS-driven firing in the mPFC and the BLA (Maren & Hobin, 2007).

We now generally agree on the context specificity of fear responses, and the important function of the vHPC providing contextual information directly to the AMG (and to mPFC). vHPC-AMG interaction can be thereby accounted as a prominent contextual modulator of fear, such that the HPC represents the meaning of a CS in a particular context and this information modulates the function of reciprocal PFC-AMG circuits involved in either the expression or suppression of conditioned fear response, according to the emotional valence of a context (Maren 2013). (HPC-mPFC engage contextual coding and allow AMG an adapted defensive behavior). However, still remain unanswered the precise mechanisms through which the AMG participates in guiding adapted defensive behavior in the evaluation of new stimuli, especially in the case of CFC, and allow contextual fear discrimination.

IV. Fear memory consolidation

Memory consolidation is a required process that occurs during sleep which provide optimal window for the brain to sort and reinforce newly encoded memories, without perturbation of any external stimuli (Girardeau & Lopez-dos-Santos, 2021). It subsequently generates long-lasting “memory traces” or “engrams” whose activation enable optimal recall of learned information during memory retrieval. Engram cells according to Josselyn & Tonegawa (2020), are 1 – activated by a learning experience, 2 – physically or chemically modified by the learning experience, and 3 – reactivated by subsequent presentation of the stimuli present at the learning, resulting in memory retrieval. Engram cell ensembles are localized within a brain region, and are connected with other ensembles in brainwide into forming an “engram complex”.

Memory retrieval from stored memory is thought to be reinstated during consolidation through synaptic strengthening (or weakening), by gradual transfer of newly encoded memories from the HPC to the neocortex (Frankland & Bontempi, 2005; Squire, 2015). This process referred as “system consolidation” facilitates long-term storage of memory, and is most effectively occurring during slow wave sleep (Born & Wilhelm, 2012). In addition, it is said to take place particularly for detailed episodic memory, since they are easily compromised following HPC lesions (Joo & Frank, 2018, Sutherland, 2001). Although optimal behavioral outcome informs that consolidation and retrieval indeed occurred, we cannot however precisely deduce when or how.

For decades, brain-wide network oscillations have been intensively studied from large amplitude LFPs recorded in the extracellular space. Depending on the behavioral state, brain oscillatory rhythms occur in various frequency, and mediate neural communication and plasticity within and across distinct brain regions (Headley & Pare, 2019). Past studies have suggested that the stability of fear memories is dependent on interactions in the AMG-HPC-mPFC network. Thus, understanding the hippocampal network oscillation dynamics within and across other regions during distinct phases of fear learning is important to develop memory phase-dependent treatment options.

In the following section we will mainly focus on two oscillations: 1 - **Theta** oscillation (4-12Hz) during rapid eye movement (REM) sleep and 2 - hippocampal sharp-wave ripples (**SWR**) and cortical **delta** oscillation (0.5-4Hz), coordinated during slow wave sleep (SWS) (Headley & Pare, 2017), and will try to provide clear information on their respective involvement during fear memory consolidation.

1. Theta oscillation

Theta oscillations are dominant hippocampal oscillation during exploratory behavior as well as during rapid eye movement (REM) sleep (Jouvet, 1969; Kudrimoti et al., 1999). As described above, synchronization at theta frequency between HPC and other cortical and subcortical regions (such as the mPFC and AMG) have been demonstrated to be crucial for adaptive memory encoding and retrieval in various behavioral tasks with spatial and non-spatial feature (Siapas, 2005; Popa, 2010; Likhtik 2014). In this section, we will mainly bring theta during REM sleep into focus, which from multiple evidence thought to be required during emotional memory consolidation.

Electroencephalogram (EEG) and LFP activity during REM sleep closely resembles awake activity. During REM sleep, the coherence in theta between HPC, mPFC and AMG increases after aversive learning (Popa, 2010), and the enhanced theta synchronization in the HPC-AMG-PFC network has been demonstrated both after auditory cued FC (Popa, 2010; Totty, 2017), and CFC (Ognjanovski, 2014; Boyce, 2016). Importantly, theta coherence between AMG and HPC during REM sleep was predictive of the behavioral performance, i.e., freezing level during retrieval (Popa, 2010). Consistent with this idea, targeted disruption of theta oscillation during REM sleep in the dHPC CA1 by optogenetically targeting GABAergic septo-hippocampal neurons impaired contextual fear memory (Boyce, 2016). In accordance to these findings, REM sleep deprivation impaired both synaptic strengthening, which affected contextual fear memory consolidation (Ravassard, 2016).

The role of theta synchronization between structures has been demonstrated during post-extinction REM sleep too, where: the mean phase difference of synchronization between vHPC and LA was indicative of extinction (Totty, 2017), and sustained AMG-HPC theta phase synchronization was observed in animal models of impaired fear extinction (Sangha, 2009). Together, these findings demonstrate the importance of theta synchronization during REM sleep in consolidation of emotionally salient memories, and suggest its potential involvement in anxiety disorders. Indeed, sleep disturbances or altered sleep patterns (REM vs. non-REM sleep) are known symptoms of such disorders (Goldstein and Walker 2014; Brownlow et al. 2015). However, to date, REM sleep is understudied compared to NREM studies, and more in-depth investigation about functional physiology related to REM sleep are required.

2. Delta oscillation and sharp wave ripples

Delta oscillation are most prominently observed during slow wave sleep (SWS), but also during quiet wakefulness, and recruit various areas that are involved for memory consolidation, including hippocampal and cortical circuits (Sirota, 2003, Kitamura, 2017). During SWS, cortical circuits are comprised of “up-” and “down-states”, marked by tonic depolarization, and hyperpolarization, respectively (Contreras & Steriade, 1995; Neske, 2016). Whereas the up-states are associated with reactivation of cortical cell assemblies activated during learning (Euston, 2007), down-states are marked with low population activity, with distinctive slow oscillation, during which consolidation occurs.

However, delta modulation in hippocampal activity seems to be weaker than in cortical circuit, without clear up- and down-states. In early work, the large, brief deflections observed in the hippocampal LFP observed during SWS were referred as large irregular activity (LIA) (O’Keefe & Nadel, 1978; Vanderwolf, 1969). Within the LIA, specific pattern known as sharp wave ripples (SWR) are defined, primarily in rodent, which also generalize in other species as well, up to in humans (Buzsáki, 1983). SWR occur in temporal proximity with cortical delta oscillation, such that they precede the cortical up-state (see Buzsáki, 2015 for a more exhaustive review). SWR consist of a sharp wave at a rate of 0.01-2Hz followed by a set of superimposed high frequency ripples (~100-250 Hz) (Buzsáki, 1992). Sharp wave events are generated through synchronous bursts of CA3 PN in the stratum radiatum, which further trigger a massive activation of CA1 PN in the pyramidal cells layer, as well as INs. The coordinated interplay between activated PN and local INs manifests as fast oscillatory events, called ripples (Buzsáki, 1989; Buzsáki, 1983, Buzsáki, 1986). During learning, a subset of CA3 and CA1 neurons are coordinated by theta oscillation and allow encoding of new information. During subsequent sleep period, SWR reactivate CA1 ensembles in such a way that pairs of CA1 PN that cofired during learning maintain this correlation and their connections are strengthened during sleep (referred as sleep reactivation), and this finally leads to memory consolidation (Buzsáki, 1989; Girardeau & Lopez-dos-Santos, 2021).

Similar to REM sleep associated theta enhancement, emotional learning like CFC also strengthens delta oscillation power, and ripple frequency during SWS (Ognjanovski, 2014). Considering the central role of hippocampal INs in generating SWR, optogenetic inhibition of PV+ IN during post-learning SWS, reduced contextual fear memory, but not during REM sleep (Ognjanovski, 2018). Similarly, opto-inhibition of CA1 PN during SWS-induced SWR, impairs reinstatement of the cells assemblies previously activated during exploration task, that led to memory impairment upon re-exposure to the same environment (van de Ven, 2016). This is indicative of a lack of consolidation of spatial maps, and suggests that SWS-mediated replay

of cellular activity pattern enhance memory consolidation, i.e., sustained memory (Barnes & Wilson, 2104). In addition to fear memory, involvement of dHPC SWRs has been also demonstrated during spatial memory. For instance, selective disruption of SWR in the dHPC during post-learning sleep decreased spatial memory (Girardeau, 2009), or facilitating coordination between cortical delta oscillation and SWR by electrical stimulation of cortical circuit in close temporal proximity with SWR, improved spatial object recognition task (Maingret, 2016).

Surprisingly, in comparison to dHPC SWR, it is still unknown whether ventral SWR, which very likely be a critical factor for the persistence of contextual fear memory, have differential impact on recruiting cortical and subcortical areas during consolidation. For instance, heightened ventral SWR and intrahippocampal communication was observed in *ex vivo* slice preparations after CFC (Çalışkan, 2016). Plus, interesting observation found is that the ventral SWRs have been shown to be more local (Patel, 2013) and preferentially recruit local neurons that send long-range projections to cortical and subcortical regions, including the mPFC and AMG (Ciocchi, 2015). In the other hand, vHPC theta oscillation during REM sleep appear to be less correlated with associated cortical and subcortical areas during fear memory consolidation (Patel, 2013).

Finally, reactivation in CA1 has been described to be stronger and longer lasting, with more frequent SWRs during and after novel experience (Cheng, 2008). Likewise, increase in SWR rate is induced immediately after emotionally salient memory, such as a reward delivery, especially if this occurs in an unfamiliar location (Singer, 2009). Together, these findings strongly indicate that heightened hippocampal SWRs is associated with novel information and reward, and are essential for memory consolidation. Together, these findings suggest that oscillations that occur during both REM sleep and SWS regulate fear memory consolidation.

3. Interaction HPC-AMG-PFC - role in fear memory consolidation

In the following section, we will review major findings dedicated on the role of hippocampal SWR in relation with the cortical area and then with the AMG.

All major findings for long-term memory consolidation involve interaction between the HPC and the neocortex. In the neocortex, unit activity alternates between periods of high activity (“up” state) associated with spindles (10-15Hz oscillation) that originate from the thalamus, and silence (“down” state) reflected as slow oscillation (< 1Hz). SWRs preferentially occur during up-states, especially at the transitions between down and up-states (Tang & Jadhav, 2019), and are closely followed by cortical delta

wave (temporal proximity ~130ms). Delta wave reflect the down-states of the slow oscillation, when cortical neurons stop firing, then in most cases, they are closely followed by spindles with delta-spindle complex most probable ~140ms after SPWs (Maingret, 2016). This fine temporal relation between SWRs and cortical oscillatory patterns is instrumental for communication between HPC and PFC. Indeed, in this very study, Maingret et al., artificially enhanced coordination between hippocampal SWR and cortical delta wave-spindle complex during sleep by timed electrical stimulation. This resulted in improved spatial memory performance on the next day, mediated by increased prefrontal responsivity to the task. They thereby demonstrate a prominent role of a hippocampo-cortical dialog during sleep in memory consolidation. Interestingly, study from Khodagholy et al., (2017) reports that ripple oscillations also occur in cortical regions, especially in the PFC and parietal cortex. Although the mechanisms of their genesis are currently unclear, they showed increased coupling with HPC SWRs during post-learning sleep. In summary, these studies indicate the importance of hippocampal-prefrontal reactivation for sustained memory, thus may be proposed as a key mechanism that support learning. However, involvement of SWRs from the vHPC, especially during fear memory learning is still not fully explored.

Although greater number of studies have put the focus on the hippocampal-cortical interaction, it has been shown that SWRs can synchronize activity within the AMG, particularly in the BLA where a subset of neurons is modulated during SWRs. For instance, during an aversive spatial experience, a subset of BLA cells is reactivated during occurrence of dHPC SWRs, during post-learning SWS, but not during REM sleep (Girardeau, 2017). During auditory cued FC, theta coherence between AMG and dHPC during REM sleep was indicative of the next day performance (Popa, 2010). Furthermore, amygdalar INs seem to increase their firing during REM sleep-like state, when hippocampal (vHPC) theta oscillations dominate (Bazelot, 2015). These observations implicate that amygdalar influence on hippocampal network oscillations during fear memory consolidation might be differential during REM sleep and SWS.

In conclusion, increased excitability and plasticity triggered by the aversive experience predisposes individual engram cells to be reactivated during consolidation-related network activities. Such off-line consolidation of fear memories appears to be one of the most critical phases for adaptive behavioral metaplasticity.

AIM OF THE THESIS

As established in the introduction, the mPFC, the vHPC and the AMG appear as critical structures in the integration of contextual and emotional content of an environment. Most importantly, the three areas share functional connectivity, and their dynamic interaction further allow modulation of fear behaviors that are adapted to the situation at hand. Such mechanisms rely on fundamental cognitive ability that bases on the disambiguation and recognition of a given stimulus/environment, and the memory recall from previously learned meaningful experience, which means that optimal memory consolidation is also a key in this process.

Given the essential role of the context, a major scientific challenge is to understand how the brain processes and reconstitute contextual information between context that is associated with different emotion. Although, we are now quite well informed on how the brain processes contextual information, precise mechanisms through which discrimination between a previously threatening context from a neutral context still remain under investigated, especially when involving the tight interaction between the mPFC, vHPC and AMG. In addition, little is known about the possible influence of the hippocampal onto the mPFC and the BLA, in the frame of fear memory.

We believe that the mPFC is a good candidate that is well positioned to allow both threat assessment and gate fear responses, through dynamic interaction with other two subcortical areas. The main goal of this thesis project is to visualize how the brain deals with such moment of context ambiguity. The questions that motivate us are:

1. What are the functional mechanisms instructing the PFC during contextual fear expression?
2. What are the functional mechanisms within the interconnected tripartite circuit that mediate appropriate behavior according to the contextual valence?
3. At cellular level, what will determine if a neuron is implicated or not in contextual discrimination?

In order to answer these questions, we defined several main objectives: first, implement and validate a novel behavioral paradigm that allow contextual discrimination. Second, perform extracellular recording simultaneously in the three structures, along the contextual learning process, i.e., fear acquisition, retrieval, and discrimination. Third, investigate hippocampal influence onto the AMG and mPFC during memory consolidation. In addition, because our behavioral paradigm offers the possibility to generate diverse groups of behavioral performance, we aim to exploit differential neuronal activities between groups.

MATERIAL AND METHODS

Subject details

We used male C57BL6/J mice (Janvier), aged 12-14 weeks, weighting 30-35g at the time of behavioral testing and electrophysiological recordings. Animals were group-housed under stable temperature (22 ± 2 °C) and humidity ($55 \pm 10\%$) control, on a 12 h light-dark cycle (lights on at 7 a.m.) and were provided with food and water ad libitum. 3 days before surgeries, each mouse was isolated in individual cages and manipulated on a daily basis, followed by another session of handling 5 days prior to behavioral testing and recording. All procedures were performed in accordance with standard ethical guidelines (European Communities Directive 86/60-EEC) and were approved by the committee on Animal Health and Care of Institut National de la Santé et de la Recherche Médicale and the French Ministry of Agriculture and Forestry (agreement #A3312001).

Behavioral apparatus

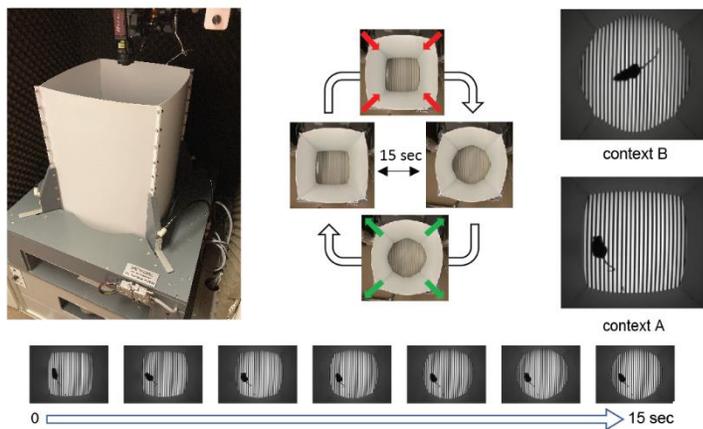


Figure 1. Behavioral apparatus description.

The behavioral apparatus used consisted of an open ceiling square chamber with a grid floor and a camera placed on the top for video recording. The 4 corners of the walls are motorized to allow forward and backward movement, which transforms into “circle” context and back to “square” context, respectively. This context transition takes 15 seconds to operate.

The Contextual fear conditioning (CFC) protocol was performed in a behavioral apparatus (Imetronic) that allowed a gradual transition between 2 different contexts (Figure1), where the transition is fully automatized, so that it prevented from physically interacting with animals during recordings. **Context A** was used during the **fear conditioning** session, and consisted of an open-ceiling cube with rigid but flexible walls (28cm length x 50cm height), which looking from above had “square” shape. The grid floor was connected to a shocker (Coulbourn Instruments) for mild electric foot-shock delivery: foot-shocks of 0.7mA intensity and 1sec duration are delivered with various inter-shock intervals. They served as the unconditioned stimulus (US). **Context B** was used for **Habituation**, and looking from above, exhibited “circular” shape (28cm diameter x 50cm height cylinder). During **Retrieval**, the context shape change was

made possible via the 4 motorized corners of the walls, that in 15 seconds, smoothly move towards or backwards the center. Context was cleaned with 70% ethanol between different mice. Above the arena was placed a camera for the tracking of mice movement and further detection of freezing behaviors. Mice were considered freezing if exhibiting complete body immobility, except for respiratory movement, for at least 1 sec.

Behavioral protocols

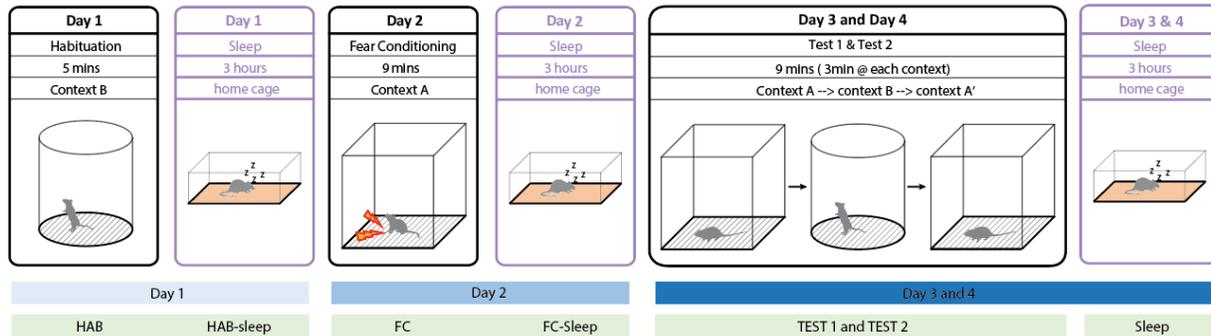


Figure 2 – Behavioral protocol used for contextual fear discrimination, including post-session sleep period.

On the 1st day, WT mice (n=25) were submitted to 5 min of habituation in the Context B where baseline activity level was measured. On day 2, mice were positioned in Context A for 9 minutes, a conditioning session during which they received 5 mild foot-shocks (US). The first US onset was set to the 5th minute and the remainder 4 US were delivered in an intermingled fashion. On the following day 3 and 4, mice were tested for conditioned fear expression, during which they were first exposed to Context A for the first 3 min, then the context transitioned to Context B (15 seconds duration), remained for 2 min 45 sec, and retracted back (15 seconds duration) to the initial Context A for the last 2 min 45 sec (Figure 2). The entire retrieval session lasted for 9 minutes in total. During the 4 days of behavior sessions (HAB, FC, TEST1 and TEST2), we characterized mice freezing behavior by offline detection of freezing episodes (described below). Mice were considered freezing when complete body immobility was observed for at least a second, and we measured the % time animals spent freezing over the context exposure time. Following each behavior session, mice were placed in their home cage to be submitted to 3 hours of recording while they are allowed to sleep (HAB-sleep, FC-sleep, TEST1-Sleep and TEST2-sleep).

Additional protocol: Habituation to context transition task

To decrease the novelty effect of motor sounds during the testing, WT mice (n=6) were submitted to 2 days of habituation, before the FC, and each habituation session lasted for 9 min. The 2 habituation sessions consisted of presenting pre-recorded motor sounds that are emitted by the moving walls during contextual transition. This artificial “transition sound” was played 2 time every minute (Figure 3, music note symbols), so that mice were exposed to the sound of motors 6 time per session. Similar to the test sessions, context transition occurred at minute 3 and 6 (Figure 3, blue symbols). Also, context presentation order was switched between Hab1 and Hab2 days, so that mice were exposed to each context in an equal amount. For these 6 mice, the rest of the protocol was similar.

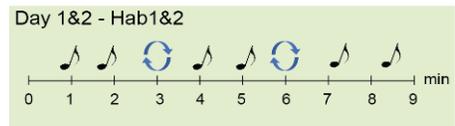
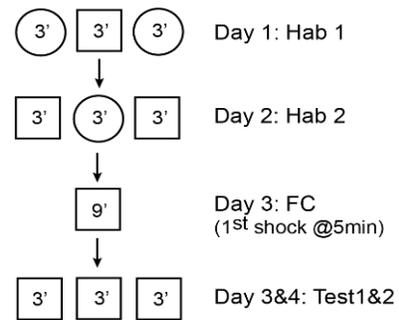


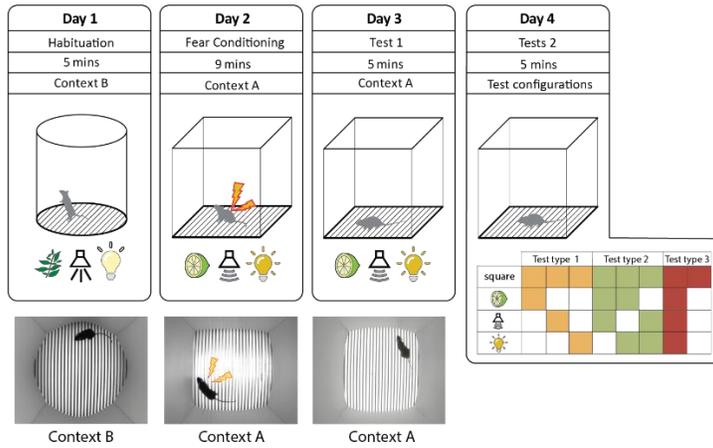
Figure 3 – Protocol used for habituation to context transition.

Green inset at the bottom indicate the timeline of different stimuli onset. Music note symbol indicate when artificial motor sound is played, and at 3 and 6 minutes, the context transition.

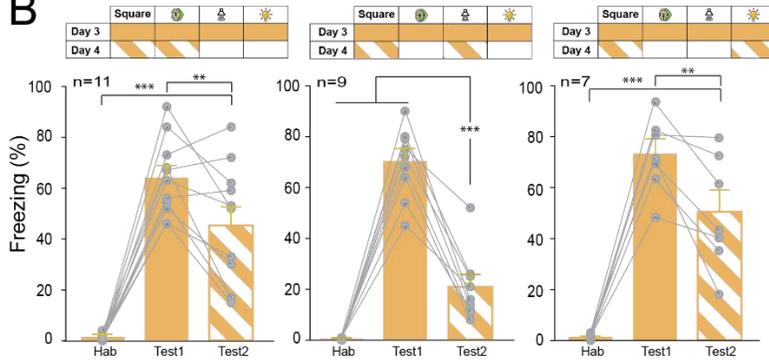
Testing for saliency among various modalities allowing context discrimination.

Prior to using the above-mentioned behavioral protocols, we first controlled for the saliency of each contextual element that was available on our behavioral apparatus. The available modalities were, the sound, light, odor and the already described contextual shape. Each of these elements could be modulated in terms of intensity and duration in an automated fashion. As described in Figure 4A, after habituation in **context B** that had circle shape, provided with thyme odor, white noise and dimmed light, mice were conditioned the next day in **context A** of square shape, provided with lime odor, 7.5kHz pips, and a brighter light. Conditioned fear response was tested on the next day, in the exact same condition and on day 4, we tested 3 main types of protocols, where we varied the number of modalities presented: single modality, double modalities, or all modalities. It is to be noted that, contextual shape was a “background” modality that was always present, as it was the arena walls. Our results demonstrated that the presentation of individual (Figure 4A) or pairs of sensory elements (Figure 4B) did not induce contextual fear memory retrieval as the freezing level observed on Test2, did not reproduce the high level of freezing exhibited on the first day of retrieval session (one-way RM ANOVA, difference in freezing level between Test1 and Test2 were always significant in every trial). It is only when all contextual elements were presented that we observed elevated level of freezing similar to Test1 (Figure 4D, left).

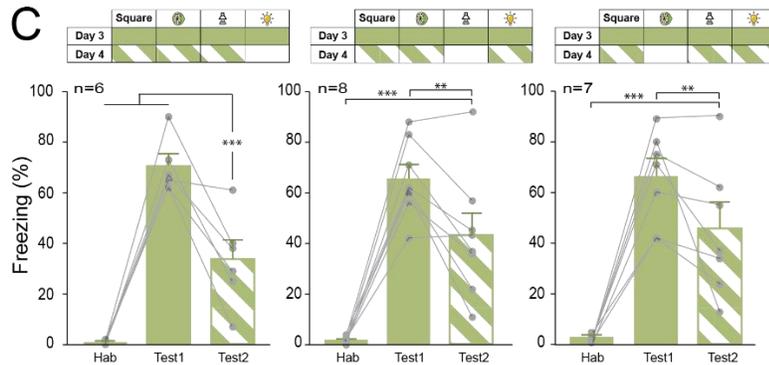
A



B



C



D

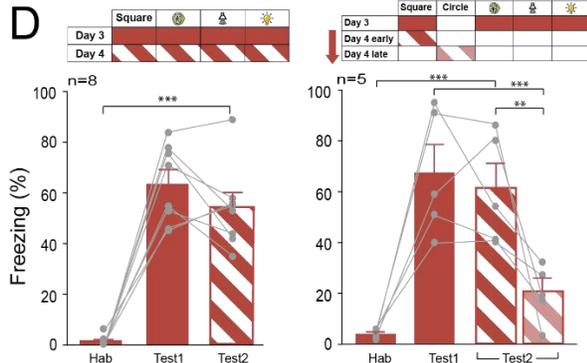
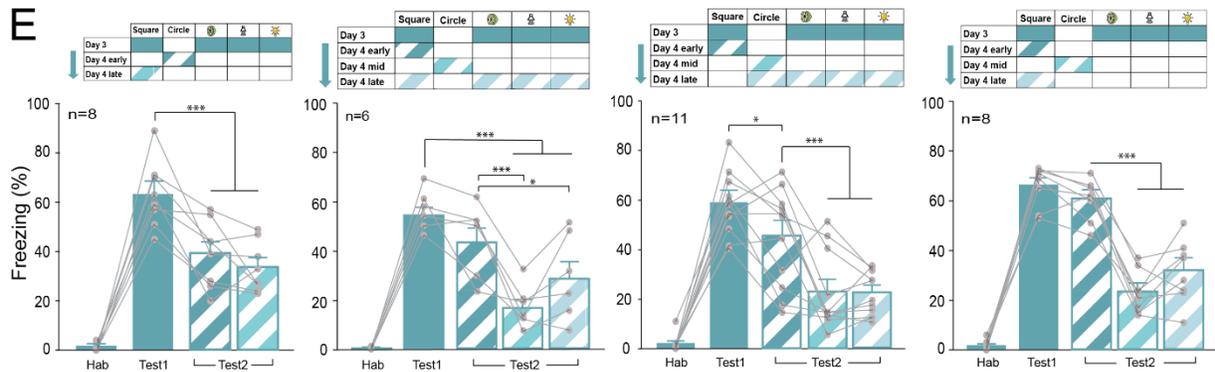


Figure 4 – Implementation of a novel behavioral paradigm to induce context fear discrimination.

A. Summary of protocols tested during which the number of sensory elements presented were varied. The first testing with all modalities is done on Day 3 to confirmed that animals exhibited conditioned fear response. In Day 4, as shown in the scheme, we either presented single elements of the context (in yellow), 2 elements of the context (in green), or presented as a whole (in red). **B.** Mice exhibited significant level of freezing on Day 3 (all $p < 0.001$), but presentation of only one sensory element on Day 4 induced a significant decrease in freezing level for all conditions ($p < 0.01$ and $p < 0.001$). **B.** Same observation for Day 3 (all $p < 0.001$). On Day 4, presenting 2 sensory elements also significantly reduced freezing level ($p < 0.01$, $p < 0.001$). **C.** Left: when presented all sensory cues mice exhibited freezing level comparable to Day 3 ($p = 0.129$, $n = 8$). Right: when only context shape was presented, freezing level was still comparable to the level on Day 3 (Day 4 early, $p = 0.59$). And mice freezing level significantly dropped when the context shape transitioned to circle shape (Day 4 late, $p < 0.01$, $n = 5$). **E.** (from left to right) **First:** On Day 4, when non-aversive context was presented prior to the aversive context, no increase in freezing level was observed ($p = 0.22$, $n = 8$). **Second:** on day4, was presented context transition from aversive to non-aversive context inducing significant decrease in freezing level ($p < 0.001$), then aversive context was presented again with all sensory elements associated, and induced an increase in freezing close to significance ($p = 0.062$, $n = 6$). **Third:** no increase was observed when sensory elements were associated with the circle context ($p = 0.93$, $n = 11$). **Fourth:** on day 4, after a significant decrease in freezing level observed ($p < 0.001$) after the first context transition, switching back to the square context induced slight freezing increase ($p < 0.071$, $n = 8$). All groups were tested with one-way RM ANOVA. Data are presented as mean \pm SEM ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).



Interestingly, presentation of context alone (the wall shape) was sufficient to induce fear memory retrieval, which suggested that the shape of the context might be perceived as the most salient modality within context. In addition, introducing context transition, i.e., transitioning from square to circle context, allowed significant decreased in freezing level (Figure 4D, right). Then we tried to characterize whether contextual transition (change in context shape) would induce context discrimination. First of all, presentation of non-aversive context (circle context) before aversive context (square context) did not induce significant change in freezing level (Figure 4E, first). Then, in the following trials, we presented square context followed by circle context which induced significant freezing level decrease (Figure 4E, second to last, Day4 mid). In the following timeline (Figure 4E second to last, Day4 late), we tried to bring back the freezing level by introducing elements of aversive contexts. When presenting all the sensory elements of the aversive context, but paired with circle shape, no significant change was observed (Figure 4E, third, Day4 late). Whereas when presenting the sensory elements paired with square context (Figure 4E, second), or square context only (Figure 4E, last), a tendency of freezing level increase was observed. These results again put forward the greater saliency of the contextual shape among other sensory elements perceived by the animals. Taking into consideration that this poor context discrimination could be related to the timing effect, i.e., context discrimination being tested only on Day4, when the fear response is not at its maximum, we pursued our behavioral testing with the actual protocol presented above (Figure 2).

Electrodes implants design - Electrode building

Each electrodes bundle consisted of 12, 16 and 4 individually insulated nichrome wires (13 μ m diameter, impedance 50-100 k Ω , Kanthal), dedicated to the mPFC, BLA and vHPC, respectively. Each electrodes bundle was fixed to an electrode guide, and an exception was made for the vHPC electrode bundle which

was fixed to a silicate tube (Thermo scientific) which smaller diameter avoid too much damaging tissues while reaching this deep brain structure. Then electrode bundle was attached to 2 different 18-pin connectors (Omnetics). Connectors were referenced and grounded via silver wires (70 μ m diameter, A-M Systems). The scheme of implants configuration and the representative positioning on mouse skull is provided in Figure 9A. Each bundle of electrodes was plated in a solution of carbon nanotubes (Cheap Tubes) and gold particles (Sifco), to improve the signal to noise ratio, and the stability of recorded signals in time.

Electrode implantation

Mice were anaesthetized with isoflurane in O₂, induction at 4% then maintained at 1.5% throughout the surgery. Body temperature was maintained at 37°C with a heating blanket and eyes were hydrated throughout with Lacrigel (Europhta Laboratories). Mice were placed in a stereotaxic frame (Kopf Instruments) and 2 stainless steel screws were attached to the skull in order to reinforce the stability of electrodes implants. Following craniotomy, mice were unilaterally implanted in the left hemisphere with an electrode array at the following coordinates relative to bregma: +1.95mm AP, -0.35mm ML, -1.35mm DV from dura for the **mPFC**; -3.16mm AP, -3.3mm ML, -4.35mm for the **vHPC** and -1.4mm AP, -3.4mm ML, -4.45mm DV for the **BLA**. Reference and ground wires were placed above the cerebellum. All implants were secured using Super-Bond cement (Sun Medical) at the surface of the skull and reinforced with dental cement (Palavit G, Kulzer) During surgery long- and short-lasting analgesic agents were injected (Metacam, Boehringer; Lurocain, Vetoquinol) both delivered subcutaneously. After surgery mice were allowed to recover for at least 7 days, during which we performed post-op monitoring on a daily basis. When necessary extra dose of analgesic (Metacam, Boehringer) was delivered.

Electrophysiological recordings

While performing the behavioral task or during immediately following resting/sleeping periods, each connector was plugged to an individual headstage (Plexon) containing sixteen unity-gain operational amplifiers. Each headstage was connected to a 16-channel PBX preamplifier (gain 1000x, Plexon). The action potentials generated by individual neurons were isolated by applying a bandpass filter from 300Hz to 8kHz for action potentials, optimizing the signal to noise ratio. Spiking activity was digitized at 40kHz by Omniplex system (Plexon), and isolated by time-amplitude window discrimination and template matching.

Electrolytic lesion and histology

At the conclusion of the experiment, mice were sacrificed and perfused. Mice were first IP injected with a lethal dose of a pentobarbituric (Exagon, Axience) mixed with an analgesic (Lurocain, Vetoquinol). As soon as there were no more reflex, electrolytic lesions were administered to verify electrode tip location in the cerebral tissue after fixation. A circulating current of 10 μ A was emitted from a generator at the pin of the implanted connector for about 10 sec, and was repeated for 3-4 others pins. Then mice underwent trans-cardiac perfusion via the left ventricle with 4% paraformaldehyde (PFA) in 0.1M PB. After perfusion, the entire implanted device composed of electrodes arrays, connectors, reference wires, glue, and cement, was pulled off of the head of the animal.

Following dissection, brains were post-fixed for 24 hours at 4°C in 4% PFA. Serial 50 μ m-thick brain sections were cut on a vibratome, then slices containing regions of interest were mounted on microscope slides along with VectaShield (Vector Laboratories) and dried. Brain slices were imaged using an epifluorescence microscope (Leica DM 5000) fitted with a 2-x or 10-x dry objectives. The location of electrodes was visually controlled and only data from correctly targeted areas were included in the analyses.

Behavioral analyses

Freezing/mobility period detection: Recorded videos of behavioral and sleep sessions were processed offline with DeepLabCut, an open-source software developed by the Mathis Laboratory (Mathis et al., 2018). We manually labeled positions for defined points of mice body, i.e., the nose, neck, center of the mouse, and the base of the tail on a consequent number of frames (about 2000 frames). This served as a base to train a network, which subsequently was able to automatically detect defined points of the body and extract their frame-by-frame X and Y positions. Notably the nose and center of the mouse were used for further analyses of freezing behavior on Matlab (MathWorks, Natick, MA).

X and Y coordinates were used to calculate the instantaneous speed in pixels/frame, smoothed over 5 consecutives frames. Freezing responses were scored when the speed of the center of the mouse was < 0.65 pix/frame in addition to the nose speed simultaneously < 0.5 pix/frame, for a minimum duration of 0.5 sec. The calibration of the camera (1 pix = 0.08cm side) and the frame rate (30 frames/sec) allowed to convert the speed in cm/s by multiplying by 0.08 x 30 = 2.4, so the freezing response threshold was equivalent to 1.56 cm/s for the center and 1.2 cm/s for the nose. These speed thresholds were set empirically, and were further controlled on live video recordings. Nonetheless, the double threshold condition allowed exclusion of immobility periods that were not freezing response related, if only center

of the mouse was taken into consideration, because the nose is moving such as when the mouse was looking, grooming, or sniffing.

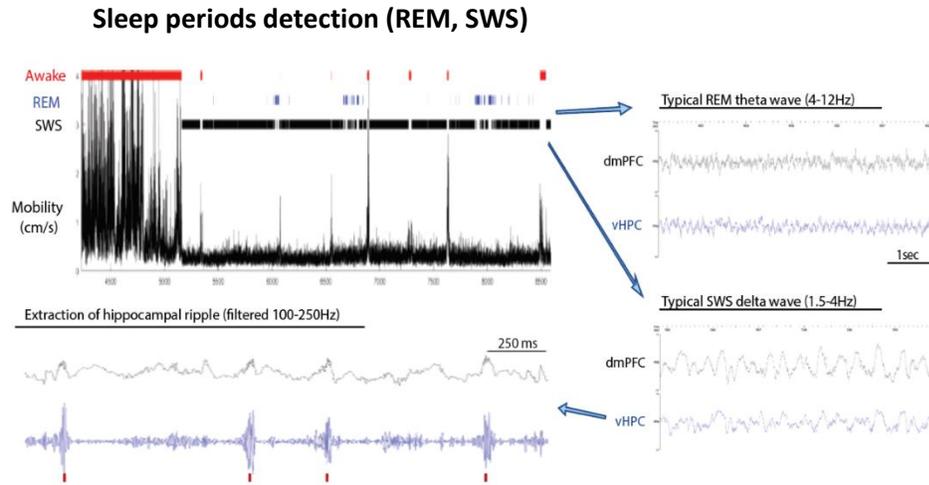


Figure 5 – Detection of sleep periods from mouse motion tracking. Example of recording of resting period that include various sleeping phases. Top left: animal tracking defines awake periods, whereas electrophysiological recordings in quietness periods (Theta to delta ratio, right panels) define slow wave sleep and REM phases. In SWS, ripples can be detected.

Different periods of sleep were detected based on the instantaneous speed of the center of the animal (in cm/s) tracked during the 3 hours of recording. Above a threshold of 0.8cm/s mice were considered awake. Among periods of quietness, we segregated into NREM/SWS and REM according to the theta/delta ratio in both the HPC and mPFC LFPs. If there was a major occurrence of theta oscillation (ranging 4-12Hz), mice were considered in REM sleep, whereas if there was a major occurrence of delta oscillation (1.5-4Hz), mice were considered being in the SWS. During the SWS, hippocampal SWP-R were detected, and the methodology for ripples detection will be described in section 8.e.

Electrophysiology analyses

Single-unit spike sorting was performed using Offline Spike Sorter (OFS, Plexon) and analyzed using Neuroexplorer (Nex Technologies) and MATLAB for all behavioral sessions. Waveforms were manually defined into an isolated cluster while visualizing in a three-dimensional space using slices positioned at crucial time points of spike waveform, and voltage features of the waveforms. The cluster of waveforms was defined as a single-unit when it did not contain spikes within a refractory period less than 1 msec based upon auto-correlation analyses, and was significantly separated from the noise and from neighboring units. Additionally, cross-correlation analyses were performed between pairs of neurons to control that a single unit was not recorded on multiple electrodes. Neurons that displayed a significant -

>10% - synchronized peak of activity as the reference neuron were considered duplicates and only one of them was considered for analyses.

To characterize the influence of locomotion on single unit activity, spike number and effective distance were collected for 1 second bins – to be coherent with the analysis criteria for freezing behavior - and correlated over the period of 9 minutes of retrieval session.

Longitudinal validation of neurons

In order to address certain questions, a longitudinal follow-up of neuronal activities along the recording sessions was performed. Several quality criteria were tested, namely, spike waveform (WF) and spike auto-correlograms (AC) similarities, that were expressed as Pearson coefficients. We then compared actual dataset with shuffled data (Figure 6). We observed that a significant number of shuffled waveforms had WF Pearson correlation coefficient above 0.95, value that is commonly used in the laboratory to consider units as being identical. The presence of false positive fall dramatically above 0,99 (Figure 6B left) suggesting that only waveforms with a Pearson coefficient above 0.99 should be considered for longitudinal follow-up. Activity-based auto-correlograms also showed higher Pearson coefficient when the WF Pearson threshold was set above 0.99 (Figure 6 B right). It is interesting to note that auto-correlogram analyses are highly dependent on the basal frequency (Figure 6B): If low, auto-correlograms tend to diverge and their similarity drops dramatically. This can be easily seen when plotting the distribution of AC Pearson coefficients for low spiking cells above 0,99 WF Pearson (light pink in Figure 6B right) that distribution was close to shuffle. This suggested that auto-correlogram Pearson is less usable than WF Pearson as being sensitive to other parameters. The WF 0,99 selection criteria was further confirmed by amplitude coherence analysis showing that WF amplitude matching remain similar to what is expected by chance until waveform similarities were above 0.99 (Figure 6C).

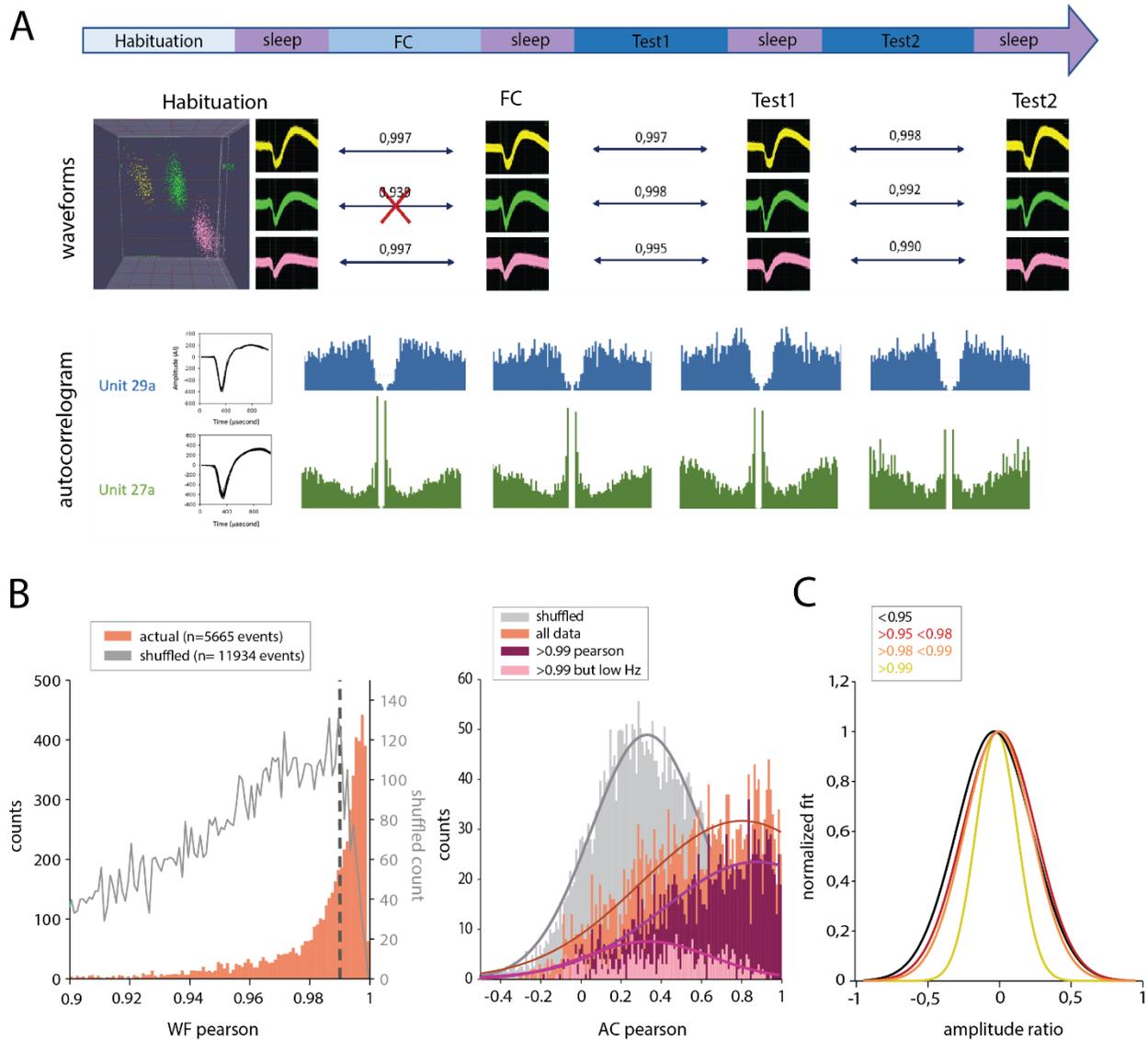


Figure 6 – longitudinal validation of recorded single-units

A. PFC and BLA units were followed along the recording sessions according to several quality criteria. Both spike waveform and spike auto-correlogram must be preserved between recording in case of similar unit. **B.** Correlation of waveforms (WF) and auto-correlograms (AC) similarities expressed as Pearson correlation coefficients, and compared to shuffled distribution (units randomly taken from other wires, in gray). Left: Only waveforms with a Pearson coefficient above 0.99 should be considered for longitudinal follow-up as the probability of false positives fall after reaching this threshold. Right: activity-based auto-correlograms are highly dependent on the basal frequency and improve if selecting units with WF >0.99, except if having too low activity level. **C.** WF >0.99 selection criteria was further confirmed by WF amplitude analysis showing that intersession matching is improved only if WF similarities reach the 0.99 criterion.

Context responsive unit identification

Bootstrap resampling method was used to identify units that were significantly activated within a particular context. For each recorded unit, we considered the inter-spike intervals from its original spike train that were shuffled 1000 times over the 9min of context exposure. We then created a surrogate

distribution of expected spike counts (from shuffled ISI) that occurred in a designated time window, i.e., 15-180s for context A, 195-360s for context B and 375-540s for context A'. We did not consider the first 15 seconds of each context to discard the effect of freezing response establishment (in context A) and the effect of context transition (in context B and A'). For each surrogate distribution created, we determined confidence intervals (CI) at 2.5th and 97.5th percentile, and units that fell outside of the CI were considered to be context responsive. This method identified units that may be significantly modulated during one or two of the three contexts (A, B and A'). To identify units that were exclusively modulated during a single context (Figure 10 in results section), we set conditions such that it should be active in one context while not active in other contexts.

Hippocampal SPW-R detection

SPW-R detection was performed by band-pass filtering at 100-250Hz field potential recorded in HPC CA1 pyramidal layer. The filtered trace was afterwards integrated (i.e., absolute value trace) and events detected as SPW-R if peaking at ≥ 4 s.d. + mean of integrated trace, with a minimum duration of 15ms-150ms around the peak (half-width under the peak) and the distance between 2 peaks had to be >45 ms. To detect the beginning and the end of a ripple, we subtracted 2 standard deviations (s.d.) to the normalized peak. All events detected during REM sleep or periods of mobility were discarded.

Single-unit activity presentations

At some occasions, the mean activity around a specific event was normalized to a reference baseline period, and was expressed as z-scores to account for variability of the baseline value. Z-score values were obtained using the following formula: $\frac{x - \text{mean}(\text{baseline})}{\text{s.d.}(\text{baseline})}$. Neurons were classified as significantly modulated if there were at least three significant bins or two consecutive significant bins following event onset. The significance was set > 1.96 for activated neurons or < -1.96 for inhibited neurons. Other specific analyses are listed below:

- **Single-unit activity during freezing and mobility:** for each neuron, we considered periods of 1 second of freezing and normalized the neuronal activity to 2 seconds before the freezing onset (ON period). As for the modulation during non-freezing periods, we considered 2 seconds of non-freezing events and normalized to preceding 1sec of freezing (OFF period). For both ON and OFF freezing periods, time bin size was set at 100ms, and we also cautiously discarded overlapping

events, i.e., when considering freezing episode, the preceding 2 seconds of non-freezing period should not include another period of freezing.

- **Display of contextual specificity of bootstrapped contextual neurons (as in Figure 20):** after segregating mobility periods and freezing periods, we calculated for each neuron considered the ratio of its firing rate (in Hz) in context B over in context A, and plot the distribution of different ratio values coming from the same contextual cell population. As an example, if one neuron has a preferential firing in context B, its frequency ratio B/A will be >1 , whereas a neuron with a preferential firing in context A, will have a ratio <1 .
- **Activity modulation by HPC SWP-R:** for each neuron considered, its firing rate was aligned to the peak of the ripples and was followed from -500ms to 500ms with a time bin of 10ms, and averaged across all detected ripples. Then neuronal activity was normalized to the “basal” period from -500ms to -300ms, excluding events that are too close to be independent.

Statistics

Analyses were performed with Matlab and Sigma plot 12 (Systat). For all datasets normality was tested using the Shapiro-Wilk normality test ($\alpha < 0.05$) to determine whether parametric or non-parametric analyses were required. Parametric analyses included Student t-tests and one- and two-way repeated-measures ANOVA followed by Holm-Sidak's or Bonferroni's, multiple comparison post hoc tests if a significant main effect or interaction was observed. For parametric data, correlation analyses were made using Pearson's correlation. If datasets did not meet normality assumptions non-parametric analyses were used (mainly non-parametric Mann-Whitney test). If significance was observed, these non-parametric analyses were followed by Dunn's multiple comparison post hoc tests. Comparison of the cumulative probability plots from 2 independent groups were tested using 2-sample Kolmogorov-Smirnov test. The asterisks in the figures represent the P-values of post hoc tests corresponding to the following values * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ based on mean \pm S.E.

RESULTS

1. Implementation of behavioral paradigm for contextual fear conditioning and discrimination

To investigate the role of the brain structures involved in the contextual fear discrimination, we first aimed to develop a novel behavioral paradigm that allows animals to learn the fearful nature of a context. By introducing another context that is sufficiently distinct, animals next learn to discriminate the non-fearful context from the fearful context. Our behavioral paradigm has been adapted from the previously published study in the Herry laboratory (see Rozeske et al., 2018, for further details), and was further validated by additional control experiments that consisted of testing different combination of sensory modalities in order to characterize the saliency of each element in triggering stable fear responses. We kindly ask the reader to refer to the methods section for a complete description of the all process of paradigm optimization. It came out that the most salient element perceived for the fear memory retrieval was the “visual” aspect of it (i.e., the transforming context shape), that, solely, induced significant and elevated level of freezing which was stable in overall exposure time.

On the first day, animals were allowed to freely explore context B (“circle” context) for five minutes. Typically, tested animals did not exhibit any fear-related behavior (Figure 7A and B). On the following day, animals were submitted to the contextual fear conditioning in context A (“square” context, 9 min in total), in which they received five temporally shuffled foot-shocks starting at 5 minutes of contextual exposition (Figure 7A), which induced a progressive increase in the freezing level (Figure 7B). When tested for fear retrieval on the next days, animals exhibited a variety of behaviors along the square-circle-square contextual exposition (Figure 7C and D). In order to segregate each retrieval session according to the performance type, we made a first distinction between animals which learn the contextual fear conditioning (CFC), and animals which did not. Former group was marked with a progressive increase in the freezing behavior during the first minute that reached significantly high level of freezing (one-way RM ANOVA $p < 0.001$, compared to habituation) which maintained over the 3 minutes of aversive context exposure (Figure 7D, in blue, green and yellow). On the other hand, animals that do not learn the task show low freezing level as compared to the “learner” groups, (two-way RM ANOVA, $p < 0.001$ between low freezing and learners), with sparse number of freezing bouts (Figure 7C, in red) and maintained this low level during the 9-minute retrieval session (Figure 7D and E, low freezing sessions, in red, $n=11$). For simplicity, we will call this behavioral group “low freezing” or “L sessions” for the rest of this manuscript.

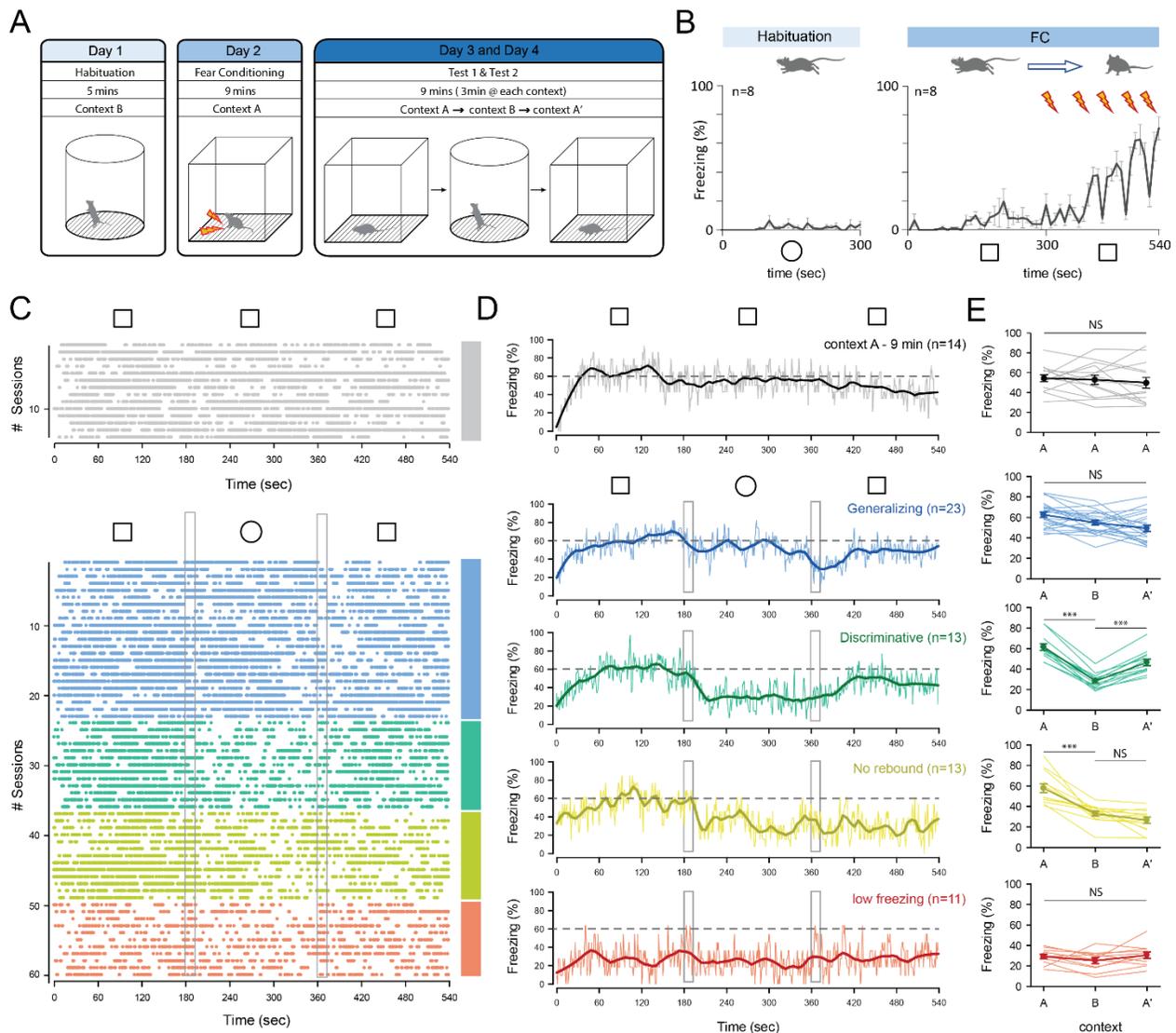


Figure 7 – Characteristics of the various behavioral patterns observed during context fear discrimination.

A. Behavioral protocol used to induce contextual fear discrimination. **B.** Typical average level of freezing observed during habituation (n=8), and fear conditioning (n=8). Lightning symbols indicate the onset of the aversive US, starting at the fifth minute. **C.** Freezing epochs that occur during the two retrieval sessions (test1 and test2). In black, are shown freezing epochs during exposure to square context for 9 min (n=14 sessions). In blue, freezing epochs during generalizing sessions (n=23), in dark green, during discriminative sessions (n=13), in yellow, during discriminative no rebound (D' sessions, n=13), and in red, during low freezing sessions (n=11). **D.** Average freezing level during the 9-minute retrieval sessions. Light color lines indicate average freezing dynamics in 1 second time bin. Darker lines indicate smoothed average dynamics. Gray squares indicate context transition timing that lasts for 15 seconds each time. **E.** Average freezing level during context A, B and A' exposure. No significant difference in average freezing level between contexts observed in control group (one-way RM ANOVA, p=0.143, in black), as well as in G sessions (one-way RM ANOVA, p=0.07, in blue), and in L sessions (one-way RM ANOVA, p=0.233, in red). However, significant difference was observed at each transition in D sessions (one-way RM ANOVA, p<0.001 for each transition, in dark green). In D' sessions, significant decrease in freezing level was observed at the first transition, but not at the second, as freezing level did not rebound (one-way RM ANOVA, p<0.001 and p=0.121 for first and second transition, respectively, in yellow). Light colored lines indicate individual data and darker colored lines indicate the average. (***)p<0.001

The next distinction to make was among the learners along the first contextual transition. When context A gradually transitioned to context B, some animals exhibit their ability to discriminate the 2 contexts, with a significant decrease in freezing level aligned to the transition (Figure 7D-E, one-way RM ANOVA, $p < 0.001$ in green ($n=13$) and yellow ($n=13$)). This decreased freezing level, comparable to the one exhibited by the no learners in context B (Figure 8E-F) was maintained during context B, marked with shorter duration of freezing bouts compared to in context A (Figure 7C, see also Figure 18). On the contrary, in some other retrieval sessions animals did not show any significant change in freezing behavior (Figure 7C-E, one-way RM ANOVA, $p=0.07$, in blue, $n=23$). This latter group will be called “generalizing” or “G sessions”.

The last distinction to make was among the discriminative sessions during the second contextual transition (context B to context A'). In some sessions, animals showed the ability to recognize the threatful context and their freezing behavior level “rebounded” to a level that was significantly higher to the level in context B (Figure 7D and E, one-way RM ANOVA, $p < 0.001$, in green, $n=13$). This group with significant freezing level drop and rebound at the second transition, will be called “discriminative” or “D sessions”. Opposing to the latter group, some animals were not able to discriminate the second time. They maintained similar freezing level as in context B without freezing level rebound (Figure 7D and E, one-way ANOVA, $p=0.121$, in yellow, $n=13$). This group will be called “discriminative no rebound” or “D' sessions”. Finally, the changes in freezing behavior observed in the different groups were attributed to the contextual transition, as we observed no change in freezing level when mice were exposed to context A for 9 minutes (Figure 7C-E, one-way RM ANOVA, $p=0.143$, $n=14$, in black).

In addition, when considering the second transition some sessions were difficult to be classified according to the criteria described above. For instance, 8/23 generalizing sessions exhibited significant decreased freezing level in context A', possibly reflecting a natural tendency of animals to explore further a given context when no salient stimuli are presented (see the progressive decrease in the “square only” group in Figure 7D). Nonetheless, they all at least exhibited high level of freezing in context A and B, thus were included as part of generalizing sessions. Following the same idea of a natural decrease in freezing level with time, and to keep our behavioral segregation as simple as possible, other “borderline” sessions were included as part of discriminative sessions (3/13) during which their freezing level in context A' was not significantly different to context B. However, they all show a strong freezing level rebound after the second transition, associated to the transition onset, before that freezing level lowered down. Some specific cases might be further analyses and possibly considered separately from the other typical sessions. Unbiased

methods for group clustering may be attempted in the future, such as K-mean strategies focusing on the key behavioral readouts or paradigm changes.

When defining each behavioral group, we had to consider behavioral outcome from each retrieval session (Test1 and Test2) separately. Indeed, while greater portion of animals that belonged to G or D/D' group in Test1 conserved their category on the next day (Test2), some animals switched their behavioral performance (Figure 8A), sometime between extremes (G→L for example). As the mean level of freezing was similar between the two tests (data not shown), we attributed these changes to individual changes, favoring the optimization of the contextual discriminative. Indeed, more numerous D sessions have being observed on day 2.

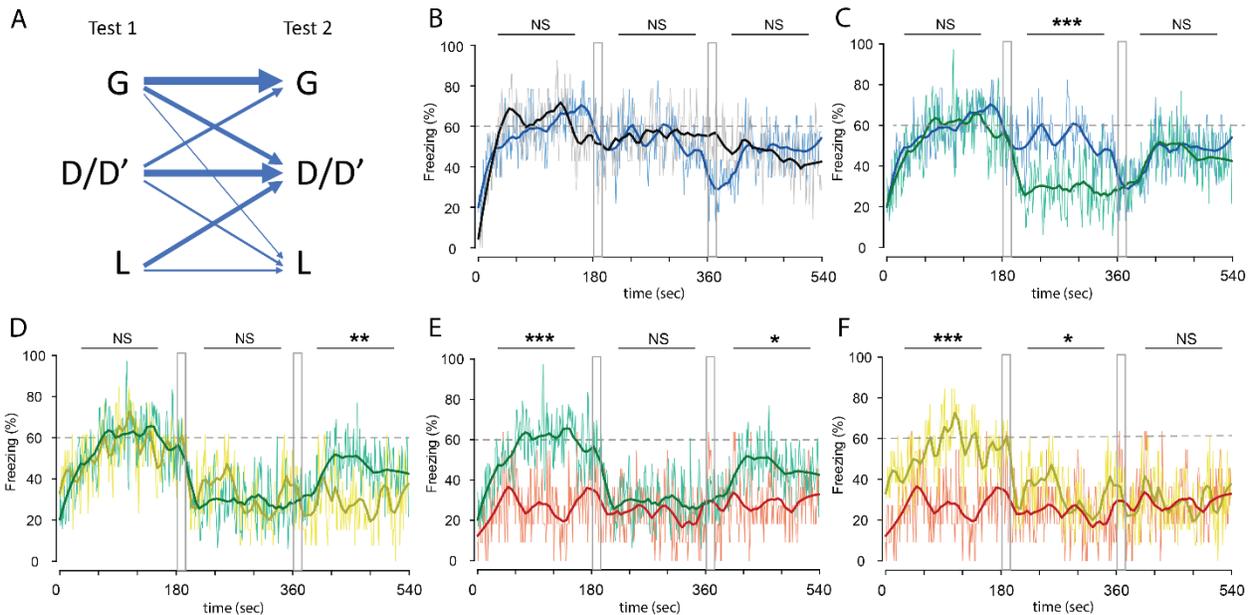


Figure 8 – Differential freezing level dynamics according to the behavioral performance.

A. Great majority of G and D/D' sessions conserve their behavioral performance on the next day (58% of G sessions and 55% D/D' sessions). **B.** Comparison of the average freezing level between G sessions (in blue, n=23) and control groups (9 minutes context A, in black, n=14), with marked effect of the transitions observed in G sessions. No significant difference observed between 2 groups in each context (two-way RM ANOVA, $p=0.61$; $p=0.572$ and $p=0.874$ in context A, B and A', respectively). **C.** D sessions (n=13) exhibit significant difference in freezing level during context B (two-way RM ANOVA, $p<0.001$), whereas exhibit similar freezing level as G sessions in context A and A' (two-way RM ANOVA, $p=0.892$; 0.665 , respectively). **D.** D' sessions (n=13) follow similar freezing dynamics as D sessions in context A and B (two-way RM ANOVA, $p=0.921$; $p=0.28$, respectively), however maintained low level of freezing after the second transition, with significant difference observed to D sessions (two-way RM ANOVA, $p=0.002$). **E.** The low level of freezing observed in D sessions during context B was similar to the freezing level of L sessions (n=11, two-way RM ANOVA, $p=0.11$). In other contexts, difference in freezing level was significant (two-way RM ANOVA, $p<0.001$ in context A, and $p=0.014$ in context A'). **F.** D' sessions do not show any freezing rebound at the second transition and exhibit freezing level that is similar to the L sessions in context A' (two-way RM ANOVA, $p=0.54$) ($p<0.001$ in context A and $p=0.037$ in context B). All tests were followed by Holm-Sidak multiple comparison post hoc tests (* $p>0.05$, ** $p<0.01$ and *** $p<0.001$).

Globally generalizing sessions exhibited freezing level dynamic that was comparable to animals that were submitted to the context A for 9 minutes, demonstrating that animals without discrimination maintained increased freezing level over 9 mins. However, G sessions were marked with the effect of the transition, with noticeable freezing level drops at the transition. This means that they detected the transitions and reevaluated the situation to maintain their fear response (Figure 8B). On the other hand, discriminative sessions were characterized with lowered freezing level in context B, that was significantly different to generalizing sessions (Figure 8C, two-way RM ANOVA, $p < 0.001$), indicating that animals adapted their threat perception in line with the context change perceived. Then, once the aversive context was presented again (context A'), freezing level came back to a level that was equivalent to the generalizing sessions. As described above, the significant difference observed between D and D' sessions occurs in context A' (Figure 8D, two-way RM ANOVA, $p = 0.002$), during which D sessions showed significantly higher level of freezing than low freezing sessions (Figure 8E, two-way RM ANOVA, $p = 0.014$, in context A'), whereas D' sessions remained at a lower level of freezing (Figure 8F, two-way RM ANOVA, $p = 0.54$, in context A'). Finally, both D and D' sessions exhibited significantly increased freezing level in context A that was comparable to L sessions, (Figure 8E and F, two-way RM ANOVA, $p < 0.001$ in both context A). However, in context B, D sessions showed similar low freezing level as in L sessions (Figure 8E, two-way RM ANOVA, 0.11 in context B), whereas D' sessions showed slightly more elevated freezing level than L sessions, which was however, close to non-significant level (Figure 8F, two-way ANOVA, $p = 0.037$).

Overall, our behavioral protocol allowed to induce contextual fear discrimination characterized by a dynamic change in freezing behavior along the gradual contextual transformation. For animals that efficiently learned the CFC, the discriminative ability was observed in half of the animals. Most of all, our fear conditioning protocol generated various types of behavioral phenotypes which are, according to us, very interesting to be considered for analyses aiming at defining neurophysiological signature of fear discrimination.

2. contextual fear discrimination is associated with optimized context-specific activity patterns of PFC (and BLA) neurons

To dissect the neuronal mechanisms underlying contextual fear discrimination, we performed single-unit recordings within the dmPFC, the vHPC and the AMG simultaneously in freely behaving mice submitted to our behavioral paradigm. Due to the number of areas to target and the spatial constraint associated with

such approach, we needed to design electrode implants configuration and implantation methods that fitted on the mouse head. A total of thirty-one mice were implanted with extracellular electrodes in the left mPFC, vHPC and BLA, with more or less success in the targeting of all three structures (Figure 9A-C). After recording of the electrophysiological signals on behaving animals, were followed offline signal processing steps, including offline unit sorting based on the recorded waveforms (Figure 9D), and auto-correlograms (figure 9E), as well as duplicate elimination (see methods).

After these different qualitative steps, we managed to retain about 650 PFC and 400 BLA neurons per recording session, numbers that were stable along the 4 recording days (Data not shown). We were interested in correlating the spiking activity of these recorded neurons while the animals are exposed to the changing context during the retrieval sessions.

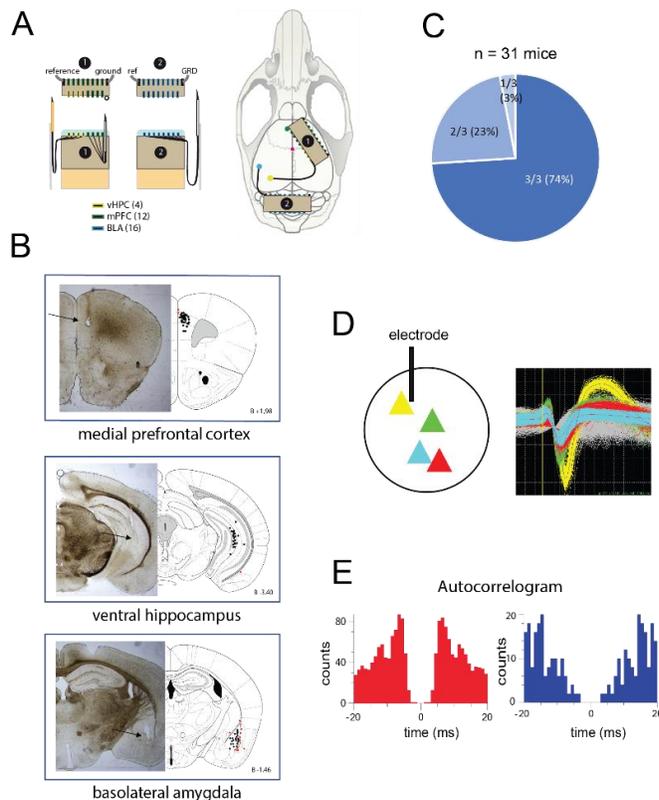


Figure 9 – Single-unit recordings in multiple brain areas.

A. Schematic of the recording strategy simultaneously targeting 3 brain regions. **B.** Electrolytic lesion verifying electrode placement in the mPFC, vHPC, and BLA. Left: example of sagittal brain section with the electrolytic lesion visualization. Right: schematic representation of the identified electrolytic lesion sites in black. Red dots indicate mistargeted implantation. **C.** Proportion of successful implantation among the 3 target sites. **D.** Left: schematic of implanted electrode within the recording site. Right: extracellular spikes originating from different units that display distinct waveform shapes and features. **E.** Examples of auto-correlograms for two different neurons.

In order to evaluate whether the firing activity of dmPFC and BLA neurons contained context-specific information, we based our analyses on the previous finding of high and low contextual fear states representation within the dmPFC population during contextual changes (Rozeske, 2018). Indeed, in this study, a subpopulation of dmPFC neurons were selectively activated during contextual fear discrimination,

being highly active in the non-fearful context when freezing behavior was minimal. Our first objective was to investigate whether we could find similar neuronal population - selectively activated during contextual fear discrimination - in the dmPFC and the BLA, using our behavioral protocol. We thereby isolated neurons from each structure using the same approach, namely the bootstrap resampling methods (see methods).

This analysis revealed subpopulation of dmPFC neurons that are active during the non-fearful context B (about 21%, n=273), but also revealed neurons that are active in other contexts, i.e., about 20% (n=264) and 30% (n=384) of neurons active in the fearful context A, and context A', respectively (defining A and A' cells, Figure 4A). It is to be noted that within each context-selective activity, some were identified as exclusively activated in a single context (e.g., B cells, only active in context B), whereas smaller portions of neurons were activated in multiples contexts at the same time (e.g., active in contexts A and B, defining AB cells or contexts B and A', defining BA' cells). These neurons active in multiple contexts exhibited maintained activity level of similar magnitude in the two contexts, as compared to neurons exclusively activated in a single context (Figure 10A, right). Thus, within each "context neurons" populations (A, B and A'), are comprised neurons that are exclusively active in the given context (exclusive), as well as neurons that are active in two contexts (non-exclusive). Lastly, "context neurons" showed activity patterns that were clearly differing to other "non-contextual neurons", i.e., neurons that did not exhibit any specific firing activity to a given context. These last neurons are generally less active than those exhibiting contextual preference (Figure 24). Interestingly, similar to what was observed in the dmPFC, we were also able to detect subpopulations of "context neurons" within the BLA, although in smaller proportions: 15% activated in context B (n=117), 20% activated in context A (n=157) and 23% activated in context A' (n=180) (Figure 10B).

An important issue in the data description and use is to understand whether Test1 and Test2 can be considered as independent measures even if coming from a single animal. With this regard, we first described that animals often exhibit different behaviors in Test1 and Test2 sessions (Figure 8A).

Next, we wanted to better characterize the proportion of A, B and A' and the less selective populations – AB, BA' and AA' – considering Test 1 and in Test2 separately, in both recorded structures: Importantly, the proportions of contextual cells in PFC and BLA was remarkably similar between Test1 and Test2 (Figure 10C and D), suggesting that mixing Test1 and Test2 was not introducing a strong bias in data description.

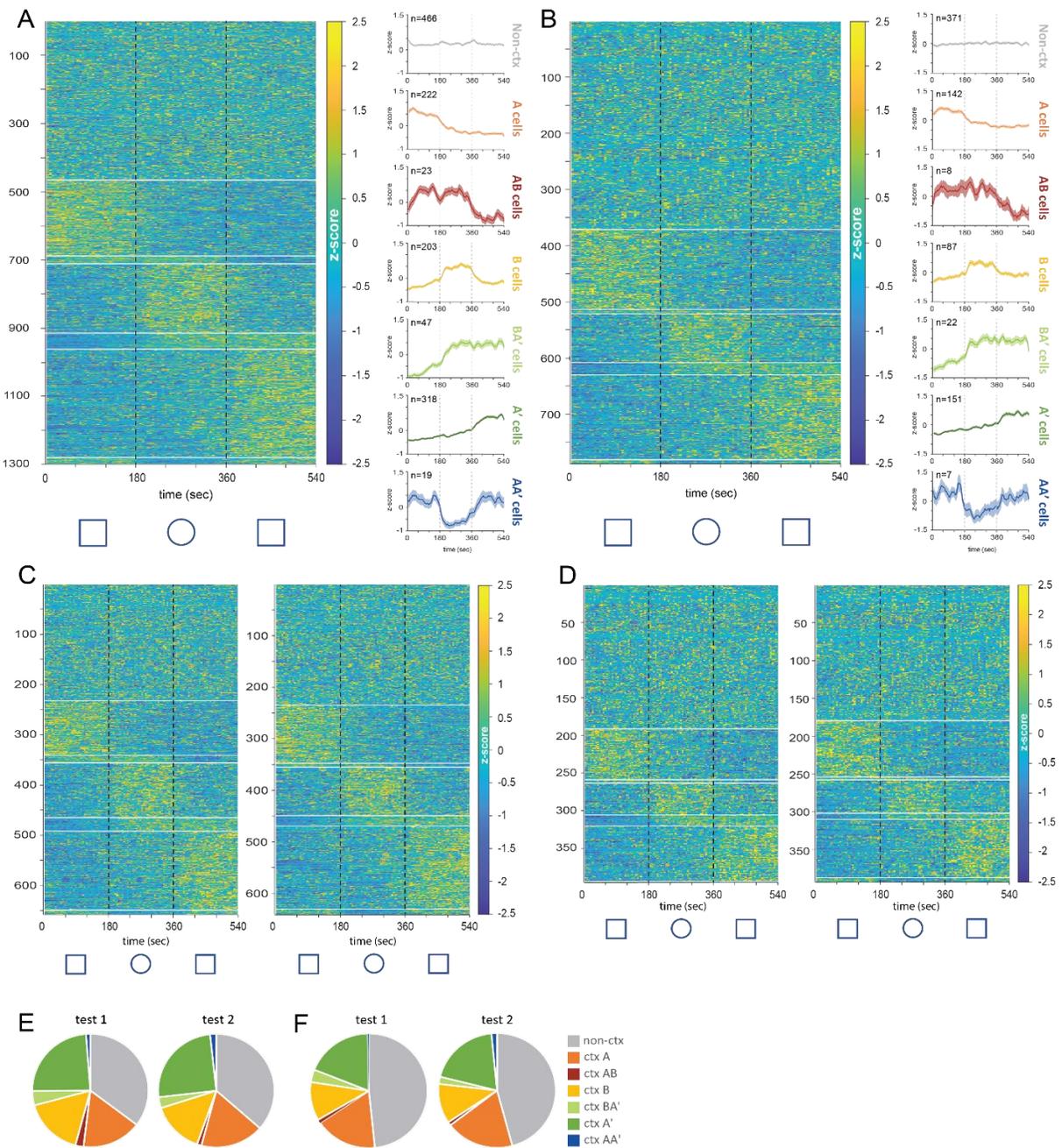


Figure 10 – Proportions of contextual and non-contextual cells are conserved from Test1 to Test2.

A. Left: PFC spiking activity during both test1 and test2 normalized to the entire 9 min period. Dashed lines in black indicate the onset of transitions, and white lines delimit each context neuron group. Right: average z-score of each group from corresponding heatmap. From top to bottom is classified in order, non-contextual neurons (in gray), context A neurons (in orange), AB neurons (in red), context B neurons (in yellow), BA' neurons (in light green), context A' neurons (in dark green) and AA' neurons (in blue). The yellow arrows show the activity rise of non-contextual cells at the onset of each transition. **B.** Same as in A. but for BLA neurons in test1 and test2. **C.** Normalized activity of contextual and non-contextual neurons in PFC test1 (left) and in test2 (right), in the same ranking order as in A and B. **D.** Same as in C but for the BLA. **E.** Proportion of neurons in each contextual category in PFC test1 and test2. **F.** Same as in E but for the BLA. Data are expressed as mean \pm

Interestingly, the activity profile on non-contextual neurons in the PFC was not completely flattened, that would be expected for complete random distribution. Instead, we observed two peaks of activity at each transition (Figure 11A, indicated with arrows), whereas only one peak was present in the BLA recordings. To understand if these activities did really correspond to responses to transition, we ranked responsive

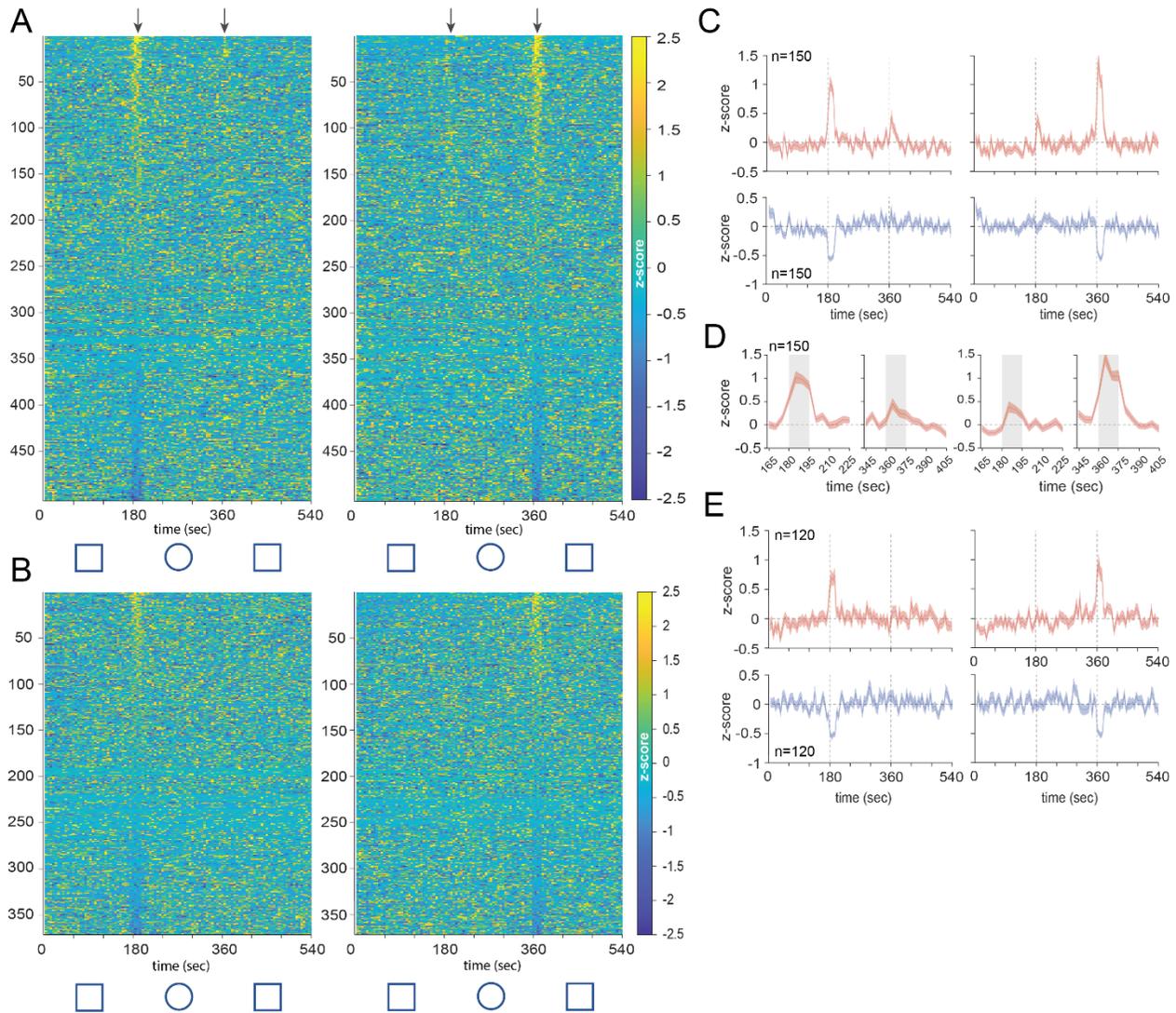


Figure 11: presence of transition neurons in the PFC but not the BLA.

A. Left: activity of non-contextual cells from the dmPFC ranked at the period of first transition (180-195s, n=466), showing visible activity pattern at the second transition. Right: activity of the same non-contextual cells ranked at the second period of second transition (360-375s). Gray arrows indicate the increased activity at the time of transitions. **B.** BLA neurons did not show activity at both transitions (n=371). **C.** Average activity level of the first third neurons in the ranking (activated, in red, n=150) and the last third neurons (inhibited, in blue, n=150). Vertical dashed lined indicated the onset of transitions. **D.** PFC neurons are activated at the onset of the transition, possibly due to an attentional shift of the animal. Gray squares indicated transition timing. **E.** Same as in C, but for the BLA (first third and last third in the ranking, n=120). Data are expressed as mean \pm SEM.

cells at transition1 (T1, Figure 11C, left) or at transition 2 (T2, Figure 11C, right) and measured responses observed at the other transition. We indeed observed that some PFC cells (Figure 11 A, C, D) – but not BLA cells (Figure 11B, E) – increased their activity rate at the time of both transitions. This was confirmed when looking closely onto the transition periods (Figure 11D, gray squares), with increased activity pattern that

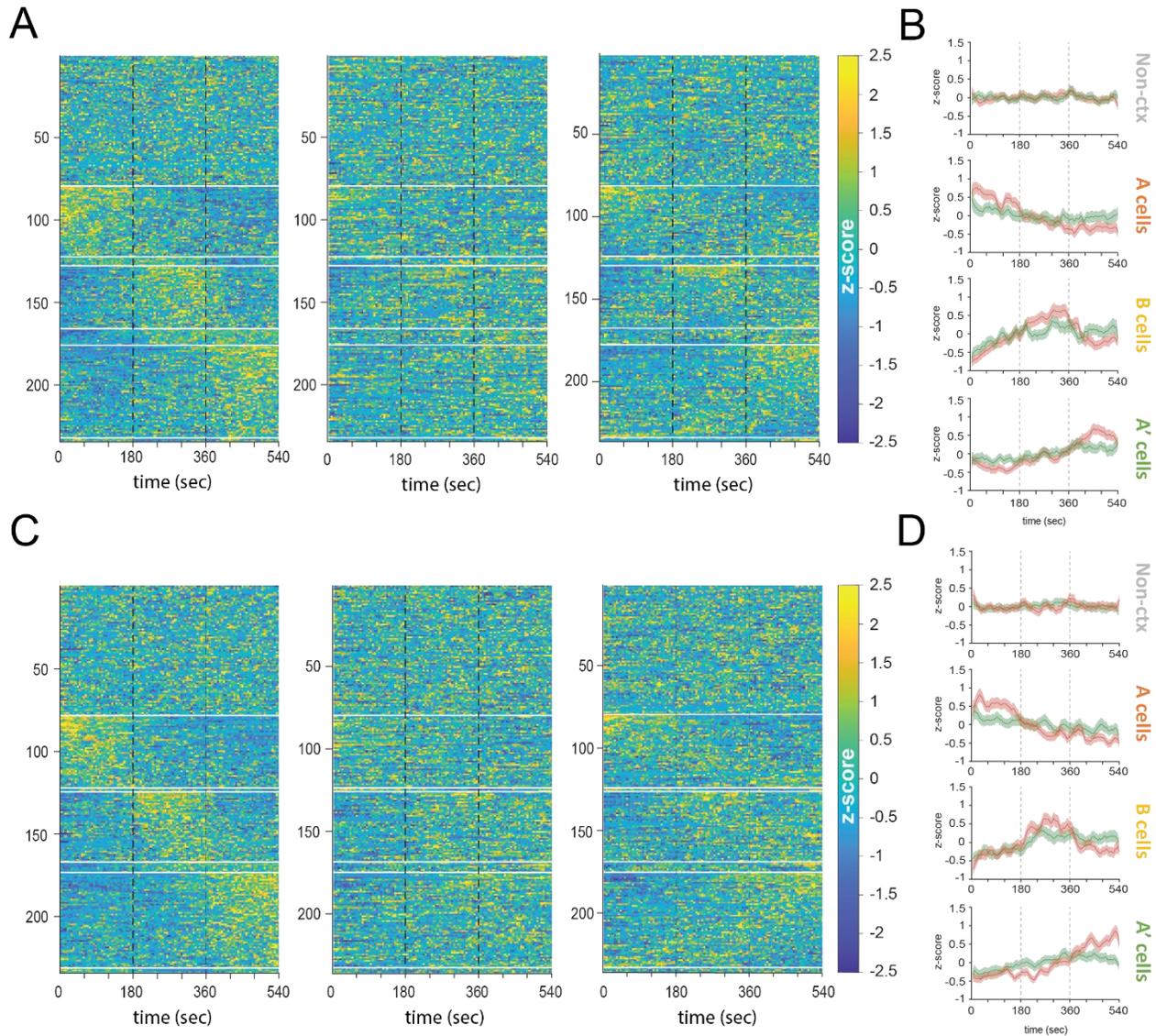


Figure 12: contextual patterns are poorly conserved.

A. Left: normalized activity of non-contextual and contextual neurons in the mPFC during Test1 (n=235 in total). Middle: normalized activity of the same neurons during Test2, ranked in the same order as in Test1. Right: activity of neurons in the same order as in the heatmap in the middle, but ranked within each segment (e.g., ranked at 0-180s for the second segment to see the proportion of neurons that conserve the context A specific activity). **B.** From top to bottom: average activity level of non-contextual, A cells (n=43), B cells (n=40) and A' cells (n=57) in Test1 (in red), and superimposed activity of the same neurons in Test 2 (in green). **C.** Same as in A, but the reference is now the activity in Test2 (n=235). **D.** Same as in B, but with Test2 as reference. Data are expressed as mean \pm SEM.

was maintained to the other transition. Together, this suggested that this can be potentially assigned to attention-related responses in the mPFC, but not in the BLA.

It remains to be determined if these cells are activated by novelty rather than by attention. Indeed, a big part of the recorded animals did not yet encounter the transitions that are detectable by the motor noise. Additional analysis must be performed in the 6 animals that were submitted to context changes and additional motor sounds during habituation periods (see methods). To pursue on the critical question of mixing Test1 and Test2 results, we wanted to test the robustness of contextual preference of units that would have been recorded in both Test1 and Test2. This so-called longitudinal analysis postulate that neuronal units that would have been recorded on the same wire, and that share enough similarities – amplitude, waveform time course, auto correlograms - in consecutive recordings would be considered as the same neurons. This then allow to compare their activity profile before, during and after the CFC procedure. As shown in the material and methods section, a combination of characterization criteria suggested us that spike waveforms recorded from the same electrode with Pearson's correlation coefficient above 0.99 more are likely to originate from the same neurons. We then compared the activity profiles of contextual and non-contextual PFC cells in Test 1 and Test 2 (n=235 neurons in total, Figure 12). To our surprise, independently of the session taken as a reference – Test1 as reference in Figure 12A-B, and Test 2 as reference in Figure 12C-D – the conservation of activity pattern between Test 1 and Test 2 was obviously very low, with no apparent match between A, B or A' cells recorded in Test 1 and Test 2. This was well illustrated by the average profiles (Figures 12B and D) that do not correspond between the deux recording periods. Isolated contextual neurons in either Test 1 (Figure 12B, in red) or Test 2 (Figure 12D, in red) exhibited clear pattern of contextual preference for the given context, with B cells showing the clearest activity pattern, particularly in Test 2. However, the same contextual neurons lost their activity patterns during the other Test (Figure 12B, D, in green).

To confirm this, we directly counted the fate of cells that were identified in Test 1 and analyzed the distribution of their identity in Test 2 sessions (Figure 13A). Results showed an average conservation of about 25-30% for contextual cell types, therefore close to their global contribution into the cell population. Another way to represent this turnover is the comparison in the B/A ratio of all contextual cells in between Test 1 and Test 2 sessions (Figure 13B). Simply said, we calculated for each neuron the ratio of its firing activity in context B over in context A. There, B cell will for instance have a ratio >1 as it will preferentially fire in context B, and at the opposite, A cell will have a ratio <1. From the distribution of calculated ratio

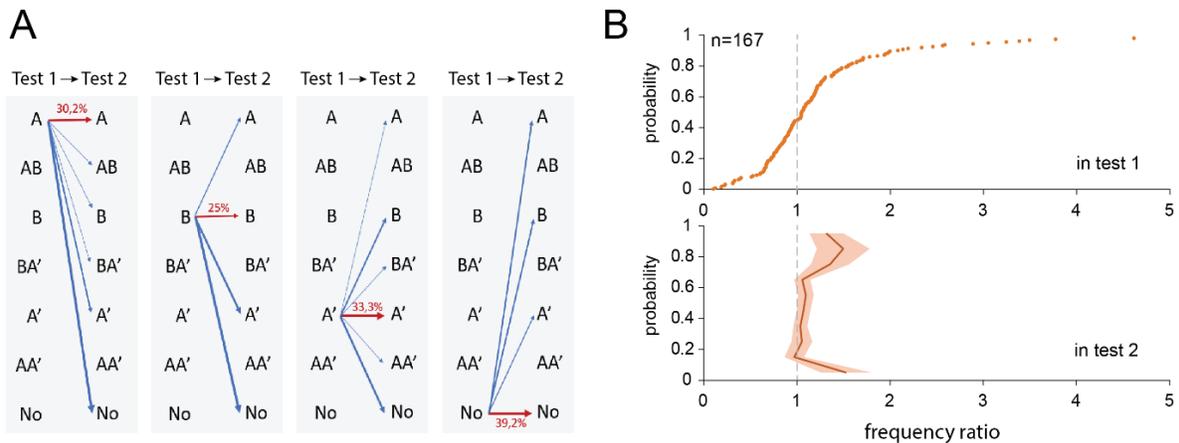


Figure 13: contextual patterns conservation is close to what expected for random distribution.

A. Proportion of each type of contextual neurons in Test1 that conserve same context specificity on the next day. The thickness of the arrows is indicative of the proportion size. **B.** Frequency ratio in context B/A of all neurons that are considered identical (pearson correlation coefficient >0.99) from Test 1 and Test2. A ratio >1 indicates preferential firing in context B over context A, and inversely if ratio <1 . Top: the cumulative probability of preferential context firing from mixed contextual neurons ($n=167$). Bottom: the same neurons from above during Test2 showing loss of preferential firing between context A and B. Data are expressed as mean \pm SEM.

values in Test1, values observed from the same cells in Test2 (ranked to Test 1 values) did not display the same distribution, and always remained close to 1, thus close to randomly distributed values. This again suggested the possibility of a very large turnover in contextual preferences of mPFC cells that occur between testing sessions. Obvious reasons that support this lack of correspondence are: first, the change in the behavioral performance that is often observed between Test1 and Test2 (Figure 8A), and second, the possibility that what we consider as the same unit from Test1 to Test2 are in reality different units.

To test for this second hypothesis, we took advantage from another aspect of our neuronal recordings, namely the unitary responses to hippocampal ripples during sleep phases. Indeed, previous results from the laboratory suggested that even if some plasticity events may significantly affect the coupling between SWS ripples and cortical units, it appears to be very stable and characteristic from one unit to another (Figure 14A). We therefore tested the stability of ripple-triggered responses of “conserved” neurons from Test1-sleep and Test2-sleep, according to their contextual preference in Test1. Results presented in Figure 14 shows a very prominent correspondence between ripple responses of preserved neurons in sleeping sessions consecutive to Test1 and Test2 (see methods), suggesting that they are indeed massively the same units. Thus, our data suggest that longitudinal analysis can be performed using the 0,99 criteria, and ii) a very strong turnover exist in contextual coding in the PFC, including neurons active in context B (B cells) supposedly mediating contextual fear discrimination (Rozeske, 2018).

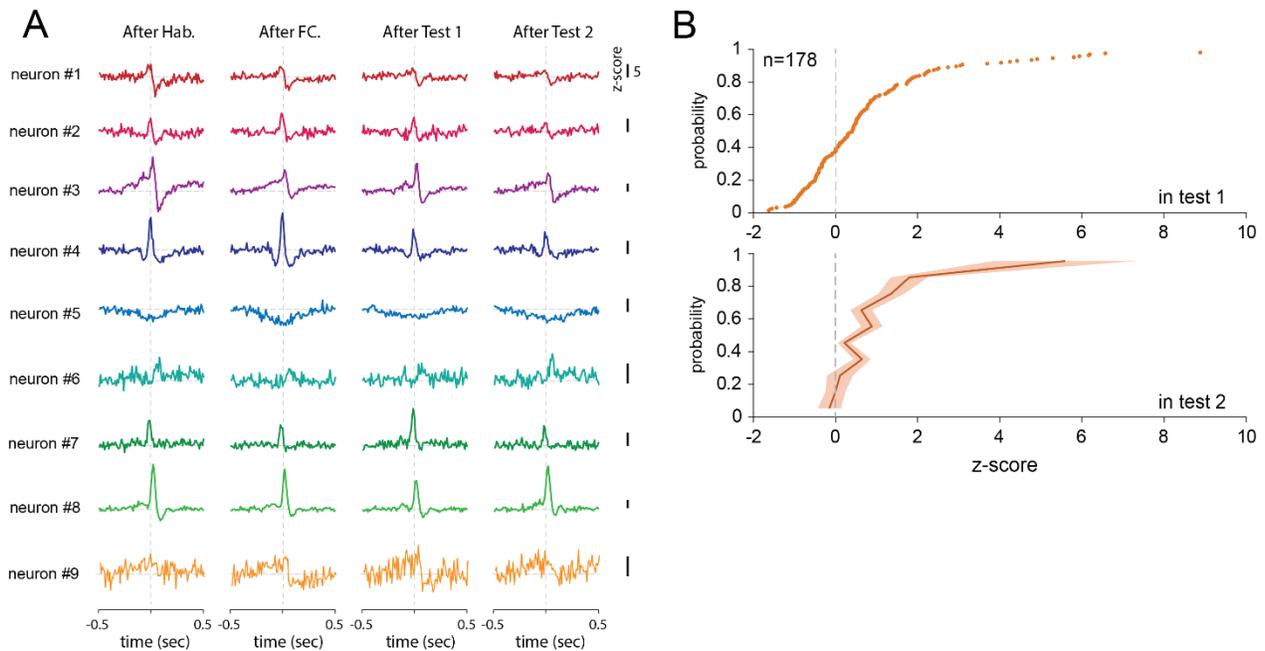


Figure 14: Lack of contextual patterns conservation is not due to a lack of conservation of PFC units.

A. Examples of conserved PFC and BLA neurons (Pearson correlation coefficient >0.99) that are modulated by the hippocampal ripples during SWS, followed from post-habituation sleep to post-Test2 sleep. Conserved neurons preserve the general pattern of modulation. Black vertical lines on the right indicate the z-score scale. **B.** Top: Activity modulation of PFC neurons by hippocampal ripples during post-Test1 sleep ($n=178$). Bottom: activity modulation of the same neurons as above, during post-Test2 sleep. Data are expressed as mean \pm SEM.

As mentioned above, another reason possibly explaining the lack of conservation in the cellular contextual preference is the change in behavioral response that is often observed between Test1 and Test2 (Figure 8A). That would implicitly suggest a strong link between contextual preferences and behavioral outputs. In the next section, we thus wanted to explore the link that exist between contextual cells and behavioral readouts.

3. Quantitative and qualitative contribution of contextual neurons according to the behavioral performance.

Based on previous reports, a number of predictions can be tested to bridge the animal performance and the presence of specific cellular patterns. For example, contextual fear expressions – here measured as augmentation of freezing behavior in context A - has been previously linked to the establishment of 4Hz oscillations in the prefrontal cortex (Dejean, 2016). Even if the entrainment of cells in such oscillations must have variable effect on their mean spiking discharge, we can anticipate that expression of contextual

fear may generate specific activity profiles in D, D' and G sessions as compared to L sessions. Similarly, the neuronal activation in the safe context associated with contextual discrimination led to modulation of the PAG (Periaqueductal Gray) activity through their direct projections (Rozeske, 2018). As compared to above cited previous reports, we here have the interesting possibility to compare between various categories of behavior, as for the specific activity of contextual neurons that may be associated with. We first quantified the proportion of various categories of contextual cells according to the nature of the behavioral response (Figure 15). Quite surprisingly, animals that did not exhibit contextual freezing were not devoid of PFC A cells, showing rather an increase in their contribution (Figure 15A). We would have postulated that A cells being active during period of high freezing level, they could potentially have been associated with fear expression. Some results presented later on in the manuscript (Figure 29) suggest that there are indeed differences that exist between Low freezing and High freezing sessions, notably in the coupling between

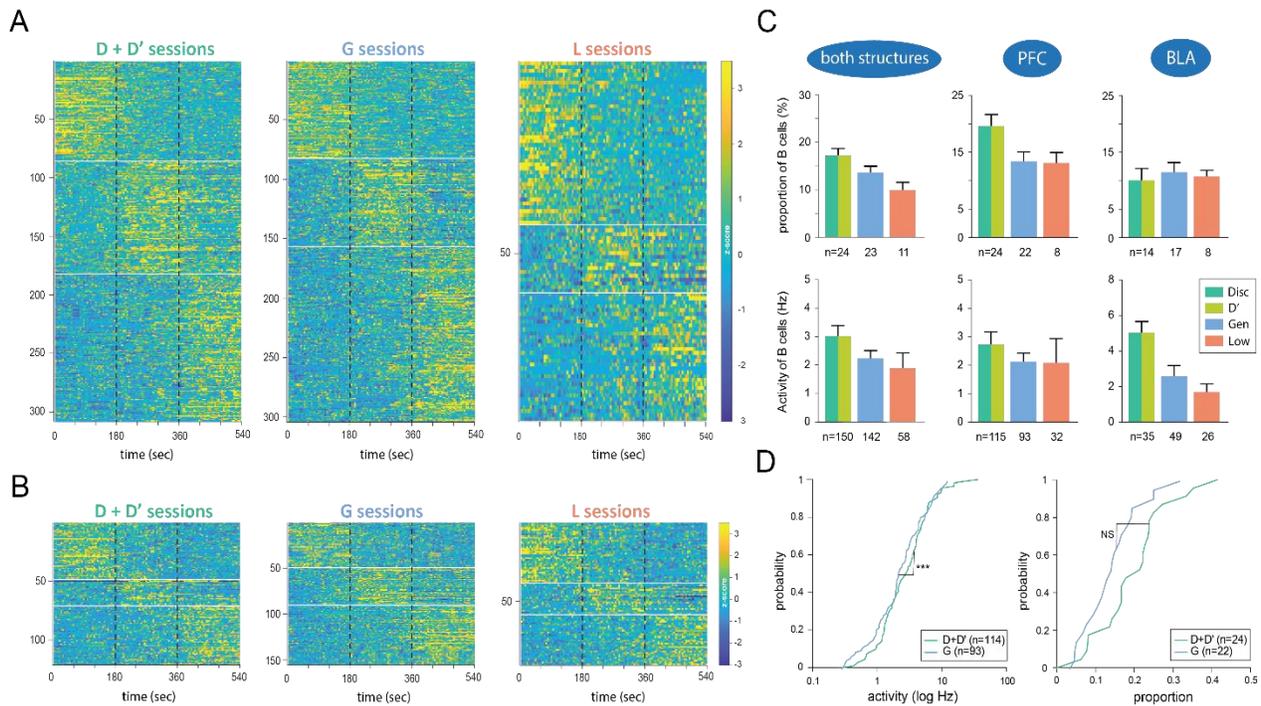


Figure 15: The presence of discrimination behavior is associated with higher presence and activity of B cells.

A. Normalized activity dmPFC A cells, B cells and A' cells and comparison of their respective proportion according to the behavioral outcome, i.e., between D/D' sessions (n=308), G sessions (n=304), and L sessions (n=93). In comparison, D/D' sessions show greater proportion of B cells compare to others. **B.** Same as above, but for the BLA. D/D' sessions (n=121), G sessions (n=155) and L sessions (n=90). **C.** Top: Proportion of B cells according to the behavioral performance, in both structures (left), in the dmPFC (middle) and in the BLA (right). Number of n at the bottom indicate numbers of each session type considered. Bottom: mean activity level of B cells (in frequency) in each behavioral session. Numbers at the bottom indicate the number of B cells considered. **D.** Left: activity of PFC B cells. B cells in D/D' sessions (n=114) are significantly more active than in G sessions (n=93) (two-sample Kolmogorov-Smirnov test: $p < 0.001$). Right: Proportion of B cells in D/D' sessions (n=24) are not significantly different from G sessions (n=22) (two-sample Kolmogorov-Smirnov test: $p = 0.1628$). Data are expressed as mean \pm SEM (***) $p < 0.001$.

the vHPC and the BLA, but here results suggest that presence of A cells is largely independent from the level of expression of contextual fear during exposition to threatening context. In contrast, our results confirmed the link between the quantity and quality of B cells and the contextual fear discrimination (Figure 15B). Indeed, we detected a significant increase in B cells activity in DD' sessions as compared to G sessions, in both the PFC and the BLA PFC (Figure 15C-D, activity level of DD' sessions were significantly elevated as compared to G sessions, two-sample Kolmogorov-Smirnov (KS) test, $p < 0.001$). For comparisons dealing with the contextual fear discrimination, we mixed D and D' sessions as they both contain this phenomenon (Figure 7). A strong tendency for an increase in the proportion of B cells compared to behavior groups is also present in the PFC (Figure 15C-D).

In general, even though more complementary analyses will be necessary to further identify meaningful contextual cells with regards to the behavioral outcome, for example by understanding their relationships within the local network, their incoming inputs and their synchrony with BLA neurons, we would like to propose that PFC B cells, as first demonstrated by Rozeske and collaborators, may play a specific role in controlling contextual fear discrimination. Nevertheless, a crucial or complementary role of other contextual cells could be envisioned, as contextual fear expression may also rely on activity of A or A' cells, that if their activity persists in the B context - therefore defined as AB and BA' cells - could enter in competition with B cells in controlling freezing behavior.

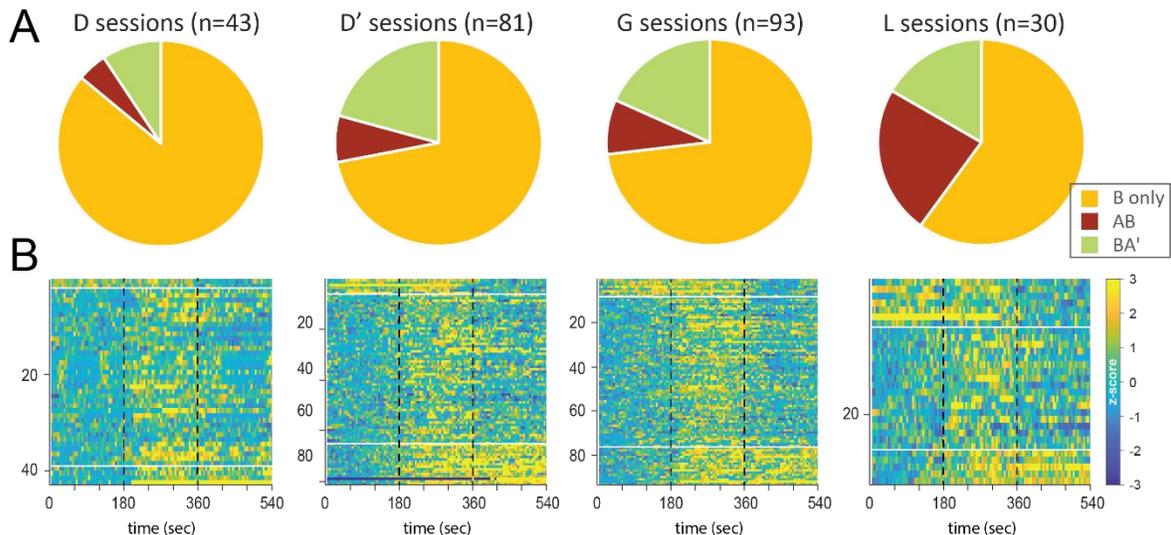


Figure 16: Precision of activity patterns of B cells in different behavioral groups.

A. Proportion of cells exclusively active in context B (“B only”), and cells active in context A-B and context B-A’ according to the behavioral performance. D sessions present the most prominent proportion of neurons exclusively active in context B, suggesting a possible link with the behavioral outcome. B. Activity pattern of AB neurons, B only neurons and BA’ neurons from the respective pie-chart above.

We directly tested if the precision of B cells activity patterns would explain the various types of behavioral sessions (Figure 16). In good accordance with a tight control of contextual fear discrimination by B cells, B cells in D sessions are found more exclusive than in D', G or L sessions that all showed less acute discrimination according to the threatening level of each context.

Finally, we carefully analyzed the behavior of PFC B cells in D' sessions, for whom the freezing level remained low while the animals were re-exposed to the "threatful" square context (Figure 17). As compared to the D sessions, the level of activity of B cells remained elevated, boosted by a higher number of BA' cells (Figure 16, 17B), but also contributed by the lack of attenuation of the firing of B cells in D' sessions (Figure 17C-E, two-sample KS test, $p < 0.001$).

To summarize, our analyses suggest that the activity pattern of B cells may determine the acuteness of contextual fear discrimination and the effectiveness of adapting behavioral response according to the threatening status of a given context.

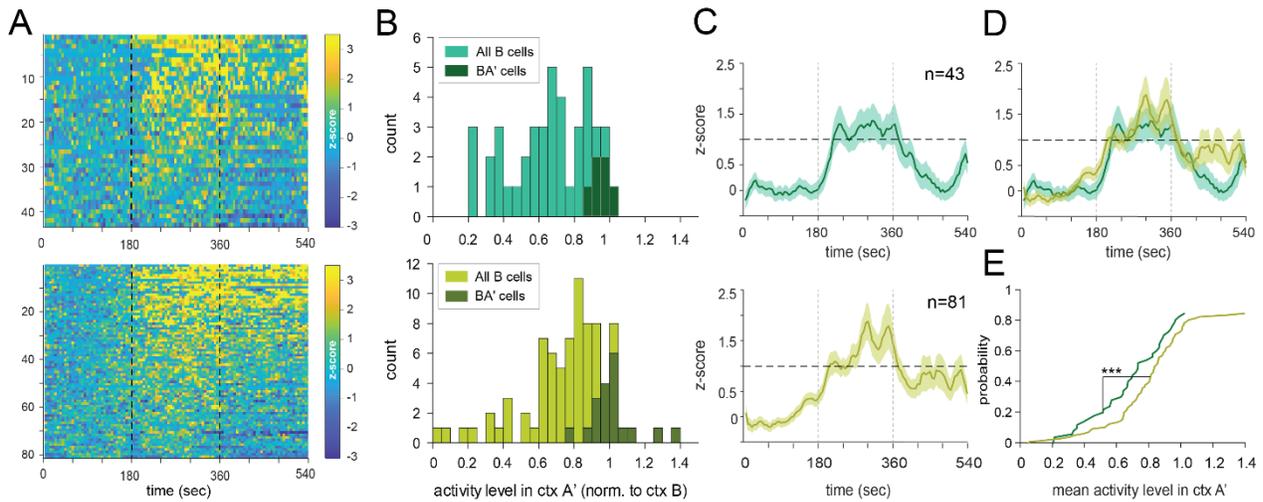


Figure 17: Precision of activity patterns of B cells in D and D' sessions.

A. Top: normalized activity of B cells in D sessions ($n=43$). Bottom: normalized activity of B cells in D' sessions ($n=81$). **B.** Activity level of B cells in context A' (normalized to activity level in context B) from D sessions (top) and D' sessions (bottom). D' sessions show remained activity in context A' as compared to D sessions, and present more neurons active during context B-A' ($n=19$ BA' cells in D' sessions, $n=6$ BA' cells in D sessions). **C.** Average activity of all B cells in D sessions (top) and D' sessions (bottom), with clear activity maintenance observed during context A'. **D.** Merged graphs from C. **E.** Average activity level while in context A' in D' sessions (light green, $n=81$) is significantly higher than in D sessions (dark green, $n=43$) (two-sample Kolmogorov-Smirnov test: $p < 0.001$). Data are expressed as mean \pm SEM (** $p < 0.001$).

4. Contextual cells activity modulation according to animal behavioral state.

Cells activated in a neutral context after threatful context exposition seems to be closely associated with the development of contextual fear discrimination (see above and Rozeske et al., 2018). However, nobody yet explored the reasons for their change in activity: Is this a consequence of the stop of the threatful information, do they receive a safety signal? Among the interesting questions we want to explore is the way by which the rising in activity of the B cells control the freezing behavior. Because PFC B cells seems to well correlate with the presence of discrimination, the next paragraphs will thus extensively explore the relationship between these cells and the freezing behavior.

At first, we wanted to better characterize the behavioral difference that exist between D/D' and G sessions. Interestingly, our analysis showed that in G sessions, freezing episodes did not evolve significantly between A and B context, their number (figure 18C, Student t-test, $p=0.305$) and duration (Figure 18A, B, D) remained comparable between the two contexts (Figure 18D, Student T-test, $p=0.758$, $n=23$). An exception however exists at the time of transition, at which a noticeable drop in freezing level is observed (Figure 7), probably the transition itself bringing a perturbation, thus interrupting the freezing behavior for a brief period of time. However, animals evaluate the situation and the freezing level was quickly reestablished. In contrast, as expected, a striking change in the structure of freezing behavior was observed between A and B contexts in D/D' sessions. In essence, the number of episodes between the two contexts maintained (Figure 18A, B, D, Student t-test, $n=0.221$), however their duration was significantly shorter in context B than in context A (Figure 18D, Student t-test, $p < 0.001$, $n=21$).

This suggested that rather than freezing initiation, the activation of B cells could facilitate the termination of the freezing episodes. This pushed us to further examine the coupling of B cell's activity with freezing episodes according to types of behavioral performance and context.

Among the interesting questions we aim to explore is the way by which the rising in activity of the B cells controls the freezing episodes, but also how contextual cells are shaped by freezing episodes. Indeed, strong modulation of neuronal activity by freezing episodes can generate context specific profiles, especially when freezing levels are changing abruptly, i.e., in discriminative sessions. In this situation, A cells "profiles" can be artificially generated if cells happen to be positively modulated by freezing - or negatively modulated by mobility - as freezing that is higher in context A than in context B. Similarly, B cells "profiles" can be generated for cells that would be inhibited by freezing – or activated by mobility –

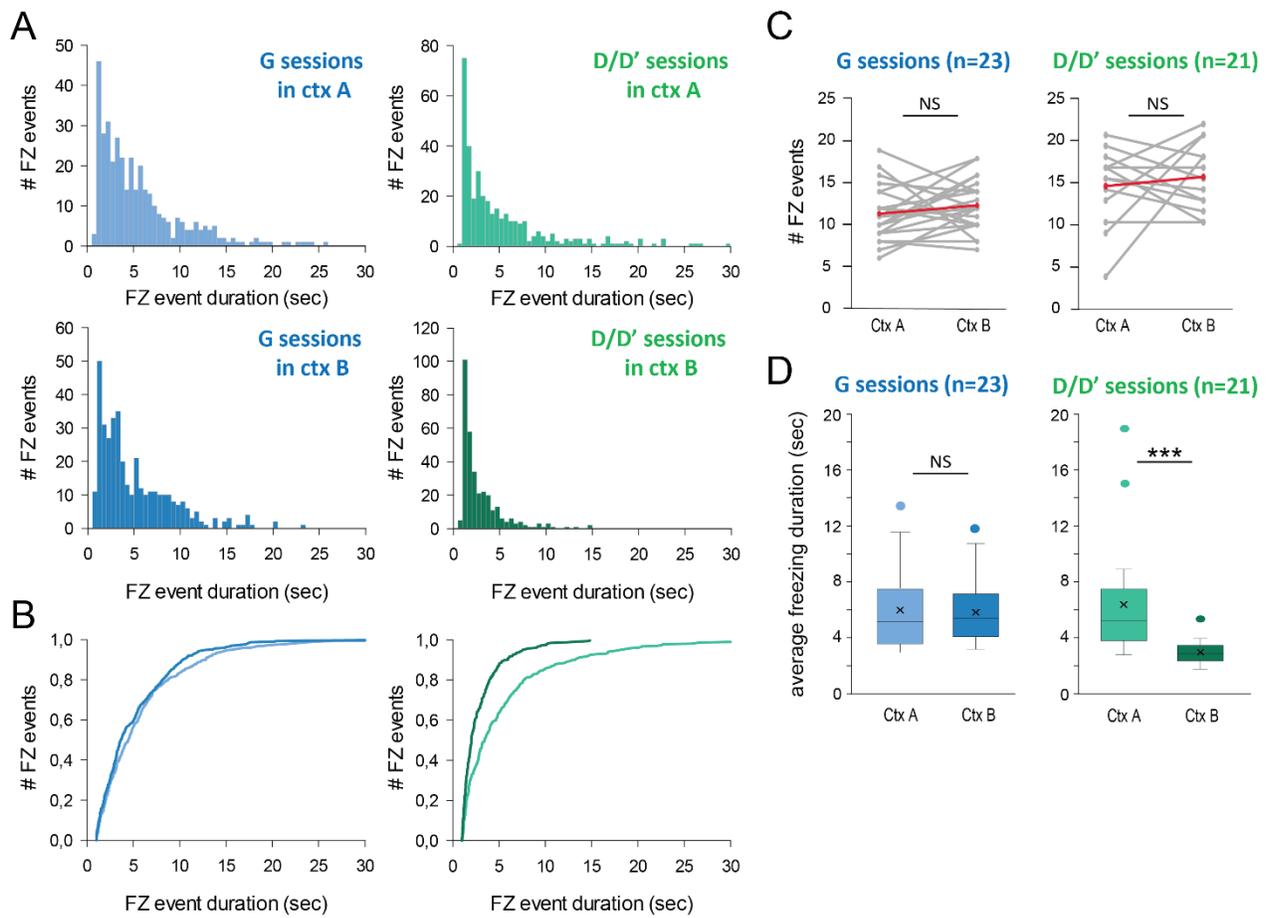


Figure 18: Behavioral description of freezing behavior.

A. Left: Numbers of freezing events of various durations in context A and context B in G sessions (n=23). Right: numbers of freezing episodes in context A and context B in D/D' sessions (n=21). **B.** While in G sessions, there is no difference in distributed durations of freezing episodes between context A and B, D/D' sessions exhibit longer duration of freezing episodes in context A (light green) than in context B (dark green). **C.** Numbers of freezing events between context A and context B is not different for both D/D' (Student t-test, $p=0.221$) sessions and G sessions (Student t-test, $p=0.305$). **D.** D/D' sessions exhibit in average significantly longer freezing episodes in context A compared to in context B (Student t-test, $p<0.001$), while G sessions exhibit freezing episodes of equivalent duration between the two contexts (Student t-test, $p=0.758$). Box-whisker plots indicate median, interquartile range, and 5th-95th percentile of the distribution. Crosses indicate the means. (** $p<0.001$).

if the animal freeze less in the B context. All these effects would be independent on the context, but rather linked to the freezing behavior or to a correlation with locomotion.

To get insights on the crosstalk between freezing and contextual cell's activity, we thus concentrated our attention on the relative activities of recorded cells in context A and context B, this in various behavioral groups, and correlated their activity separately in freezing (FZ) and mobility periods (MB). Interestingly, results from our first analyses go against the above-mentioned statement that contextual cells would

originate in changes in freezing levels. In Figure 19, we compared the ratio of activity in FZ/MB periods between each cell type. First, all categories of contextual cells did exhibit very similar activity ratio distributions between freezing and activity periods, suggesting that they are not specifically biased by freezing episodes (Figure 19).

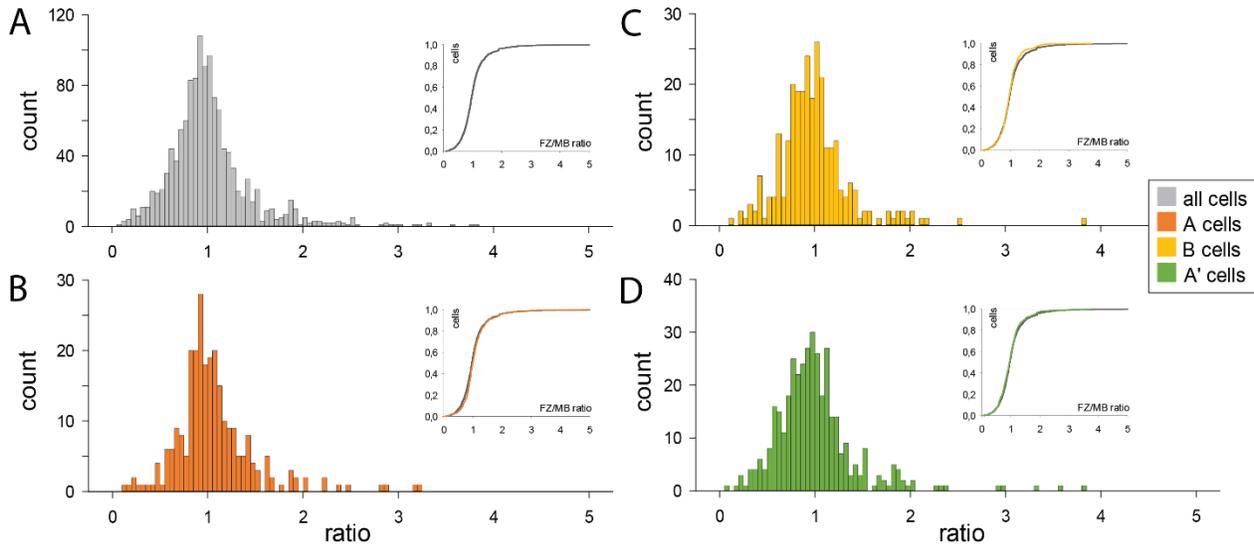


Figure 19: No specific effect of freezing episodes on a particular contextual cell's population.

A. Activity ratio between freezing and mobility periods (FZ/MB ratio) for PFC non-contextual cells (n=466). **B.** Activity ratio in PFC A cells (n=264). **C.** In PFC B cells (n=273). **D.** In PFC A' cells (n=384). Insets show the cumulative probability of each contextual cell in comparison to non-contextual cells (superimposed).

Second, to test for a scenario of creating “false” contextual cells due to changes in freezing levels, we compared the distribution of B/A activity ratios in all contextual cell’s categories in periods of freezing and mobility (Figure 20). Results presented showed that the vast majority of A cells (Figure 20, second row) and B cells (Figure 20, third row) kept their identity in both behavioral states: in mobility, 83% of A cells have greater activity in context A than in context B (and 80% of A cells during freezing events). On the other hand, B cells showed 89% and 78% of activity preference for context B during mobility and freezing periods, respectively. Both showed significant difference in this preferential activity on a given context, as compared to non-contextual cells that show – as expected - a homogenous ratio distribution in both periods (two-sample KS test: A cells vs. non-contextual cells, $p < 0.001$ (both in mobility and freezing), and B cells vs. non-contextual cells, $p < 0.001$ (both in mobility and freezing). Lastly, A' cells show preference for the context B, effect that is mainly explained by the presence of the non-exclusive BA' cells (Figure 20, last row). In the same line, a part of the borderline cases observed in A cells and B cells was contributed by non-exclusive contextual cells (AB cells, indicated in black bars). Globally, distribution curves showed

that contextual cells massively retain their contextual preference, independent on the behavioral – freezing or mobility - state of the animal.

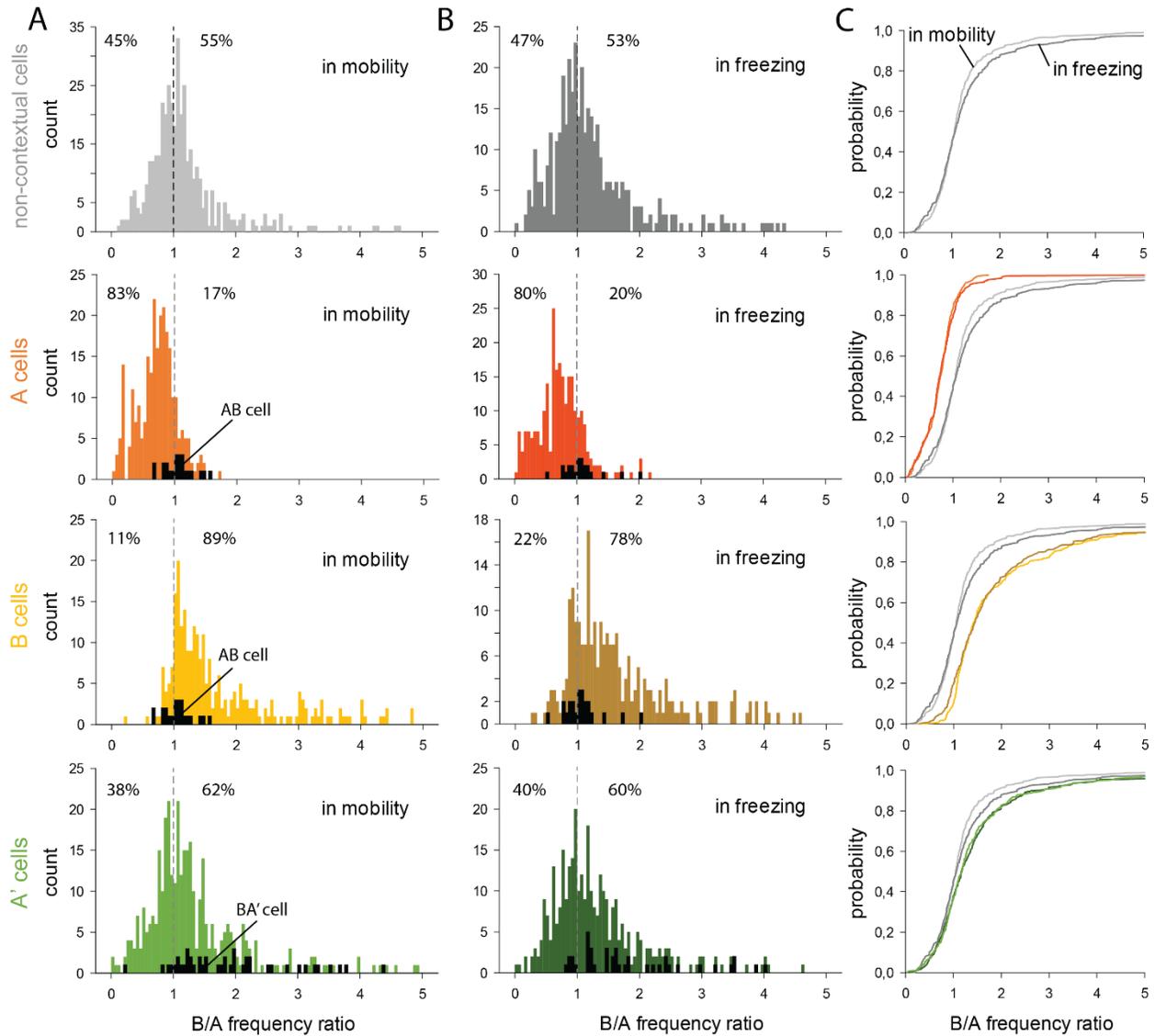


Figure 20: Contextual cells display contextual preference independent on the behavioral state.

A. Frequency ratio in context B/A during mobility periods, for non-contextual neurons ($n=466$, in gray), for A cells ($n=264$, in orange), for B cells ($n=273$, in yellow), and for A' cells ($n=384$, in green). A ratio that is >1 indicates preferential neuronal activity in context B, whereas ratio <1 indicates preferential activity in context A. A cells conserve their preferential firing in context A during mobility (83% with ratio <1), and B cells in context B (89% with ratio >1). Percentages indicate the proportion of cells displaying a preference in context A (B/A ratio below 1) or in context B (B/A ratio above 1). **B.** Same as in A, but during freezing episodes (non-contextual cells in dark gray, A cells in red, B cells in brown, and A' cells in dark green). **C.** Distribution of frequency ratio compared between mobility and freezing periods, in order, non-contextual cells, A cells, B cells and A' cells. The 2 gray curves conserved in all graphs are from non-contextual neurons. Two-sample KS test compared pair of curves indicated with arrows (ctx cell vs. non-ctx cells in mobility; ctx cell vs. non-ctx cell in freezing), for all pairs considered difference was significant, $p<0.001$.

The notion of contextual cells is complex, as context preference can be consecutive to various reasons. Among them, the threat/safety perception is of importance as we postulated that it is somehow encoded and represented within the PFC, especially by some neuronal populations displaying contextual preference such as B cells (as shown in Rozeske et al., as well). We therefore tested if B cells would behave

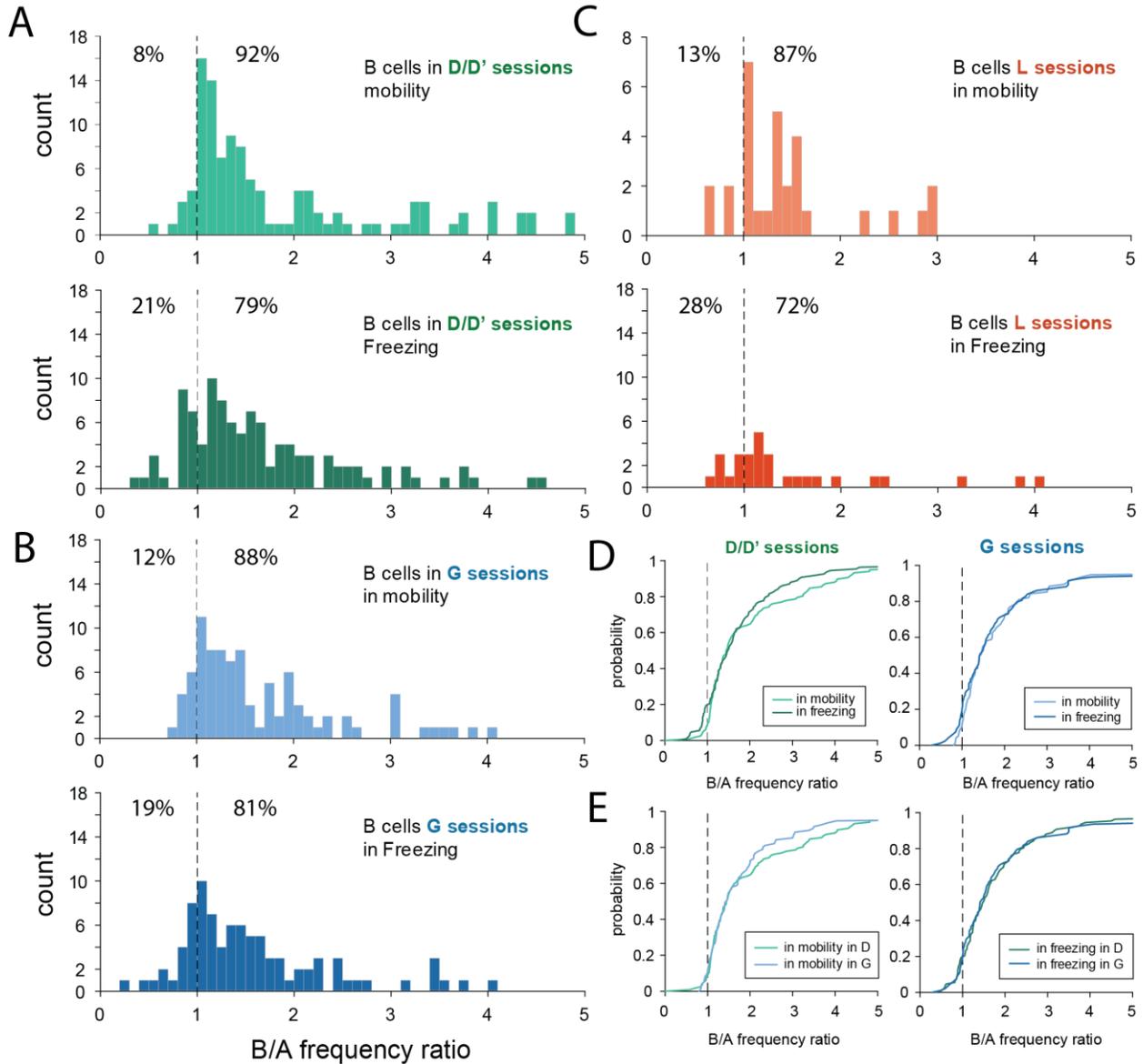


Figure 21: Contextual B cells in discriminative sessions display contextual preference independent on the behavioral state. 21

A. Context B/A frequency ratio of PFC B cells in D/D' sessions (during mobility (top, n=111) and freezing (bottom, n=105) episodes. **B.** Same representation as in A for G sessions (during mobility (top, n=91) and freezing (bottom, n=88) episodes. **C.** Same representation as in A for L sessions (during mobility (top, n=30) and freezing (bottom, n=29) episodes. In A, B and C, percentages indicate the proportion of cells displaying a preference in A (B/A ratio below 1) or in B (B/A ratio above 1). **D:** cumulative plots of B/A ratio distributions in D/D' sessions (left) and G sessions (right). Two-sample KS test: $p < 0.001$ in DD' sessions and $p < 0.001$ for G sessions. **E:** cumulative plots of B/A ratio distributions in mobility sessions (left) and during freezing episodes (right). Two-sample KS test: $p < 0.001$ in mobility and $p < 0.001$ during freezing. (***) $p < 0.001$

differentially to freezing events in discriminative (D and D') and generalizing (G) sessions, and compared contextual preference of B cells during these periods in various behavioral groups (Figure 21).

Interestingly, an effect of freezing was detectable on B cells from the discriminative sessions that was not visible in other groups. As shown in the Figure 21A, the contextual preference of B cells in D/D' sessions is larger in mobility than in freezing (Figure 21D, left, two-sample KS test, $p < 0.001$). Interestingly, this observed difference when directly compared to contextual preference of B cells in G sessions, was solely visible in periods of mobility but not during freezing episodes (Figure 21E, left, two-sample KS test, $p < 0.001$). Indeed, G sessions did not show any change in context preference during mobility, at least visually (Figure 21D, right and Figure 21E, right). However, from the statistical tests performed, the difference within group and when compared to DD' sessions, was significant. Because curves are really superimposed, we attributed this to a misconducted statistical evaluation that improved the sensitivity of such statistical tests. With that said, we suggest that B cells in discriminative sessions are particular and

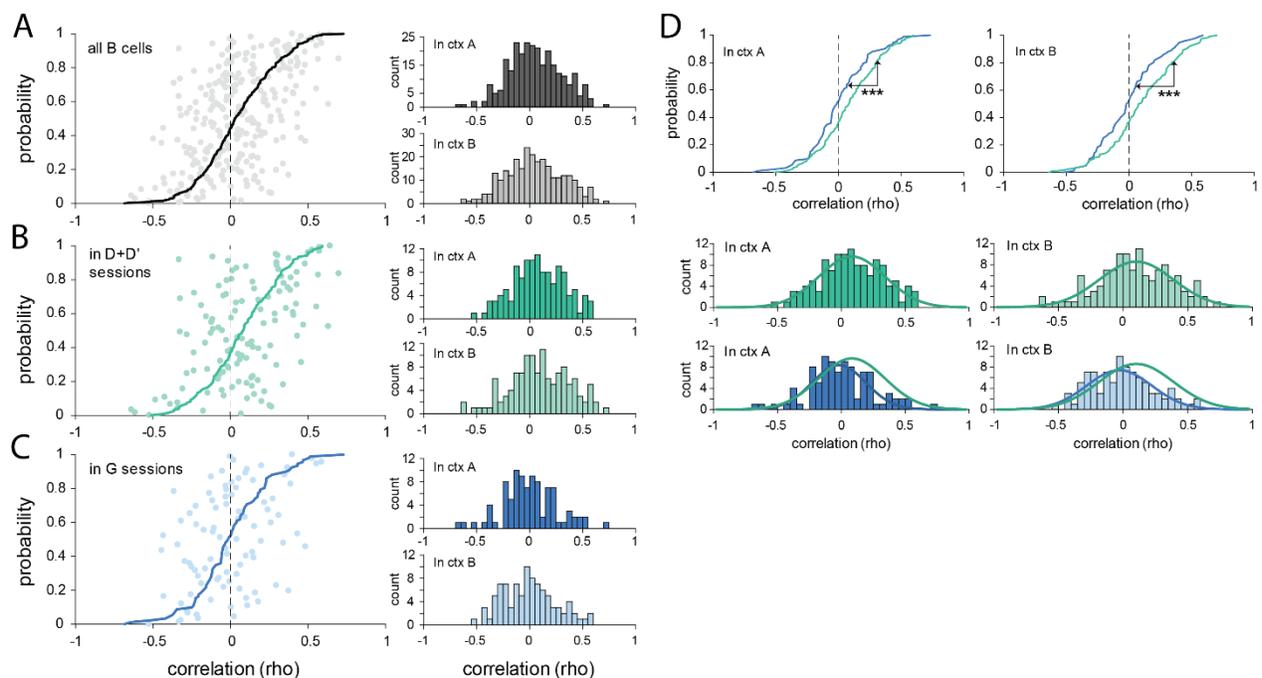


Figure 22: Correlation of B cells with locomotion in various behavioral conditions.

A. Correlation of B cells activity with animal locomotion was determined in context A and context B ($n=272$). Cells were ranked according to their correlation in A (dark line, cumulative distribution) and results in B are presented as dots. Right: histogram distribution of Pearson correlation coefficient in each context. **B:** same presentation as in A, but for B cells in D/D' sessions ($n=123$). **C:** same presentation as in A, but for B cells in G sessions ($n=93$). **D:** Cumulative (Top) and histogram (bottom) distributions of B cells correlation to locomotion in context A (Left) and context B (Right). Gaussian fits have been added to better visualize the right shift of B cells in both contexts (D/D' sessions in green, and G sessions in blue, two-sample Kolmogorov-Smirnov test: $p < 0.001$ in context A and $p < 0.001$ in context B).

get a part of their contextual preference independently from animal state. Finally, when it comes to L sessions, the number of B cells is unfortunately too low (n=30 during mobility and n=29 during freezing) to draw any conclusions at this stage.

In the case of discriminative sessions, a gain of context B preference during mobility periods could be the result of a slightly better coupling to animal locomotion. We thus directly compared the correlation to locomotion of B cells in D/D' and G sessions in both context A and B (Figure 22). Interestingly enough, we detected a slight right shift of correlation distribution for D/D' sessions (Figure 22B). When compared to G sessions, the shift to the right is significant (Figure 21D, two-sample KS test: $p < 0.001$ both in context A and in context B) indicating that B cells indeed get a stronger modulation by locomotion. However, this shift was already present in the context A suggesting that it was pre-existing prior to the contextual modulation of cells activity.

Thus, in summary, our analysis shows that i) B cells did not display a contextual preference because of the animal state (freezing or mobility), ii) their degree of contextual preference is higher in D/D' sessions, and iii) that their correlation to locomotion is higher in case of discrimination, a specific relationship that is yet observed in context A. This last point would potentially suggest that animals that will display discrimination are already in a different brain state than ones that will show generalization.

5. Contextual cells modulation during freezing and mobility reflect animal discrimination capacity.

One hypothesis is that B cells mediate discrimination by terminating the freezing episodes. Indeed, the contextual fear discrimination is exemplified by a decrease in the duration but not the number of freezing episodes, suggesting the reinforcement or the appearance of a termination signal. Our hypothesis is that this can be contributed by PFC contextual cells, especially the B cells that are showing higher activity rate in the safe context. In discriminative sessions, these B cells must have a particular time course in relation to the freezing episode. We tested this hypothesis by analyzing the activity profile of contextual cells in various behavioral groups and in the threatful and neutral context.

We first wanted to determine if the contextual specificity did confer a specific relationship of cells with freezing. For this, we combined all types of contextual cells (ctx cells in Figure 23, in green) and compared their activity changes during freezing episodes to the ones of non-contextual cells (non-ctx cells in Figure 23, in gray). Interestingly, Contextual cells exhibited higher rate of activity as compared to non-contextual cells (Figure 23C), and are in average slightly more down-regulated during freezing episodes. However,

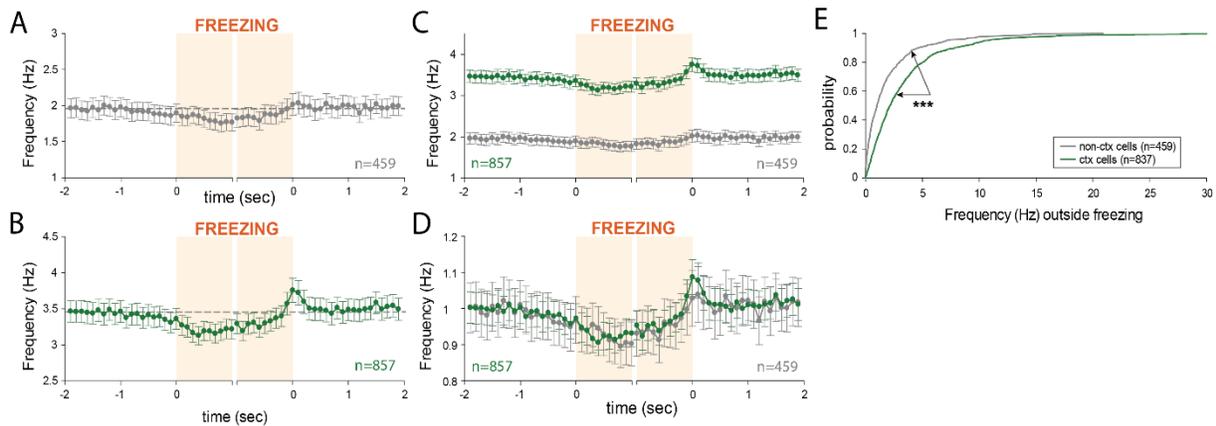


Figure 23: Contextual cells distinguish from non-contextual cells by their activity rate and response to freezing episodes. Freezing episodes have been extracted and activity of cells before, during and after the freezing episodes have been determined. Because freezing episodes are of different duration, only the first and the last second of freezing are shown (light orange box). **A.** mean spiking frequency around freezing episodes for cells with no particular contextual preference. **B.** Same presentation as in A, but for contextual cells. **C.** Same graphs as in A and B, but presented together, allowing to appreciate the difference in activity between the two groups. **D.** Same graph as in C, but with frequency normalization during the period preceding the freezing episode. **E.** Cumulative plot showing the difference in basal activity of contextual (dark green) and non-contextual cells (dark grey). two-sample Kolmogorov-Smirnov test: $p < 0,001$. Data are expressed as mean \pm SEM (***) $p < 0,001$.

when scaled to their initial frequency (Figure 23D), both cell types showed similar time course during freezing episodes, with an initial decrease and a ramp of activity rise preceding the end of the freezing episode. In the case of contextual cells, a rebound of activity is observed at the end of the freezing episode that is less present in profiles from non-contextual cells (Figure 18E, two-sample KS test, $p < 0.001$).

One intriguing possibility is that contextual cells in the PFC are controlling the duration of the freezing episodes. To test for that, we systematically compared the activity profiles of contextual cells during freezing episodes occurring in context A, B and A', this between D/D' sessions and G sessions (Figure 24). As expected, when compared, basal frequency of each type of contextual cells was greater in their preferential context, but some noticeable differences were associated with contextual discrimination: First, the A cells, are more active in the G sessions than in D/D' sessions (Figure 24A). We can hypothesize that they would engage the PFC network in a state that is reluctant to discrimination. Second, B cells in D/D' sessions are strongly inhibited by freezing episodes as compared to B cells in G sessions (Figure 24B). Their activity is also strongly enhanced at the end of the freezing episodes in almost all context (Figure 24). Notably, in G sessions, B cells well display their preference for context B (higher basal activity in context B compared to other contexts), but did not exhibit any particular modulation by freezing, again confirming that B cells in G and DD' sessions are behaving differently. In contrast to A and B cells, A' cells

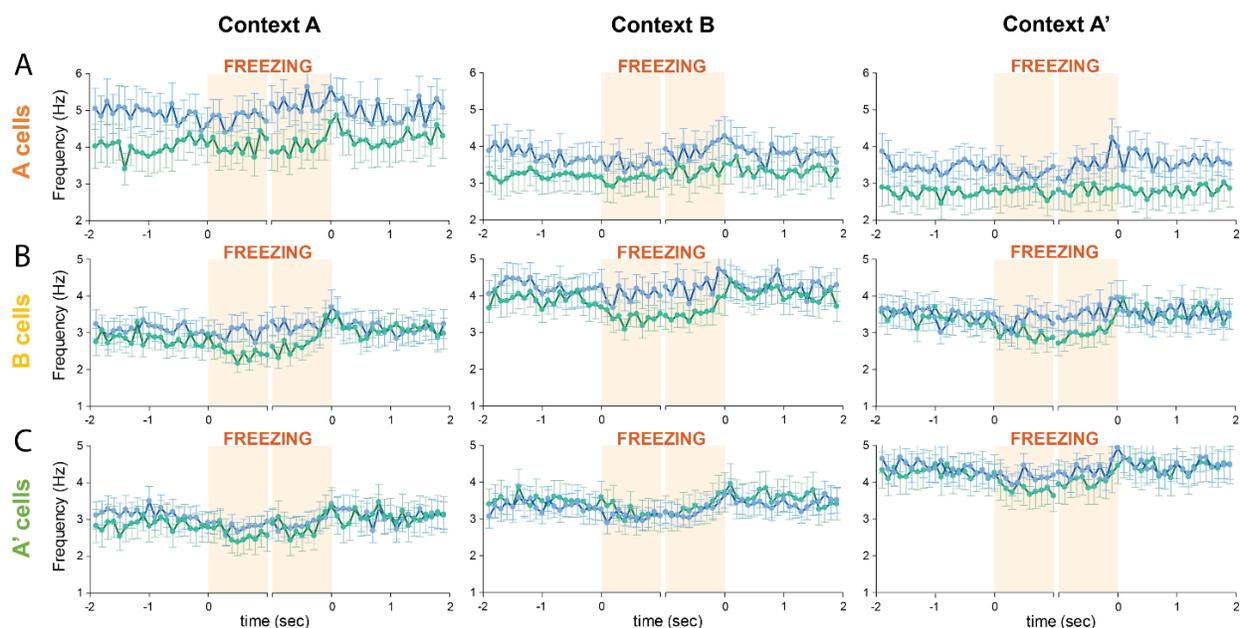


Figure 24: A and B cells have activity modulations that are depending on behavioral outcomes.

Same presentation as in figure 18. Freezing episodes have been extracted and activity of cells before, during and after the freezing episodes have been determined. Because freezing episodes are of different duration, only the first and the last second of freezing are shown (light orange box). **A.** mean spiking frequency around freezing episodes for A cells in context A (Left), context B (middle) and context A' (Right). B cells from G sessions are in blue (n=105), and those from D/D' sessions in green (n=97). **B.** Same presentation as in A, but for B cells (n=97 in G, and n=122 in D/D' sessions). **C.** same presentation as in A, but for A' cells (n=166 in G, and n=148 in D/D' sessions). Data are expressed as mean \pm SEM.

did not seem to differ according to behavioral outcome, being constantly slightly inhibited by freezing independently to context (Figure 24). To better appreciate the difference between B cell's relation to freezing in G and D/D' sessions, we normalized the frequency of cells during the freezing episode and reported their activity immediately before and after the freezing episode (Figure 25). It is clearly visible that the coupling to freezing is only affected by the session type, but that it is already present in context A, a context in which animals do always strongly exhibit conditioned fear responses (Figure 25A-B).

An interesting prediction would then be that the degree to which B cells are inhibited by freezing in context A may predict the degree of which discrimination would occur in context B. We tested this hypothesis by reporting the repartition of B cells modulation by freezing in context A and the level of freezing in context B (Figure 25C). To be able to compare between cells of different frequencies, we normalized all frequencies to the one observed when the freezing behavior was established. Interestingly, a strong correlation exists between the negative coupling to freezing of B cells in context A and the appearance of a discriminative behavior in context B. If there is a little overlap between the behavioral

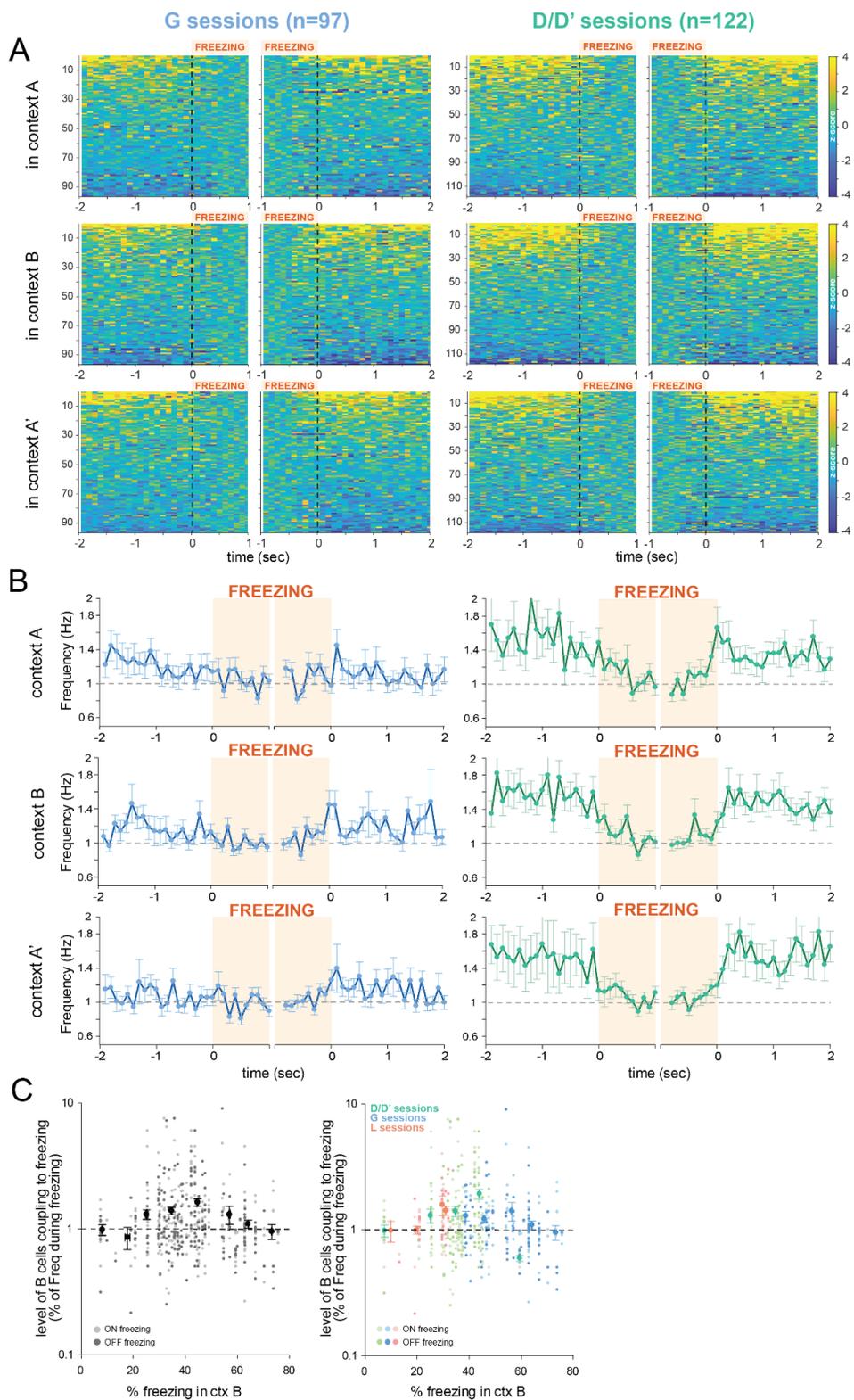


Figure 25: B cells have freezing-dependent modulations that are associated with discrimination.

A. Activity of mPFC B cells ranked at the -2 to 0 sec period before the freezing episode (Left) and by the 0 to 2 sec period after the freezing episode (right). Conditions are indicated, and frequencies were normalized to frequencies during freezing. **B.** Same data but averaged to allow direct comparisons. Note the difference in the modulation of B cells in G sessions (n=97) and D/D+ sessions (n=122) is independent on the context. **C.** To test if modulation of B cells by freezing can predict the appearance of discrimination behavior, we plotted the relationship between the % of freezing in the B context and of the ratio Freq MB/FZ of the B cells at the beginning (ON) and the end of the freezing episodes (OFF) of all sessions (Left) or color coded by session types (Right). Data are expressed as mean \pm SEM.

groups, that could be partially explained by possible discrimination still present in “high” freezing L sessions and “low” freezing G sessions. Together this result suggests that an increase in the negative coupling to freezing of B cells is a signature of a potential to elicit contextual fear discrimination. Interestingly, this negative coupling is present already in context A, at a time when B cells displayed low activity rate, but is exacerbated - in absolute value - when these cell’s activities will be boosted in context B.

6. Hippocampal modulation of contextual neurons during fear memory consolidation.

An important part of the project was to determine the physiological mechanisms that would be at the origin of the establishment of contextual fear conditioning and discrimination. We initially organize the behavioral paradigm to record not only neuronal activities during the testing sessions, but also during the sleep phases, with the aim of exploiting the natural coupling between the vHPC and cortical areas that occurs during slow wave sleep (SWS) ripples oscillations. To this aim, 4 electrodes were positioned in the CA1 region of the vHPC to record hippocampal ripples (see methods). Ripples were identified, and time-stamped to test for eventual changes in the mPFC and the BLA unitary activities around ripples. Previous reports and observations including ours suggested that because of the high synchronicity and internal frequency of ripples, they could be considered as robust trains of presynaptic activity for hippocampal axons impinging on cortical targets, thereby providing monosynaptic excitatory drive that can be isolated temporally from secondary – inhibitory - responses. Given that, to the same ripples isolated from the vHPC, were correlated activity recorded from two different brain regions (mPFC and BLA).

We first compared the responses observed in BLA and mPFC (Figure 26). Interestingly, this direct comparison goes well with the idea of a local response to an incoming, coherent excitatory drive: in both regions, the principal response rises and extinguishes within 100 msec, building up on a slower response that starts earlier (see introduction for more details, Figure 26). Interestingly, the major phasic response

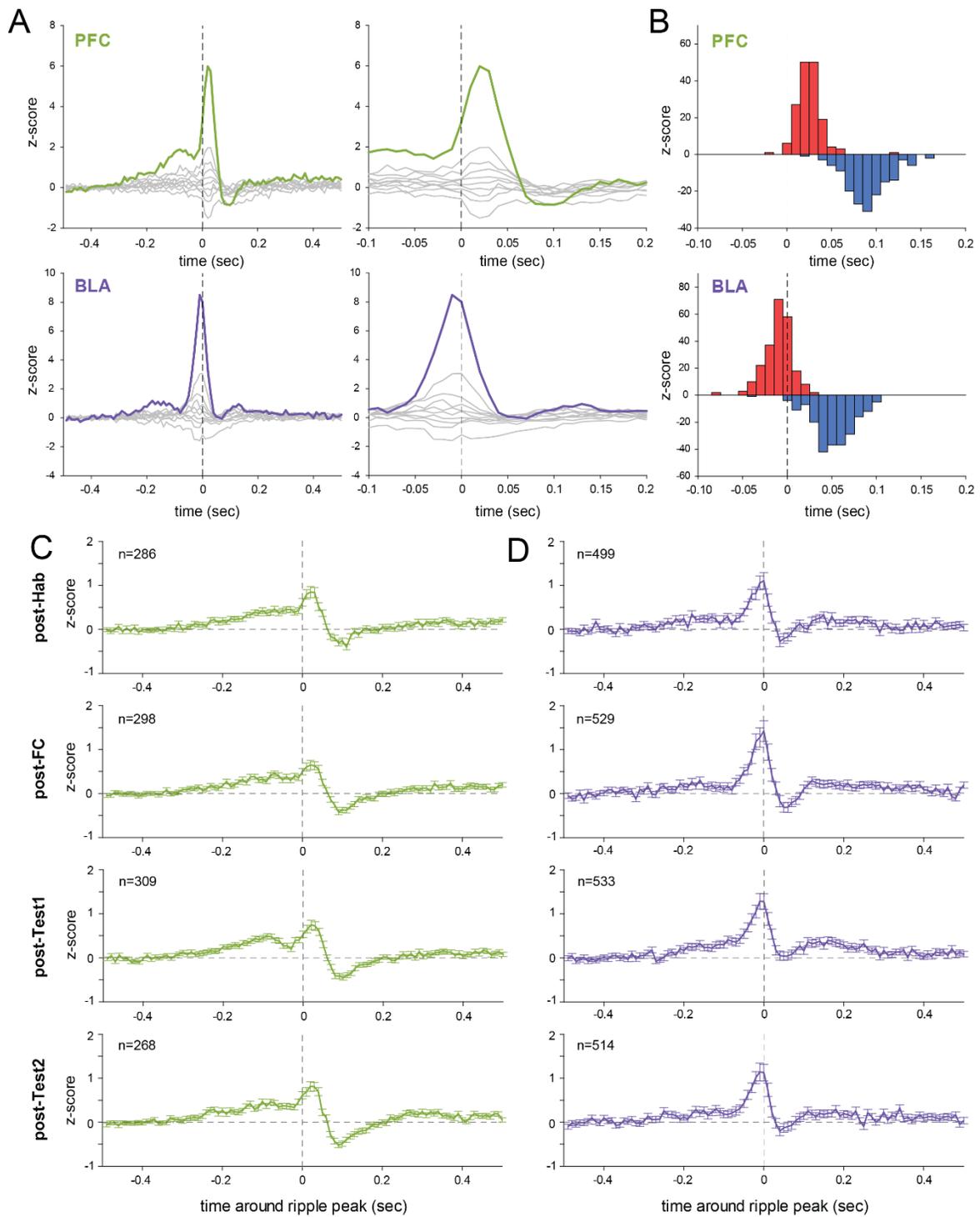


Figure 26: Characterization of PFC and BLA responses to ripples along the CFC paradigm.

A. ripple-mediated responses in the mPFC and the BLA were collected and grouped by their Z score at +10 to +30 msec (PFC) or -20 to 0 msec (BLA). The time 0 corresponds to the ripple peak. The 10% higher responses were averaged and their mean highlighted in colored lines. **B.** the timing of the positive (red bars) and negative peak (blue bars) were collected for the 10% higher responses, illustrating the excitatory/inhibitory sequences that are conserved, although being delayed in the PFC to about 30 msec. **C and D.** Averaged Z-score for all recorded PFC (Left, C) and BLA (Right, D) cells in the various sleep sessions. The number of recorded cells is indicated at the top left corner. Data are expressed as mean \pm SEM.

displayed significantly longer delays for the mPFC than the BLA, with the peak of response observed at 10 to 30 milliseconds after the ripple peak (Figure 26A-B, in green), whereas at -20 to 0 milliseconds for BLA cells (Figure 26A-B, in violet). In addition, average responses recorded during the 4 consecutive resting periods were quite stable with days (Figure 26C-D, see also Figure 14).

However, as shown in the Figure 27, one has to be precautionous when directly comparing the amplitude of average responses expressed as Z-scores, as they strongly depend on the number of ripples that are considered. Indeed, if the shape of the response is maintained among a large range of ripple numbers, the response amplitude is deeply affected, being larger when more ripples are included, due to the decrease of the variance in basal activity (Figure 27A and B). In our dataset, for a yet unknown reason, but that can be due to the CFC protocol - specific effect of learning on the number or frequency of ripples within SWS - we observed that the number of ripples was slightly lower after habituation than in the other sessions (Figure 27D), a phenomenon that can affect Z-score amplitudes. However, when considering relative changes based on the same ripple detection dataset, for example comparing BLA and PFC responses within a single recording session, no correction was needed (Figure 27C) as the same ripples are considered.

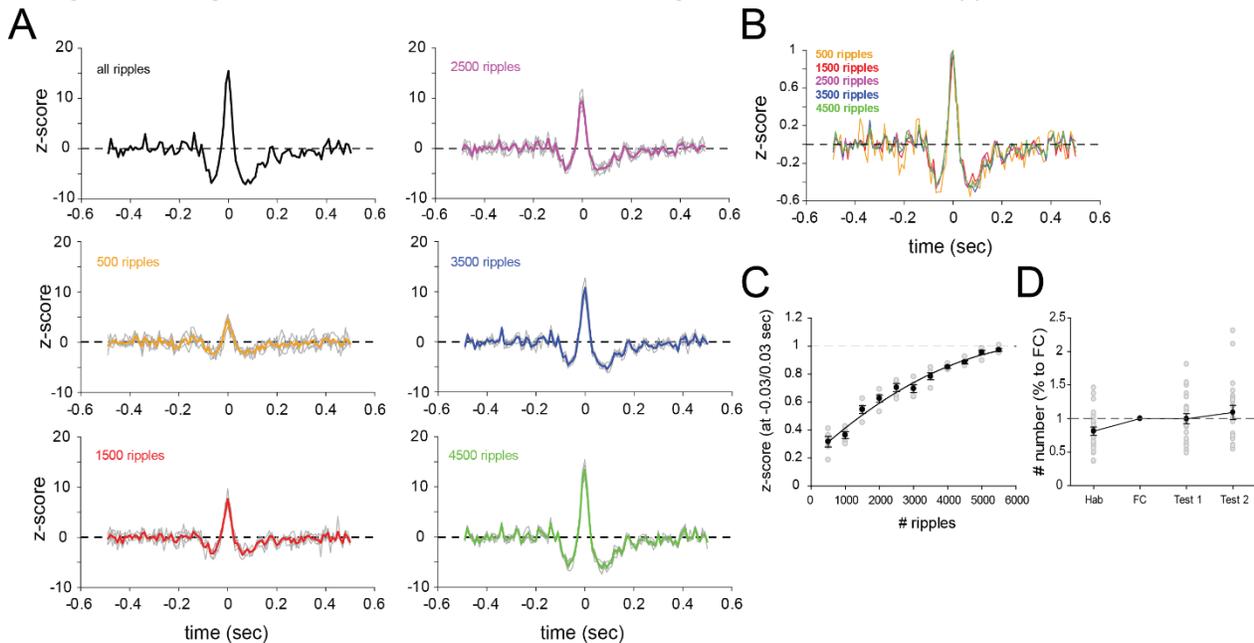


Figure 27: Effect of ripple number on cortical response amplitude and time-course.

A. ripple-mediated responses for a mPFC unit that is produced if considering a different number of ripples. A randomized choice of ripples has been performed 5 times for each condition (grey lines), and the average time course is shown as a colored line. The number of considered ripples is indicated. **B.** all obtained profiles have been normalized to their maximum to allow comparing their time course. **C.** Relation between the ripple number and the calculated Z-score showing the strong dependency of Z-score measure on the contributing ripples. This relationship is likely to depend on the initial frequency on each unit. **D.** The detected ripples number have been plotted for each sleeping session (grey dots) and the average calculated (black dots). A tendency to an increase in the ripple number is observed, possibly due to CFC learning. Data are expressed as mean \pm SEM.

Next, we wanted to test if variations in the hippocampal coupling would be predictive of the development of either contextual fear or contextual fear discrimination. For this purpose, we compared the response of BLA and mPFC neurons to ripples along the CFC procedure by splitting the data according to behavioral groups as defined in Test 1 (Figure 28). To our surprise, if the average ripple response was very coherent and homogeneous in the PFC, with no major differences observed between D/D', G and L sessions, the responses observed after FC in the G group in the BLA appear to be larger than in the other groups (Figure 28A). The responses observed in D/D' session in Test 1 (i.e., post-FC sleep) are somehow intermediary, laying between L and G groups (Figure 28B). Thus, it is possible that the strength of vHPC → BLA projections would constitute a key determinant for the expressions of contextual fear. Strikingly, this difference was already present before the CFC procedure, suggesting that animals may not be equivalent in their susceptibility to elicit contextual fear. In case of generalizers, it can be also postulated that excessive activation of BLA cells by vHPC inputs lead to pathological fear, as being expressed in a “safe” context.

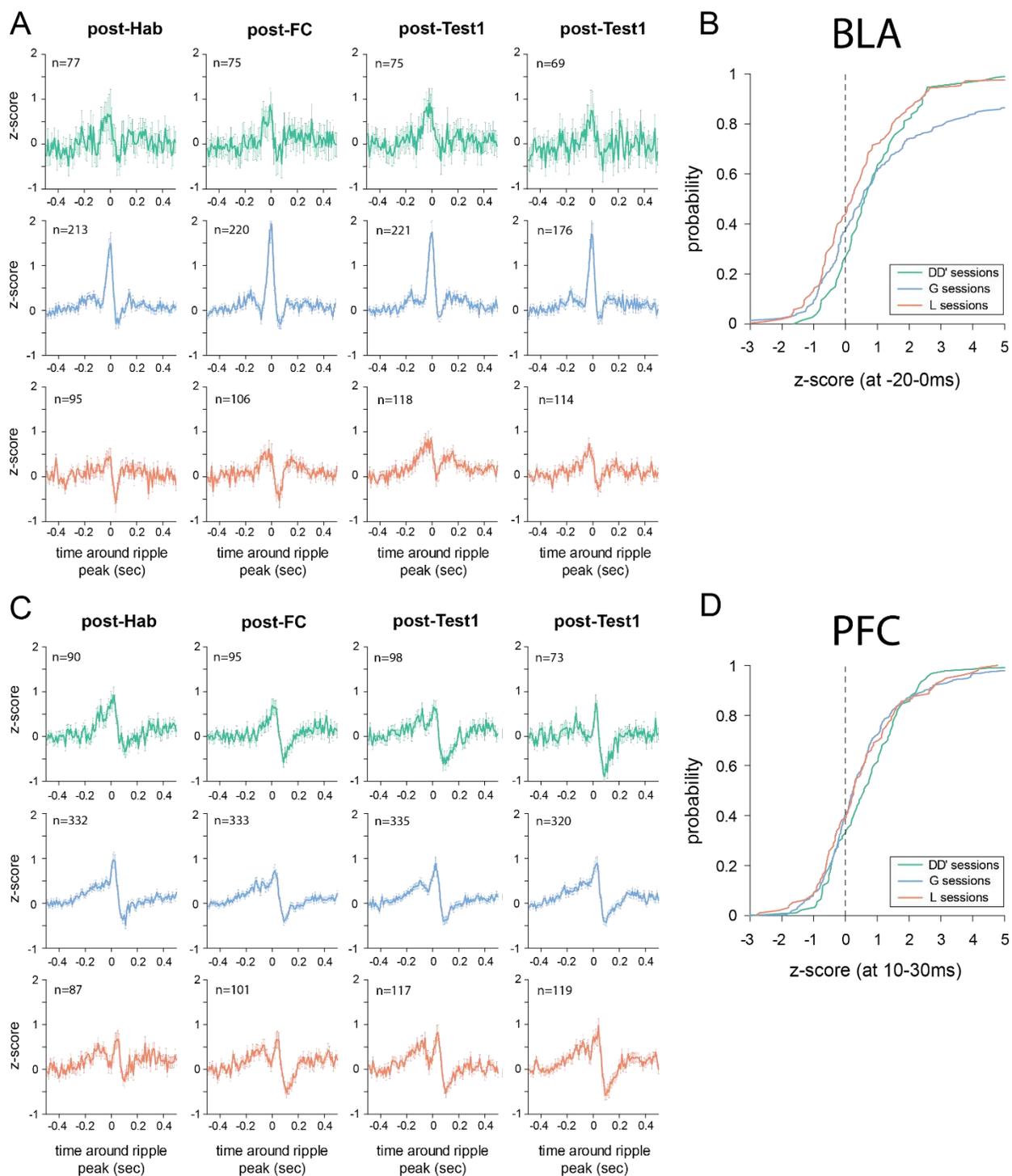


Figure 28: Expression of contextual fear is associated with a larger connectivity between the vHPC and the BLA.

A. Average responses of BLA cells aligned to detected ripples in the four recorded resting sessions of the various behavioral groups: D/D' in green, G in bleu, and L in red. The number of contributing cells is indicated. **B.** The same data are shown as a cumulative plot of Z-score peaks. two-sample KS test: $p < 0,001$ between G and DD' sessions; $p < 0,001$ between G and L sessions, however $p = 0.39$ between DD' and L sessions. **C and D.** same presentation as in A and B for PFC neurons. Two-sample KS test: $p < 0,001$ between G and DD'; $p < 0,001$ between G and L; $p = 0.63$ between DD' and L. Data are expressed as mean \pm SEM.

Finally, following the simple idea that cells that will have an increased firing rate in a specific context must be under direct contextual control by vHPC, we compared the responses between A, B and A' cells along the CFC procedure in both the mPFC and the BLA (Figure 29). We also wanted to test if a specific level of vHPC coupling would confer a particular contextual pattern to the cells. We therefore classified PFC and BLA cells according to their contextual pattern in Test 1 (see previous Figures in results part 2) and measured their responses to vHPC ripples in sleeping periods following Hab, FC and T1. Given the high turnover of contextual patterns between T1 and T2 (Figure 12), we did not consider T2 results at this stage.

Our results were very reminiscent from what was obtained by group separation: in the mPFC, no difference was observed between cell categories after FC (Figure 29B). When compared to non-contextual neurons, B cells did not show any significant difference (two-sample KS test, $p=0.218$). A and A' cells showed better coupling to vHPC ripples, as compared to non-contextual neurons (two-sample KS test, $p=0.02$ and $p=0.0016$, respectively). Globally, the monosynaptic coupling with vHPC did not seem to clearly define contextual patterns of PFC cells. In contrast, BLA A cells appeared to be more responsive than others to vHPC ripples (Figure 29D, two-sample KS test, $p<0.001$), a phenomenon that was again yet existing before fear conditioning (Figure 29A). In general, cells with a contextual pattern do exhibit a slightly higher coupling to the vHPC input than non-contextual ones (Figure 29C, two-sample KS test, $p=0.02$). Further analysis will be necessary to understand if the evolution of patterns with CFC procedure will depend on the behavioral outcomes during the testing sessions. That will require to combine – and therefore split neuronal populations in – subgroups of cells with contextual specificities in various behavioral sessions. There, a lack of n numbers can be anticipated as neuronal sampling is for now quite low, a number of sleep analysis being impossible in several animals. However, longitudinal analysis of interesting units will be performed in the future to evaluate if common evolutions will emerge, possibly linking vHPC modulations with contextual specificities and finally behavioral outcomes.

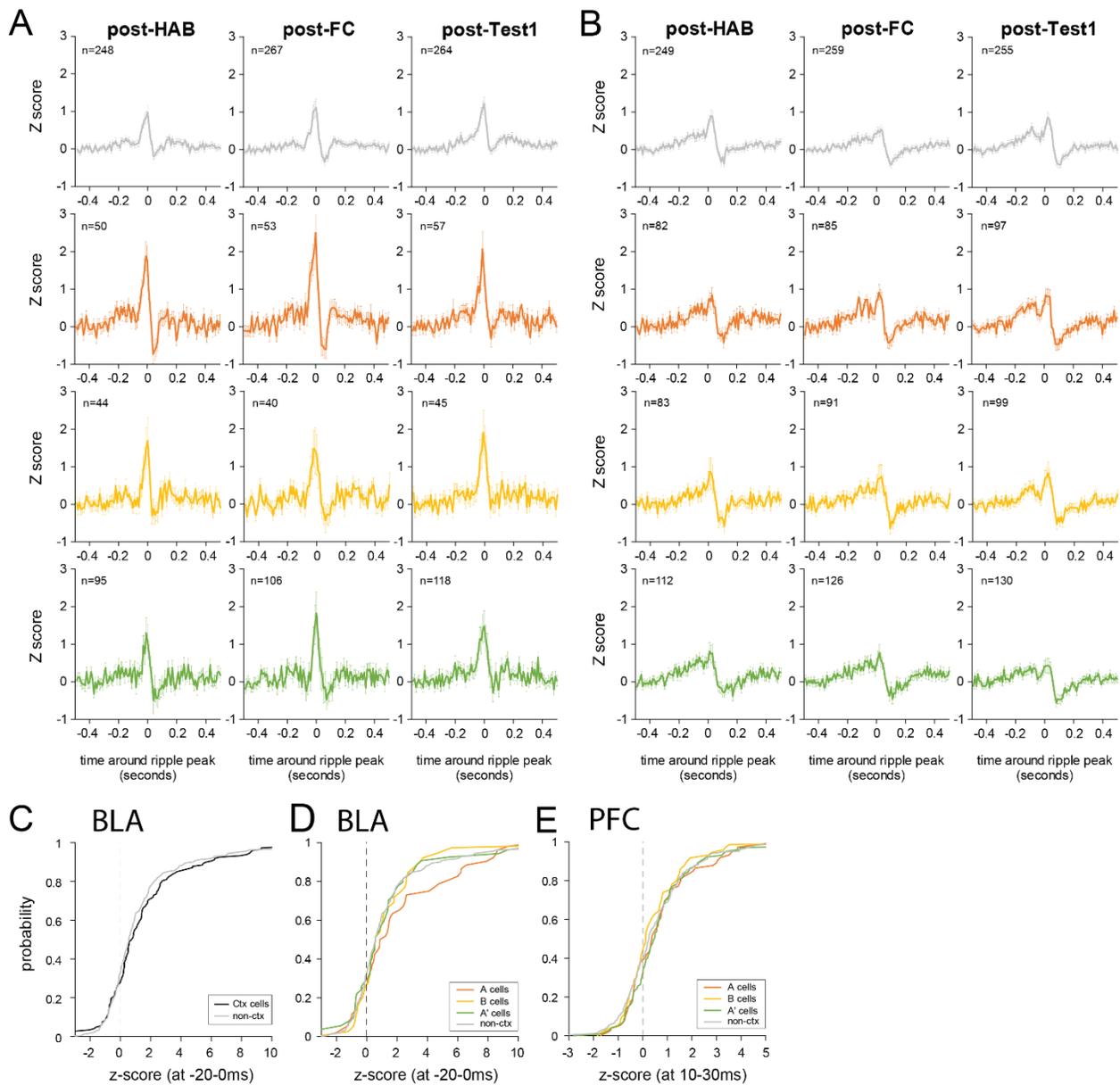


Figure 29: BLA contextual A cells receives more vHPC inputs than other cell groups.

A. Average responses of BLA cells aligned to detected ripples in three recording resting sessions as indicated. A (orange), B (yellow) and A' (green) cells were considered separately. The number of contributing cells is indicated on the top left corner. **B.** same presentation as in A for PFC neurons. **C.** Cumulative plots summarizing the ripple coupling for non-contextual (non-Ctx) and Contextual cells (A, B and A' cells) in the BLA. Two-sample KS test: $p=0.02$. **D.** same presentation as in C for all contextual BLA cell categories. Two-sample KS test: $p<0.001$ between A cells and non-ctx cells as well as between A' cells and non-ctx cells; $p=0.02$ between B cells and non-ctx cells. **E.** same presentation as in D, but for PFC contextual and non-contextual neurons. Two-sample KS test: $p=0.02$ between A cells and non-ctx cells, $p=0.218$ between B cells and non-ctx cells; $p=0.0016$ between A' cells and non-ctx cells. Data are expressed as mean \pm SEM.

CONCLUSION AND DISCUSSION

This work has been conducted during 4 years starting from previous work that was published in 2018 by Rozeske and colleagues. The original goal was to better characterize the neurophysiological events that were leading to contextual fear discrimination. To this aim, we built a new device with progressive changes in context, and recorded for several regions, vHPC, mPFC and BLA at the same time. This multisite recording strategy was achieved using electrode bundles as typically used in the host lab (collaboration between Humeau and Herry groups). However, in the course of the thesis project, and with a common interest of the two labs in understanding how memory consolidation would contribute to contextual fear memory, we decided to implement sleep recording and longitudinal following of neuronal units along the CFC protocol over the 4 consecutive days. All these added constraints complexified the initial paradigm especially at the analytical level. Thus, we consider that the dataset exploitation presented in this manuscript is not yet at its final stage, and we are planning to run numerous additional analytical steps to reinforce the key conclusions. Some of them are discussed in the paragraphs below:

Part 1: After a lot of different testing steps (see methods), we end up with a new paradigm aiming at evaluating contextual fear discrimination in mice. Because arena shape appears to be the more salient index generating the discrimination, we simplified the protocol and focused on this particular modality. Using this test, only a fraction of the testing sessions was leading to clear discrimination (Figure 7). However, we took advantage of the other behavioral response to study the associated neurophysiological signatures and try to isolate the key features of contextual discrimination. **We therefore validate a new protocol for contextual fear discrimination allowing to isolate neuronal activities specific for contextual valence shifting.** We also noticed that behavioral outcome was not very consistent from Test1 to Test2, a given animal possibly displaying different behavioral responses to context sequences (Figure 8). Together with the fact that contextual specificity of unitary activities was also quite diverging from Test 1 to Test2 (Figure 12), we decided to consider individually behavioral sessions, and to not pair Test1 and Test2 sessions from a given animal. We are aware of potential risks to consider twice the same neurons for analysis in the below mentioned analysis, but given the poor conservation of i) behavioral performance (around 50% between test1 and test 2, if considering D and D' sessions in the same group), and ii) contextual pattern of activity (around 30%, Figure 7), we are feeling confident in not introducing a major bias.

Part 2: From the seminal paper of Rozeske and colleagues, some PFC neurons that project to the periaqueducal grey (PAG) matter were specifically activated when the animal was switched from a threatful to a neutral context. However, no mechanistic explanation was presented to understand how these neurons would be activated and if some other contributors would exist for example in the amygdala, a central region for contextual fear encoding. **We here show that i) contextual cells exist in both PFC and BLA, although in lower proportion, ii) the global contextual encoding is preserved in PFC (and BLA) but is not contributed by the same cells (PFC) and iii) we identified a population of PFC cells that react specifically to the transition.** To demonstrate the turnover of contextual representation, we compared the stability of contextual patterns to another feature of recorded cells, namely their response to hippocampal ripples (Figure 13 and 14). We know that longitudinal analysis using this single wire strategy is not well accepted, thus we did not emphasize it along the manuscript. However, using very stringent parameters, far above what is classically used (see methods), we think to convincingly show that the contextual preference is not a very stable feature of PFC cells. Remains to be determine the contribution of behavioral outcomes in this turnover, and if BLA cells exhibit the same feature. Regarding the transition-specific PFC cells - cells that are activated by both transition within a given session (Figure 11) - , it would be interesting to know if they would reflect attentional rise of the animal, and if yes if they would be specific for threatful context change. As they could potentially be more stable than contextual cells in our recording, they would be another way to test for longitudinal aspects of our recordings. Another great information would be to know if discrimination failure – generalization - is associated with a lack of activation of these transition cells, that would possibly indicate that the animal did not actually perceive the context change.

Part 3: In this part of the manuscript, we wanted to better understand the specificity of discriminative sessions regarding the contextual coding of the PFC cells. Indeed, “safe context” cells identified in the Rozeske manuscript were only described in discriminative sessions, that do not allow separating contextual aspect from discrimination. Here we describe that **i) B cells - defined as being more active in a neutral context after threatful context exposure – are present in discriminative but also non discriminative sessions, ii) in discriminative sessions, B cells tend to be more active, more numerous, and display a more specific contextual pattern.** Here, the question was really to discuss the singularity of context B specific “discrimination” cells – the “Rozeske” cells – as compare to context B specific cells that are detectable in non-discriminative sessions. By comparing and correlate some of their feature with the behavioral outcomes, we aimed to better define how these cells could potentially contribute to modify

the freezing behavior. Importantly, we could show that B cells in discriminative sessions tends to be in higher proportion and more active than in the G sessions. When carefully looking at their activity pattern, we observed that in case of discrimination, B cells profiles are more restricted to the safe context than in other sessions. Also, an interesting difference occur in the A' context in case of D' sessions that show a persistent activity of the B cells therefore associated with a persistence of the safety perception (even if the threatful context is present, Figure 17). These results tend to suggest that B cell's activity in tightly associated with discrimination behavior. Actual analyses will be soon further tested using a ROC analysis to demonstrate that B cells activity predicts or not animal behavior in various conditions. Some analysis at the population level can also be envision, to determine if patterns of cells activation are recurrently found associated with the development or termination of the freezing episodes, possibly including A, B and A' and no CTXT cells as a population ensemble.

Part 4: When we considered exploring the mechanism by which B cells could contribute to express fear discrimination, we first had to characterize freezing behavior in discriminative sessions. We here show that **in discrimination sessions, the duration but not the number of freezing episodes was decreased in the neutral context**, suggesting that discrimination affect the termination rather than the initiation of freezing behavior. In generalization sessions, both parameters were maintained in B context as high as in A context. We then analyzed the relationship between freezing episodes and the context preference of contextual cells in order to exclude some possible confounding factors. We then described that **contextual preference of cells is massively independent on the freezing behavior** that only marginally contribute to determine cell's group attribution. This indicate that freezing per se is not strongly affecting cell's activity rate. By carefully examining B/A activity ratios in various animal state - freezing VS mobility - and behavioral groups, we observed that in discriminative session B cells tends to have a better contextual specificity during mobility, a phenomenon that is not observed in G sessions (Figure 21). In fact, during freezing episode, B cells lost this improved contextual accuracy, that was then similar between G and D/D' sessions (Figure 21). A part of the improved context accuracy can be attributed to a better correlation of B cells with locomotion in the DD' sessions (Figure 22). However, above-mentioned ratios were independent on the time spent in one or the other state (mobility or freezing), and interestingly enough, this increase in the correlation to locomotion is already present in the context A, and therefore cannot really explain the specific improvement of context specificity of B cells in DD' sessions.

Part 5. Freezing episodes are interrupted or shortened during context discrimination (Figure 18). It was thus essential to comprehend how contextual and non-contextual cells were behaving during freezing

episodes. Our results showed that **i) Frequency of A cells is poorly modulated by freezing behavior, but is higher in G sessions, ii) Frequency of B cells is decrease during freezing episodes, only in D/D' sessions, iii) this effect is independent on the context valence, being present all along the testing time.** Our first observation is that contextual cells have higher rate of discharge than non-contextual cells, a difference that is preserved during freezing (Figure 23). The difference is highly significant and quite impressive. A possible reason for this difference is their “selection” as being contextually modulated: This would potentially mean that they have specific position in the neuronal network, such as receiving direct excitatory inputs conveying contextual information, or being under the control of a disinhibitory network. Another possibility would be an important contribution of local interneurons – often high spiking cells - as being contextually modulated. We did not have yet the time to fully sort putative interneurons from our data set, but this would certainly be an interesting analysis to perform. Finally, the low level of activity of non-contextual cells could also be due to the inclusion of very low spiking cells or even inadequately sorted cells. This can be controlled by analyzing the distribution of waveform amplitudes of both categories of cells, or more interestingly by comparing the activity rate of cells that would have gain or lost their context specificity between the two tests. Interestingly enough, contextual cells did not arbor stable frequency modulation during freezing episodes: they globally adopt a sequence of inhibition followed by a ramp of frequency increase that eventually overshoot their initial frequency. This rebound is absent from non-contextual cells (Figure 23). If the underlying mechanism is not yet known, it would be interesting to test if there is a causality link between the time course of this modulation and the duration of the freezing episode. Indeed, one can postulate that when a lot of neurons are far from their initial frequency during freezing – possibly being entrained by 4 Hz oscillations- they must behave as an attractor destabilizing the network state, thereby leading to a shortening of the episode. It would then become attractive that B cells in D/D' sessions could play this role, as they are deeply inhibited by freezing. In this line, B cells do exhibit this freezing-mediated inhibition only when discrimination is actually present (Figure 25). In contrast, A cells, that are more active in generalization sessions did not exhibit this freezing mediated inhibition, and may simply support the freezing behavior itself, as being again more active in context A. In good accordance with that, PFC A cells are less numerous in the L sessions (data not shown).

Part 6. One of the reasons to build up this project was to identify some determinants for contextual fear expression and discrimination, and to identify the hierarchy between BLA and PFC is these processes. This is why we included in our protocol some recordings during sleeping phases. First because consolidation of memory that occurs during sleep is one of the most intriguing phases of memory processing, with

sequences of slow wave sleep (SWS) and rapid eye movement (REM), phases that may play complementary roles in the process of memory selection and stabilization (Girardeau & Lopes-dos-Snatos, 2021). Second, because during slow wave sleep, we have access to endogenous recurrent activations of hippocampal structures – the so-called ripples (Buzsaki, 2015) - that through their projections, activate numerous brain regions, including the prefrontal cortex and the basolateral amygdala (Girardeau, 2017; Khodagoly, 2017). A previous project in the lab showed that coupling between dorsal part of the hippocampus (dHPC) and the mPFC was affected after spatial rule learning, thus we systematically measured the coupling between hippocampal ripples and mPFC and BLA units along the CFC protocol. Doing this, we showed that **i) both structures display hippocampal-mediated reactivations during sleep, ii) BLA but not mPFC cells showed behaviorally correlated reactivations.** Ripple-mediated reactivations are complex in their time course, and should be manipulated carefully. Z-score expression of activity changes is very popular in *in vivo* experiments, but is very sensitive to sampling rate. That for example lead to strong Z-scores when looking for high spiking cells such as fast spiking interneurons. It is thus not surprising that Z-score amplitude is sensitive to ripple number (Figure 27), and the number of contributing ripples is fluctuating from a session to another, due to day-to-day changes in animal sleeping, and changes in the settings for ripple detection (see methods). Thus, longitudinal analysis and day-to-day comparison of Z-score amplitudes must be corrected in some ways from this effect even if the global number of detected ripples is quite stable along the recordings. The second reason to not emphasize the conclusions that can be raised from longitudinal following of unitary activities is the use of single wire strategy that did not fully convince for unit conservation along days. However, we were still able to examine the vHPC→PFC and vHPC→BLA coupling in various days and in animals with various behavior (Figure 28) or in cells that activity profile was determined during the Test1 (Figure 29) and that were then retrospectively analyzed if respecting the quality criteria for longitudinal conservation (see methods).

The first observation is that animals that will display G sessions in Test1 have an exacerbated reactivation of BLA neurons during SWS after FC. Animals that will undergo DD' sessions have intermediate scores, and animals that do not express contextual fear (L sessions) have the lower score. The number of ripples in each category in the post FC resting period (post FC: DD': 2366 +/- 388, $n=5$; G: 2864 +/- 403, $n=13$; L: 2851 +/- 333, $n=6$) suggest that these differences are real, with a slight underestimation of D/D' coupling. Thus, expression of CFC in Test1 seems to be correlated with the connectivity between vHPC and BLA, rather than vHPC→PFC connectivity that is not different between groups (Figure 28 C-D). Importantly, this difference was yet present during habituation sessions, and persist after CFC testing, suggesting that the

level of vHPC→BLA connectivity determine the CFC capacity of the animal and could be a major determinant to act on fear encoding capacity in individuals. This interesting hypothesis should be directly tested by inactivating vHPC→BLA inputs in vivo at different time points of the CFC paradigm. It is nevertheless reinforced by the second observation that BLA A cells are the ones receiving the most vHPC inputs, independently of the behavioral readout (Figure 29). Not surprisingly, BLA – but not PFC - contextual cells in general are well reactivated by vHPC ripples, the A cells being more reactivated than the others. Again, this increased coupling is yet present in the post-habituation recording, suggesting that this excitatory input could be a determinant for future contextual cell preference. Again, further analysis will determine if A, B and A' cell populations include local interneurons that can heavily contribute to average Z-scores, and we will also compare how these cells evolve between Test1 and Test2 as a lot of them change their fate. This will be a key analysis to understand the level of causality between these inputs and the cell-specific activity modulations during CFC expression and discrimination.

Final conclusions

Altogether, our data support the notion that contextual information that are crucial for CFC expression and discrimination are first reaching the BLA, as BLA cells received differential inputs from vHPC according to behavioral outcomes, whereas PFC cells do not. However, during testing, a close relationship exists between contextual PFC cells and CFC expression and contextual discrimination. Thus, it is likely that the examination of BLA-PFC reciprocal connectivity during testing will help in understanding the information transfer especially during freezing episodes that are shortened during contextual fear discrimination. Some important analyses that remain to be done, to determine:

- How do contextual cells in both regions synchronize to the 4Hz oscillations that are controlling freezing behavior?
- What is the contribution of interneurons in the described contextual cell populations?
- Is the turnover of BLA contextual cells different than in the PFC?

Some of these important questions especially those dealing with longitudinal analyses - such as predisposition to CFC, contextual cells turn over, etc... - will require new experiments using dedicated strategies, such as high-density silicon probes, and/or calcium imaging that are under testing in the lab.

Furthermore, to establish a causality link between specific cell behavior and behavioral outcomes, genetic strategies such as Cal-light will be tested to inactivate specific cell populations. The important turnover of contextual specificities will certainly be an important parameter to consider at that time.

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