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PET metabolic imaging of time-dependent reorganization of olfactory cued fear memory networks in rats

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**Running title:** microPET imaging of odor fear memory

## **Abstract**

Memory consolidation involves reorganization at both the synaptic and system levels. The latter involves gradual reorganization of the brain regions that support memory and has been mostly highlighted using hippocampal-dependent tasks. The standard memory consolidation model posits that the hippocampus becomes gradually less important over time in favor of neocortical regions. By contrast, this reorganization of circuits in amygdala-dependent tasks has been less investigated. Moreover, this question has been addressed using primarily lesion or cellular imaging approaches thus precluding the comparison of recent and remote memory networks in the same animals. To overcome this limitation, we used microPET imaging to characterize, in the same animals, the networks activated during the recall of a recent vs remote memory in an olfactory cued fear conditioning paradigm. The data highlighted the drastic difference between the extent of the two networks. Indeed, while the recall of a recent odor fear memory activates a large network of structures spanning from the prefrontal cortex to the cerebellum, significant activations during remote memory retrieval are limited to the piriform cortex. These results strongly support the view that amygdala-dependent memories also undergo system-level reorganization, and that sensory cortical areas might participate in the long-term storage of emotional memories.

## **Keywords**

[<sup>18</sup>F]FDG micro PET; Cued Fear conditioning; Recent memory; Remote memory; System-level consolidation; Odor

## Introduction

It has been known for a long time that new memories are gradually stabilized from an initially labile state to a more permanent state through consolidation processes (Müller and Pilzecker 1900). Consolidation involves reorganization at both the synaptic and system levels (Dudai 2004). Synaptic consolidation refers to cellular and molecular modifications at the synaptic level and occurs over relatively short periods of time following learning acquisition, while system consolidation involves large-scale circuit reorganization and can encompass several days or months following the initial learning episode (Frankland and Bontempi 2005).

According to the standard memory consolidation model, recent declarative memories are initially hippocampus-dependent but are progressively reorganized as time passes (Winocur et al. 2010; Squire et al. 2015). Following training, there is continued interplay between the hippocampus and relevant neocortical regions, with the hippocampus becoming gradually less important over time for storage and retrieval, and a more permanent memory developing in distributed regions of the neocortex. This model is supported by results from cellular imaging and pharmacological inactivation studies in animals (Bontempi et al. 1999; Frankland et al. 2004; Maviel et al. 2004), showing that recall of recent spatial and contextual memories is associated with activation of the hippocampus, and inactivating the hippocampus preferentially disrupts the recall of these recent memories. By contrast, as these memories get older, their recall is predominantly associated with activation of cortical regions such as the prefrontal, frontal, anterior cingulate, retrosplenial and temporal cortices and inactivation of the prelimbic or anterior cingulate cortices block recall of remote memory.

Of note, system consolidation has been mostly investigated using hippocampal-dependent tasks like spatial learning paradigms (Bontempi et al. 1999; Maviel et al.

2004), or contextual fear conditioning (Frankland et al. 2004). By contrast, the reorganization of circuits with the passage of time in amygdala-dependent tasks like cued fear conditioning, has been much less investigated (Bergstrom 2016).

In cued fear conditioning, a discrete sensory cue predicts the arrival of a foot-shock and information about the sensory cue and shock converges in the lateral amygdala nucleus via thalamic and cortical inputs and triggers synaptic modifications necessary for memory consolidation (LeDoux 2000; Maren 2001). The lateral amygdala then communicates with the central amygdala nucleus, which sends projections to different structures in the brainstem and in the hypothalamus that orchestrate conditioned fear autonomic and motor responses. While the amygdala plays a key role in the acquisition, storage and retrieval of cued fear memories, there are now a series of studies examining the role of sensory cortices in both recent and remote cued fear memory retrieval (Grosso et al. 2015a, 2015b). Most of these studies concern the auditory cortex. First, electrophysiological and pharmacological studies have shown that auditory stimuli paired with footshock induce robust changes of synaptic strength in the auditory cortex (Quirk et al. 1997; Weinberger 2007; Letzkus et al. 2011), and that some of these learning-related changes are driven by the amygdala (Chavez et al. 2009) suggesting that the amygdala may strengthen emotional memory trace formation occurring in the auditory cortex. Second, Sacco and Sacchetti (2010) performing localized excitotoxic lesions of different sensory cortices, demonstrated temporally graded deficits in the retrieval of remote fear memory. Indeed, lesions of the auditory cortex disrupted remote, but not recent, auditory cued fear memory. In the same way, lesions of the piriform cortex and visual cortex disrupted remote but not recent olfactory and visual cued fear memory, respectively. Similarly, a pharmacological study showed that blockade of NMDA receptors in the posterior

piriform cortex decreased remote memory performance while recent memory was unaffected (Hegoburu et al. 2014). These data support the view that emotional memories, like hippocampal-dependent memories, undergo some kind of system-level reorganization, with the sensory cortices being involved in remote but not recent fear memory retrieval. Importantly, in contrast to what happens for hippocampal-dependent memories showing a dispensable role for the hippocampus in remote memory retrieval, the amygdala continues to be crucially involved in fear expression during remote memory retrieval (Bergstrom 2016).

So far, system-level reorganization of amygdala-dependent cued fear memories has been investigated primarily through lesion or inactivation experiments and, to a lesser degree, using autoradiography or histochemistry imaging techniques, thus precluding the comparison of recent and remote memory networks in the same animals. To address this question, we performed 18F-fluorodeoxyglucose ([<sup>18</sup>F]FDG) positron emission tomography (PET) imaging in rats engaged in an odor fear conditioning paradigm. The key advantage of PET compared to other imaging approaches is that the PET radiotracer allows the whole-brain function to be examined with limited confounding effects of anesthesia. Indeed, the animal is awake and freely moving outside the PET scanner during [<sup>18</sup>F]FDG uptake, allowing the assessment of brain activation when the animal is engaged in a behavioral task (Jang et al. 2009; Sung et al. 2009). Moreover, [<sup>18</sup>F]FDG PET imaging can be used for serial examination of metabolic function in the same animal (Kornblum et al. 2000). In a recent study, we brought the first evidence that [<sup>18</sup>F]FDG PET was a powerful tool to study odor representation in large-scale rodent neuronal networks (Litaudon et al. 2017). Here we capitalized on this observation to investigate system-level reorganization in odor fear conditioning.

## **Material and methods**

### **Animals**

All experiments were conducted in strict accordance with the European Community Council Directive of September 22, 2010 (2010/63/UE), and received approval from the French national ethics committee (APAFIS #8682-201701261053886v3). Care was taken at all stages to minimize stress and discomfort to the animals. Data were obtained from 18 male Long Evans rats (10 weeks,  $287 \pm 20$  g at the beginning of the experiment, Charles River, L'Arbresle, France). They were group-housed at 23°C and maintained under a 12/12 h light/dark cycle (lights on from 8 A.M. to 8 P.M.). Food and water were available ad libitum. At their arrival, rats were randomly assigned to one of the two experimental groups.

### **Experimental apparatus**

The apparatus has been described in detail in a previous study (Hegoburu et al. 2011). It consisted of a plexiglass cage (diameter 20 cm, height 30 cm, EMKA Technologies) placed in a sound attenuating cage (length, 60 cm; width, 60 cm; height, 70 cm, 56-dB background noise). The bottom of the animal chamber was equipped with a shock floor connected to a programmable Coulbourn shocker (Bilaney Consultants GmbH). Three Tygon tubing connected to a programmable custom olfactometer were inserted in the tower on the top of the cage to deliver air and odorants. Deodorized air flowed constantly through the cage (2 l/min). When programmed, an odor was introduced smoothly in the air stream through the switching of a solenoid valve (fluid automation systems, CH-1290 Versoix), thus minimizing its effect on change in pressure. The bottom of the animal chamber had a port connected to a ventilation pump which could draw air out of the plethysmograph (at a rate of up to 2 l/min) thus maintaining a

constant airflow. Animal's behavior was monitored with two video cameras placed on the walls of the sound-attenuating cage.

## **Fear conditioning paradigm**

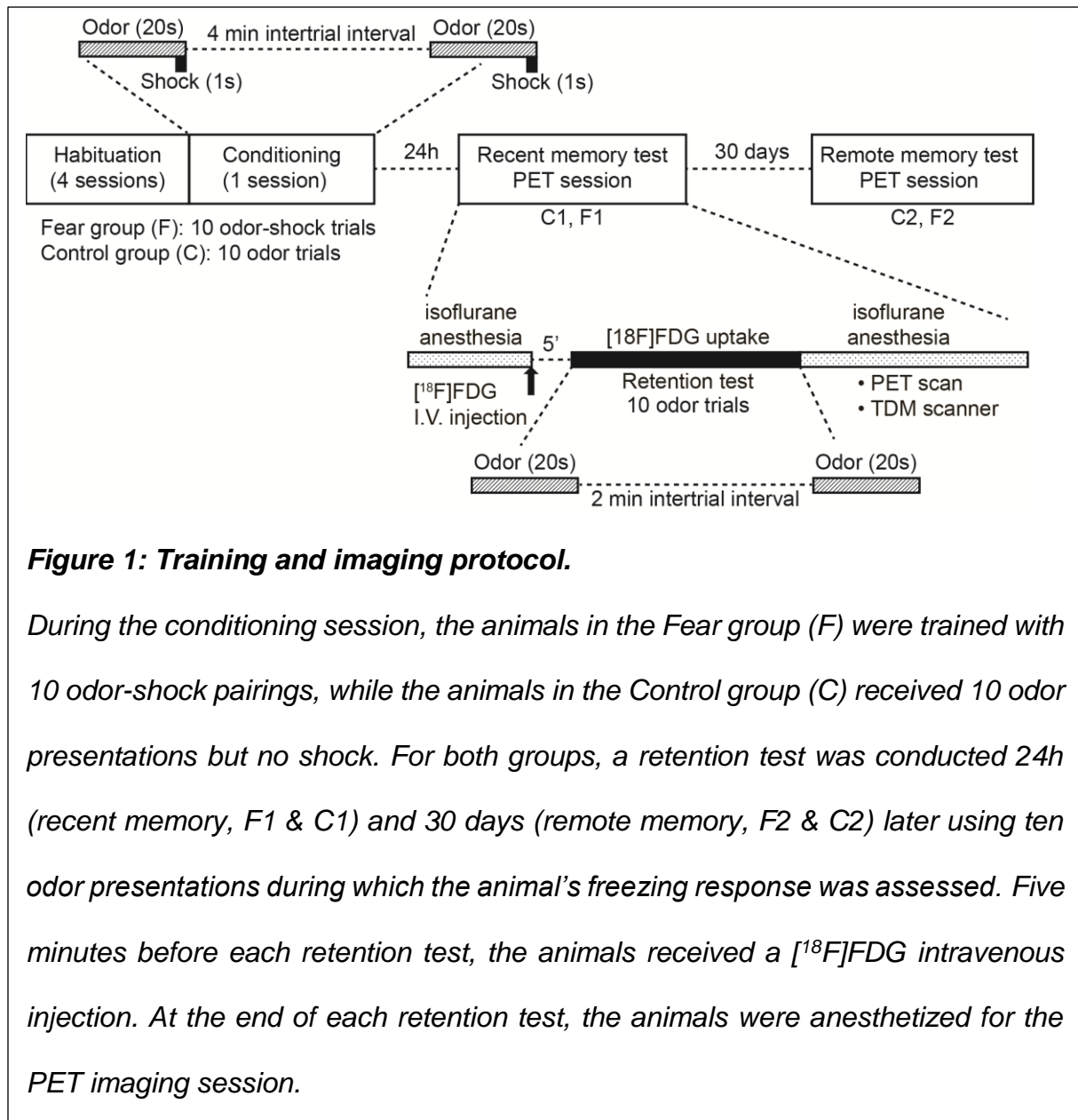
### ***Conditioning***

The training protocol is illustrated in figure 1. The animals were handled individually and placed in the experimental apparatus (two sessions of 30 min per day) during the two days preceding the beginning of the experiment to minimize stress induced by exposure to a novel environment and to familiarize them with being manipulated. For the conditioning session, rats from the Fear group (F, n=9) were allowed free exploration during the first 4 min, and then an odor (1:10 peppermint vapor to air) was introduced into the cage for 20 seconds, the last second of which overlapped with the delivery of a 0.4-mA foot-shock. The animal received 10 odor-shock trials, with an inter-trial interval of 4 min. After the last pairing, the animal returned to its home cage. Rats in the Control group (C, n=9) followed the same protocol except that they received 10 presentations of 20s-odor but no shock.

### ***Retention test***

The conditioned fear response was assessed in both groups during two retention tests: one 24h after conditioning (recent memory, C1 and F1) and another 30 days later (remote memory, C2 and F2) (Fig. 1). The testing cage was equipped with new visual cues and with a plastic floor to avoid contextual fear expression. Four hours prior to each retention test session, the rats were food-deprived but were allowed free access to water. This restriction was applied to selectively maximize and homogenize [ $^{18}\text{F}$ ]FDG uptake at the brain level (Fueger et al. 2006). Immediately before the start of the retention session, the animals were briefly anesthetized with isoflurane (5% for





induction and 2-2.5% for maintenance), and an intravenous catheter was placed in the tail vein. Immediately after bolus [<sup>18</sup>F]FDG injection ( $10 \pm 1.6$  MBq, 500  $\mu$ l), the catheter was removed and isoflurane was discontinued. Then, the rat was placed in the testing cage, and allowed a 5min period to recover from anesthesia. The learned odor was then presented ten times for 20 s with a 2-min inter-trial interval. The fear memory strength was assessed by measuring the duration of the animal's freezing response during the retention test. Freezing behavior defined as the absence of any visible movement except that due to breathing (Blanchard and Blanchard 1969) was

automatically detected using a Labview homemade software and further verified by an experimenter (for a detailed description, see Dupin et al. 2019). The animal's freezing response was quantified during each 20s odor presentation. The obtained values were then expressed as a percentage of the sampled period total duration, averaged across the trials for a given animal, and averaged among animals of the same experimental group. Freezing amounts were compared using a two-way repeated-measures ANOVA, with group (Control versus Fear) as an independent factor and age of memory (recent vs remote) as a repeated factor, followed by post-hoc Fisher tests for pairwise comparisons. For all the statistical comparisons performed, the significance level was set at 0.05.

## **PET imaging**

### ***Data acquisition***

At the end of each retention test session, the rats were anesthetized with isoflurane and placed in a prone position on the bed of the scanner, and an emission scan lasting 20 minutes was initiated (Fig. 1). To minimize head movement, the rat was head-fixed with ear bars and a tooth bar in a head holder. Its body temperature was maintained at  $37\pm0.2$  °C using a dedicated heating pad and the respiration rate was monitored using a pressure sensor. Data were acquired in list-mode using a dedicated small animal PET/CT scanner manufactured by Inveon (Siemens, Erlangen, Germany). The scanner has an axial field of view of 12.7 cm and a spatial resolution of 1.8 mm full width at half maximum (FWHM) (Bao et al. 2009). This nominal resolution means that two sources of the same activity separated by less than 1.8 mm cannot be distinguished. Nevertheless, smaller sources can be detected and quantified. A CT scan was performed in the same position immediately after PET emission scan to correct for tissue attenuation. The images were reconstructed in four frames of five

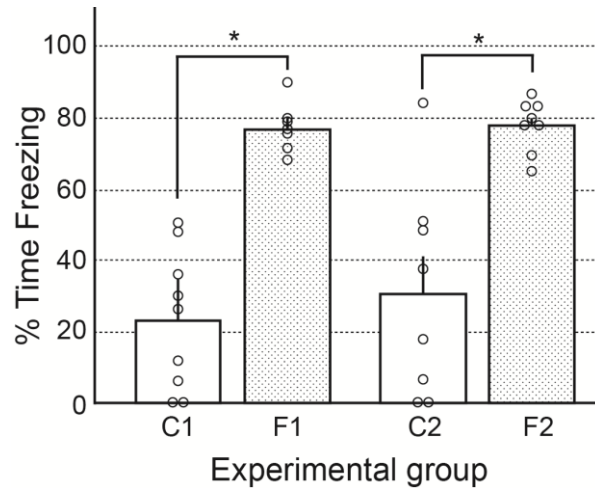
minutes each with attenuation as well as scatter correction by a 3D-filtered back-projection algorithm (Hamming filter; cut-off frequency 0.5 cycles/pixel) and a zoom factor of two. This led to reconstructed volumes of 159 slices comprising  $128 \times 128$  voxels in a bounding box of  $49.7 \times 49.7 \times 126$  mm with voxel size  $0.388 \times 0.388 \times 0.796$  mm. After scanning, the animals were returned to their home cages.

### ***Data analyses***

Data processing was carried out using statistical parametric mapping software (SPM12, Welcome trust center for neuroimaging, London; <http://www.fil.ion.ucl.ac.uk/spm/>). Individual PET images were spatially normalized to a custom PET [ $^{18}\text{F}$ ]FDG template made from the MRI template in the space of the Lancelot rat brain atlas (Lancelot et al. 2014) and resliced to a resolution of  $0.1 \times 0.1 \times 0.205$  mm. Lancelot et al. (2014) showed that the sensitivity of detection in the subject space does not differ after spatially normalizing PET images in the template space via a functional template. Furthermore, this method resulted in excellent reproducibility. The normalized brain images were smoothed using a Gaussian filter (isotropic 0.5 mm full width at half maximum) and subsequently masked to remove extra-cerebral activity. Subsequently, PET image intensities were normalized using the standardized uptake value (tissue activity concentration/injected dose  $\times$  body weight). A voxel-based SPM analysis was performed within a one-way measures ANOVA design with C1, C2, F1 and F2 as factors. Whole-brain mean [ $^{18}\text{F}$ ]FDG activity computed for each scan within the template mask of the brain was used for ANCOVA normalization. Statistical maps data were thresholded at  $p < 0.001$  (uncorrected) with an extent threshold of 100 voxels ( $0.205 \text{ mm}^3$ ). Such a cluster-forming threshold is a reasonable statistical level inference to identify regions that are localized enough to be interpretable (Woo et al. 2014).

## Results

### Behavioral data



**Figure 2: Odor induced freezing response**

Percentage of freezing (mean + SEM) in response to odor presentation in Control group (C) and Fear group (F) during recent (C1, F1) and remote (C2, F2) memory retention tests. Open circles show single data points. \*: significant difference with the control group,  $p < 0.001$ .

In both groups of rats (Control and Fear groups), two retention tests were carried out respectively 24 h (recent memory) and 30 days (remote memory) after the acquisition session. Freezing behavior measured in the two groups during each test session is shown in Figure 2. Due to technical issues, three video recordings could not be analyzed. A two-way repeated-measures ANOVA revealed a main effect of group ( $F_{1,13} = 70.467$ ,  $p < 0.001$ ) but no effect of memory age ( $F_{1,13} = 0.100$ ,  $p = 0.757$ ). Further post-hoc comparisons showed that for both retention tests, the level of freezing in response to the odor is significantly higher in the Fear group than in the Control

group. In the two groups, the level of freezing is not different between recent and remote memory.

### **PET imaging data**

First, the functional network related to recent memory was assessed by contrasting Fear group (n=9) and Control group (n=9) maps obtained during the 24h retention test (F1 VS C1 contrast). This analysis revealed several clusters showing significant increases in [ $^{18}\text{F}$ ]FDG uptake along the brain antero-posterior axis. The main activated brain areas are reported in Table 1 (all the significant clusters are reported in supplementary table 1).

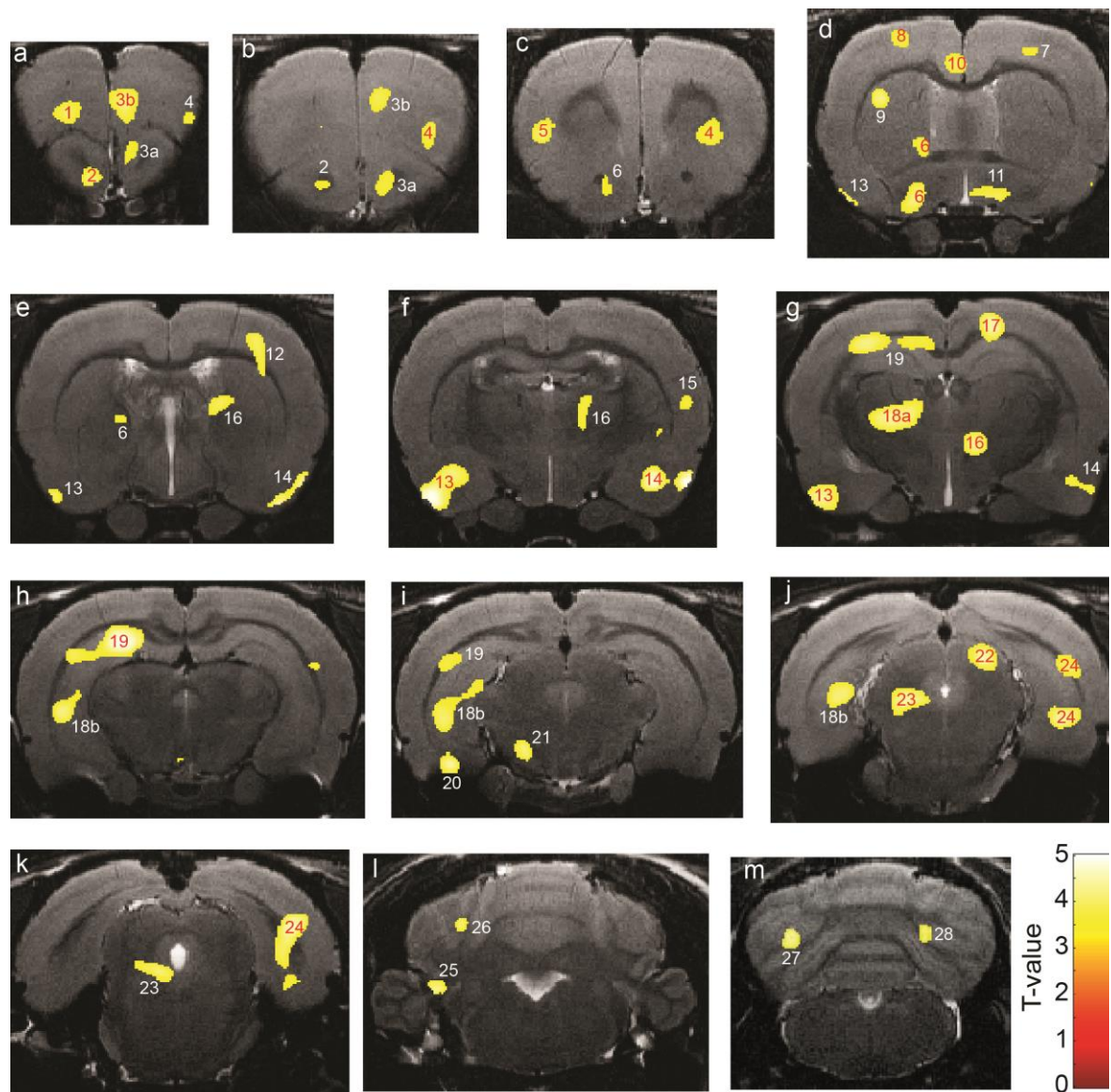
**Table 1:** Main brain regions showing significant increases in [ $^{18}\text{F}$ ]FDG uptake between Fear (F1) and Control (C1) conditions for recent memory retention test.

Brain regions	Structures within brain regions	Cluster Number	Fig. 3 panel
Primary olfactory cortex	R : AON, aPC, pPC, Ent	3a,14,24	a,b,e-g,j,k
	L : AON, aPC, pPC	2, 13	a,b,d-g
Frontal Cortex	R : PL, Cg	3b	a,b
	L : LO, Cg	1, 10	a,d
Cortex	R : S1, S2, Parietal	7,12,15,17	d,e,f,g
	L : S1	5,8	c,d
Striatum	R : CPu, Acb	4,16	b,c,e
	L : Acb, CPu	6,9	c-e
Thalamus	R : dorsal/ventral	16	f,g
	L : dorsal	18a	g
Amygdala	R : BLA	14	f
	L : BLA	13	f,g
Hippocampal formation	R : subiculum	24	j
	L : dorsal and ventral hippocampus	18b,19,20	g-j
PAG	L : PAG	23	j,k
Cerebellum	R : Crus 2	28	m
	L : Crus1, Crus2	25,26,27	l,m

Notes: R: right hemisphere. L: left hemisphere. Cluster number corresponds to clusters defined in supplementary Table 1 and to numbers indicated on figure 3 panels.

*Abbreviations: AON (anterior olfactory nucleus), aPC (anterior piriform cortex), pPC (posterior piriform cortex), Ent (entorhinal cortex), PL (prelimbic cortex), Cg (cingulate cortex), LO: lateral orbital cortex, S1 (primary somatosensory cortex), S2 (secondary somatosensory cortex), CPu (caudate putamen), Acb (accumbens nucleus), BLA (basolateral amygdaloid complex), PAG (periaqueductal gray).*

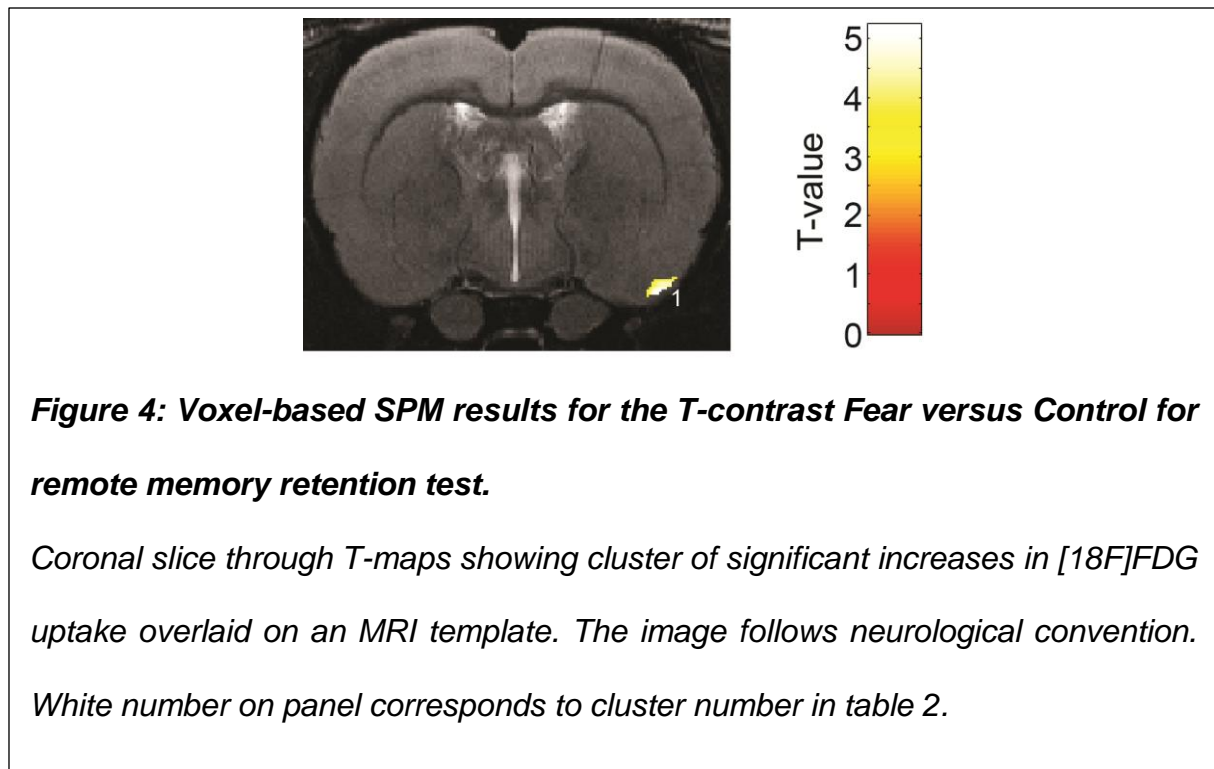
First, regional brain activations were observed in several olfactory cortical areas including the anterior piriform cortex, the posterior piriform cortex, the anterior olfactory nucleus, and the entorhinal cortex (Fig. 3a-g,j). Clusters of significant voxels were also observed in the prefrontal cortex including the prelimbic cortex and the cingulate cortex (Fig. 3a-d), in the somatosensory cortex including the barrel field cortex (Fig. 3c-f). In addition to cortical structures, significant increases in glucose metabolism were observed in the basolateral amygdaloid complex (Fig. 3f,g), the striatum (Fig. 3b,c-e), the thalamus (Fig. 3f,g) and the hippocampal formation including dorsal and ventral hippocampus and the subiculum (Fig. 3g-j). Finally, activations were also observed in the ventral part of the periaqueductal gray (Fig. 3j,k) and in several modules of the cerebellum (Fig. 3l,m). We did not observe decrease in [ $^{18}\text{F}$ ]FDG uptake.



**Figure 3: Voxel-based SPM results for the T-contrast Fear versus Control for recent memory retention test.**

From a to m: Coronal slices through T-maps showing clusters of significant increases in  $[^{18}\text{F}]\text{FDG}$  uptake overlaid on an MRI template. The images follow neurological convention, with the left side corresponding to the left hemisphere. Red and white numbers on panels correspond to cluster numbers in table 1 and supplementary table 1.





We then investigated the functional network related to remote memory by contrasting the maps obtained for the Fear group (n=9) and Control group (n=8, one animal could not be recorded due to a technical issue with the PET scanner) during the 30 days retention test (F2 VS C2). In striking contrast with the network activated during the recent memory retention test, the remote memory network was limited to posterior piriform cortex (Fig. 4, Table 2).

**Table 2:** Brain regions showing significant increases in [<sup>18</sup>F]FDG uptake between Fear and Control conditions for remote memory retention test.

Cluster Nb	Regions within the cluster	Cluster size (mm <sup>3</sup> )	Cluster Peak		Coordinates (mm)		
			T-value	p-value	x	y	z
1	Right pPC	0.27	5.18	<10 <sup>-3</sup>	5.0	-10.1	-0.9

*Notes: Values x, y, z are the coordinates of the peak activation centers in the Paxinos and Watson stereotaxic space (Paxinos and Watson 2005). The x-coordinate corresponds to the distance lateral from the midline between the hemispheres (positive values for spots located on the right side) (L, Left; R, Right); the y-coordinate denotes the dorsoventral position; the z-coordinate indicates the position relative to Bregma (positive values for sections anterior to Bregma). Abbreviations: pPC (posterior piriform cortex).*

## **Discussion**

The present study investigated the fear neural networks by questioning the existence of system-level reorganization of amygdala-dependent emotional memories as assessed using odor fear conditioning. Our study is the first to our knowledge to describe the networks involved in recent and remote fear memory in the same animals using [ $^{18}\text{F}$ ]FDG PET. The data showed that the recall of a recent odor fear memory activates a large network of structures spanning from the prefrontal cortex to the cerebellum. In sharp contrast, in our experimental conditions, remote fear memory retrieval activates significantly a very restricted network limited to a single cortical area, the piriform cortex.

## **Behavior**

The strength of recent and remote odor memories was assessed by measuring the freezing response to the learned odor during two retention tests carried out respectively 24h and 30 days after the conditioning session. The data showed that the level of freezing was high and similar during the two retention tests, which indicates that the strength of the remote odor memory was equivalent to that of the recent one. This data is important because the recent memory test consisted in the repeated presentation of the learned odor alone, which could have triggered extinction of the memory, resulting

in low levels of freezing during the remote memory test. This was not the case, suggesting that the learned association was strong and resistant to extinction processes that could have occurred during the recent memory test.

### **Recent memory network**

The recent memory network includes olfactory cortical areas and brain areas classically described as playing a critical role in cued fear memory, like the amygdala, the prefrontal cortex and the periaqueductal grey. This network also includes less classical brain areas like the cerebellum, the striatum and the somatosensory cortex.

### ***Olfactory cortical network***

Our data showed bilateral activations in primary olfactory areas. Among them, the piriform cortex is the largest area and is classically divided into two parts: the anterior (aPC) and posterior (pPC) piriform cortex.

Previous studies in the literature have highlighted that the aPC is preferentially involved in sensory processes and simple forms of memory, such as short-term habituation (Wilson 1998a, 1998b; Kadohisa and Wilson 2006), pattern completion (Barnes et al. 2008), and perceptual learning (Wilson and Stevenson 2003). In contrast, the pPC is involved in cognitive mnemonic processes, including learning and recall of associations between odorants and information from other sensory modalities (Litaudon et al. 1997, 2003; Chabaud et al. 1999, 2000; Mouly et al. 2001; Mouly and Gervais 2002; Mouly and Di Scala 2006). The activation reported here in aPC and pPC is in accordance with previous studies showing the role of aPC (Barnes et al. 2011; Chen et al. 2011) and pPC (Sevelinges et al. 2004, 2008; Jones et al. 2007; Hegoburu et al. 2009; Dupin et al. 2020) in odor fear conditioning. They are also in agreement with recent data showing that olfactory fear conditioning activates sparse and distributed ensembles of

neurons in the mouse piriform cortex and that specific silencing of these neurons impairs odor fear memory recall (Meissner-Bernard et al. 2019).

The activation observed in the anterior olfactory nucleus (AON) is more surprising. A few studies have shown that AON was activated during context-odor association (Aqrabawi and Kim 2020; Levinson et al. 2020). More interestingly, a study by Staples et al. (2005) showed that AON was activated during predator odor contextual fear conditioning. Finally, our results confirmed data from a previous study showing that fos expression is enhanced in the AON following odor fear conditioning (Funk and Amir 2000).

### ***Activation in the classically described fear conditioning network***

#### *Amygdala*

It has been known for a long time that circuits within the amygdala are essential to the acquisition, storage, and retrieval of contextual and cued (mostly auditory) fear memories (LeDoux 2000; Maren 2001; Pape and Pare 2010). Specifically, selective lesions of the basolateral amygdaloid complex (BLA) produce severe deficits in both the acquisition and expression of Pavlovian fear conditioning independent of the stimulus modality used for the CS (LeDoux et al. 1990; Campeau and Davis 1995; Maren et al. 1996; Cousens and Otto 1998). In our study, the retrieval of recent odor fear memory activates the BLA and confirms its role in the expression of odor fear memory highlighted in previous studies using electrophysiological recordings (Rosenkranz and Grace 2002; Sevelinges et al. 2004, 2007; Dupin et al. 2019, 2020), intracerebral microdialysis monitoring (Hegoburu et al. 2009) and lesion or pharmacological inactivation (Cousens and Otto 1998; Kilpatrick and Cahill 2003; Walker et al. 2005; Hegoburu et al. 2014).

### *Prefrontal cortex*

Our data showed an activation of the median prefrontal cortex (mPFC), and more specifically its prelimbic part, during recent odor fear memory recall. In rodents, the mPFC is subdivided into infralimbic (IL) and prelimbic (PL) regions, which have been described as having opposite influences on fear behavior: while the PL is involved in the expression of fear, the IL is rather involved in the suppression of fear during extinction learning and retrieval (Sierra-Mercado et al. 2011; Milad and Quirk 2012; Giustino and Maren 2015). There are now several lines of evidence linking activity in the prelimbic cortex, with the retrieval of auditory cued fear conditioning (Corcoran and Quirk 2007; Burgos-Robles et al. 2009; Choi et al. 2010; Sierra-Mercado et al. 2011; Courtin et al. 2014; Do-Monte et al. 2015). Here we show that the PL is also activated during the retrieval of a recent odor fear memory, suggesting its involvement is independent of the sensory modality of the learned CS. These data extend previous electrophysiological data showing that the PL might be involved in the encoding of the temporal relationships between an odor CS and the US during acquisition of an odor fear conditioning (Dupin et al. 2020). In addition to median prefrontal cortex, our data showed that the cingulate cortex was activated during odor fear memory retrieval as observed for auditory cued fear memory (Tang et al. 2005).

### *Hippocampus*

Here we report an activation in both dorsal and ventral parts of the hippocampus during the retrieval of a recent odor fear memory. Maren and Holt (2004), comparing the effects of pharmacological inactivation of the dorsal hippocampus or ventral hippocampus on Pavlovian auditory fear conditioning in rats, reported that inactivation of the ventral, but not the dorsal hippocampus, induced impairments in the acquisition of the learning. They also showed that postraining lesion of the ventral hippocampus

disrupted the expression of conditioned fear to the tone. Thus, the ventral hippocampus might be involved in the acquisition and expression of auditory fear memory. The dorsal hippocampus, as for it, is thought to play a critical role in contextual fear conditioning (Anagnostaras et al. 2001; Rudy 2009; Maren et al. 2013), and does not seem to be involved in cued auditory fear conditioning (Kim and Fanselow 1992; Phillips and LeDoux 1992, 1994). In the present study, although the testing cage was equipped with new visual cues and with a plastic floor for the retention test the animals might have partly recognized the initial training cage thus expressing contextual fear in addition to odor fear. This could explain why both ventral and dorsal parts of the hippocampus were activated during the recent memory test.

#### *Periaqueductal grey (PAG)*

The PAG has long been implicated as the organizer of the different behavioral components of the response to threat (LeDoux 2000; Tovote et al. 2016). Previous studies showed that activation of the ventrolateral periaqueductal gray (vIPAG) by the central amygdala (CeA) drives immobility and lesions of the vIPAG reduce the conditioned freezing response (LeDoux et al. 1988; Vianna et al. 2001), while expression of conditioned freezing is associated with increased vIPAG neuronal activation (Carrive et al. 1997). In a recent study, Tovote et al. (2016) identified an inhibitory pathway from the CeA to the vIPAG that produces freezing by disinhibition of vIPAG excitatory outputs to pre-motor targets in the magnocellular nucleus of the medulla. Our data confirm the involvement of the vIPAG during the retrieval of a recent odor fear memory that is accompanied by a high level of freezing in the trained animals.

#### ***Activations beyond the classically described fear conditioning network***

In the present study we found activation in several modules of the cerebellum. This is in accordance with the data reported in the literature showing that the cerebellum has functional connections with fear-related areas, including the PAG, the amygdala, and the prefrontal cortex (Koutsikou et al. 2014; Frontera et al. 2020; Vaaga et al. 2020). Nevertheless, only few studies have directly investigated cerebellar contributions to fear behavior. Thus, Pavlovian fear conditioning affects cerebellar plasticity (Sacchetti et al. 2004), and cerebellar lesions or inactivation modulate freezing response (Supple et al. 1987; Koutsikou et al. 2014). Activation observed in the present study in Crus 2 lobule, as also reported in a previous study (Litaudon et al. 2017), is in accordance with previous data showing that Crus 2 is activated by whisker stimulation (Sharp et al. 1989; Bosman et al. 2010) and by stimulation of the skin of the snout (Armstrong and Drew 1980), both being associated with sniffing. This result could also be related to the activations we observed in the somatosensory cortex including barrel field cortex. These activations might be related to whisker movements, which are coupled with sniffing during exploration of the environment (Welker 1964; Deschênes et al. 2012). Interestingly, Maier et al. (2020) analyzed the nose/nostril representation in the rodent somatosensory cortex using receptive field mapping and subsequent histological reconstruction. Their results reveal previously unknown cytoarchitectonic and physiological properties of the rodent nose somatosensory cortex, potentially enabling it to integrate multiple sensory modalities.

The other brain structure showing increased metabolism during the recent memory test was the striatum, and more specifically its dorsal part. Importantly, the medial posterior dorsal striatum receives inputs from the piriform cortex (McGeorge and Faull 1989) and the amygdala (McDonald 1991). The dorsal striatum has been suggested to be involved in aversive learning in general such as in auditory fear conditioning (Ferreira

et al. 2003; Kishioka et al. 2009; Wendler et al. 2014), or in a two-way active avoidance task (Darvas et al. 2011; Wendler et al. 2014) but not in contextual fear conditioning (Ferreira et al. 2003). Our present data are in line with results from Boulanger Bertolus et al. (2014) assessing the activity of the striatum in odor fear conditioning using 2-Deoxyglucose autoradiography. They revealed that, in paired rats, the medial part of the posterior striatum showed an increased activity compared to control animals. Thus, the present data extend the role of the dorsal striatum to the retrieval of a recent odor fear memory.

### **Remote memory network**

The remote memory network revealed using [ $^{18}\text{F}$ ]FDG uptake was limited to pPC. The present data are in accordance with studies showing the role of pPC in remote odor fear memory. Indeed, Hegoburu et al. (2014) assessed the role of the glutamatergic N-methyl-D-aspartate (NMDA) receptors in the BLA and pPC at discrete moments of odor fear acquisition. They showed that while NMDA receptors' activation in BLA at encoding is critically involved in odor fear acquisition, the same activation in pPC is not necessary for recent odor fear memory while it seems involved in remote memory. In addition, Sacco and Sacchetti (2010) found that excitotoxic lesions of pPC impaired remote, but not recent, fear memories in rats, leading the authors to suggest that sensory cortices support memory storage and retrieval of sensory stimuli that have acquired a behavioral salience with the experience. The privileged anatomical links between the amygdala and posterior piriform cortex (Majak et al. 2004; Sadrian and Wilson 2015) could explain the critical involvement of pPC compared to aPC, in remote odor fear memory. Projections from the BLA to the pPC may provide a pathway through which the amygdala can modulate cortical processing of olfactory information and



initiate plastic changes supporting the storage of the various attributes of the learned odor.

Surprisingly, the amygdala did not show an increased activity compared to control group. There is abundant literature showing that the amygdala is involved in fear expression, but very few studies have investigated its role over time in cued fear memories (Bergstrom 2016). In one of these studies the basolateral amygdala was lesioned 1-day, 7-days and 28-days following auditory cued Pavlovian fear conditioning (Maren et al. 1996). At each time point, the expression of cued fear was abolished. These data suggest the dependence of cued fear memory retrieval on amygdala function whatever the age of memory. In the present study, we would thus have expected an activation of the amygdala during remote memory retrieval. It could be the case that the amygdala was still activated, albeit at a lower level or for a very short period of time, that fell below the sensitivity of the [ $^{18}\text{F}$ ]FDG PET imaging technology. Indeed, we can imagine that for the recall of an old memory, the amygdala, even if it is still necessary, is involved in a transitory way and/or at a lower energy cost.

One less expected result was the lateralization of activation of the pPC. Lateralized activations of brain areas have been extensively reported in human in different tasks. Interestingly, in a review focused on olfactory processes, Royet and Plailly (2004) suggested that there is a dissociation of olfactory processes, with involvement of the right hemisphere in memory processes and the left hemisphere in emotional processes. Moreover, several studies have demonstrated that lateralization of cortical functions is not an exclusive feature of the human brain, and has been reported for a great variety of animal species (Corballis 2008). A few studies have investigated the lateralization of olfactory processes in rodents (Dantzer et al. 1990; Ods et al. 1994; Cohen et al. 2015; Cohen and Wilson 2017; Jozet-Alves et al. 2019). Specifically, such

a lateralization was recently shown in the rat anterior piriform cortex during an olfactory discrimination learning task (Cohen et al. 2015), with a predominance of the left hemisphere and a previous study using  $\mu$ PET also reported lateralized brain glucose metabolism (Litaudon et al. 2017). Regarding fear conditioning, several studies have reported a stronger involvement of the right amygdala (Baker and Kim 2004; Scicli et al. 2004; Kiyokawa et al. 2016).

## Limitations

The present study entails some limitations that need to be considered.

Indeed, some technical issues must be considered when using FDG PET. First, the temporal resolution of this technique is poor compared to that of other functional methods. Indeed, [ $^{18}\text{F}$ ]FDG is irreversibly trapped within tissues over the course of the uptake session, and the final PET image is the cumulative result of all brain activations occurring during the retention test, thus precluding to assess the time course of brain activation across the session. Second, the spatial resolution of this technique is limited, making it difficult to assess the activity of small brain areas. Despite these caveats, PET imaging has important advantages. First, compared to other imaging methods like fMRI and optical imaging that require the animal to be anesthetized or head-fixed during the whole procedure, probe uptake occurs while the animal is conscious and freely moving. Thus, this technique allows the examination of whole brain function with limited confounding effects of anesthesia even if a possible effect of anesthesia on FDG uptake during the imaging procedure could not be ruled out. Indeed, a few studies have reported that anesthesia reduces brain metabolism compared to awake animals (Toyama et al. 2004; Mizuma et al. 2010; Miranda et al. 2019). In our study, changes in brain metabolism related to behavioral response took place during the waking period and were therefore not affected by the anesthesia. Moreover, both experimental

groups (control and fear groups) were imaged in the same anesthetized conditions. Thus, even if anesthesia had an effect on FDG uptake during imaging session, it should be the same in both groups and largely removed by contrast analysis. PET should also be considered as a complementary approach to electrophysiological recording. Indeed, by assessing whole brain activity, even at low spatial resolution, this technique could help to identify otherwise unexplored areas of interest for subsequent electrophysiological recording.

## Conclusions

The present work allowed us to characterize for the first time, in the same animals involved in an olfactory cued fear conditioning paradigm, the networks activated during the recall of a recent vs remote amygdala-dependent memory. The data pinpointed to the drastic difference between the sizes of the two networks. These results bring strong support to the view that amygdala-dependent memories, like hippocampal-dependent memories, undergo system-level reorganization, and that sensory cortical areas might participate in the long-term storage of emotional memories.

Importantly, the two time points used in the present study to investigate recent and remote fear memory (1 day versus 30 days after learning) were chosen based on the existing literature on system consolidation. Indeed, following the seminal work of Bontempi et al (1999) investigating time-dependent reorganization of brain circuits underlying long-term storage of spatial memory, remote memory has been repeatedly tested 30-days after learning in rodents, and intermediate time points have been very seldom used until now. It is important to note that the neuroscience models that account for system consolidation are exclusively derived from the study of hippocampal-dependent memories. There are no such models for cued fear memory

retrieval (Bergstrom, 2016). This is the reason why we focused on time points classically used for hippocampal-dependent memories, our main aim being to investigate whether amygdala-dependent memory networks also show reorganization as time passes. However, investigating what happens at intermediate time point would be an important step further to describe how the transfer to the cortical areas is established over time.

Only very few studies have investigated rodent fear memory network using imaging methods initially developed for the human brain. Uselman et al. (2020) used manganese-enhanced MRI to analyze innate fear response to predator odor. In the only other study using PET imaging associated with auditory fear conditioning, Luyten et al. (2012) focused their analysis on the neurocircuitry of contextual anxiety. The present study went a step further in the investigation of the fear neural networks by questioning the existence of system-level reorganization using a longitudinal approach. The fact that we highlighted the neuronal network classically described in the literature for fear memory shows that the results obtained with FDG PET are reliable. In addition, by assessing the whole brain activity, our results extend the fear associated network to other less investigated structures.

Overall, our results demonstrate that PET imaging offers a powerful approach for neuroimaging in behaving animals. By enabling longitudinal studies thanks to serial brain imaging of the same subject, PET imaging supports the necessary reduction in the number of animals used in experimental procedures.

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**Declaration of interest:** None

**Data/code availability statement**

The data that support the findings of this study are available from the corresponding author, Philippe Litaudon, upon reasonable request.