

Where does the engram come from? Study of prefrontal cortex inputs during memory consolidation

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Abstract

The formation and storage of memories has been under deep investigation for several decades. Nevertheless, the precise contribution of each brain region involved in this process and the interplay between them across memory consolidation is still largely debated. Although memory encoding requires the hippocampus (HPC), it is becoming apparent that other brain regions are also important in early phases of memory formation, such as the medial prefrontal cortex (mPFC). In addition, *engram* cells have been found in the mPFC at memory encoding: these cells represent a close approximation of the “memory trace” insofar as they are activated at the time of learning, as they undergo enduring molecular modifications after learning, and as their reactivation drives memory recall. In contrast to HPC engram cells, mPFC engram cells are thought to be kept silent during a recent recall and until the memory is fully consolidated, becoming active only at remote recall. To explain the dynamics of these mPFC engram cells, we hypothesized that inputs to the mPFC could be differentially activated over the course of memory consolidation.

We first determined that the prelimbic cortex (PL), a subregion of the mPFC, was specifically activated during the encoding phase of a fear memory in mice by measuring the expression of the Immediate Early Gene cFos, transcribed upon neuronal activation. We then traced the inputs of PL cells that were active during encoding, the putative PL engram cells, in order to screen for relevant connections for further investigations. To achieve this, we used an activity-dependent monosynaptic retrograde tracing method based on rabies tracing. Then, to determine the activation patterns of these specific PL inputs, we measured their activity throughout consolidation of a contextual fear memory using an unbiased retrograde tracer coupled with cFos immunostaining. We further tested their functional relevance by chemogenetically inhibiting these projections during the different learning phases. This approach confirmed the roles of the Entorhinal Cortex and the Basolateral Amygdala inputs during the encoding phase of a fear memory to later recall remote memories. In addition, we found that the Claustrum (CLA) to PL projection was also required during the encoding phase, but to recall recent memories specifically. Moreover, the Insular Cortex (INS) to PL projection was necessary during recent recall. Eventually, we observed that the CLA and INS manipulations led to a modification of PL engram cells reactivation during recent recall.

Overall, our results suggest that there is a functional shift in PL inputs during the course of memory consolidation, which is also impacting how PL engram cells are reactivated. In addition, we observed that the activity of PL inputs is part of a broader brain network, both through the presence of axon collaterals targeting other brain regions and by the existence of parallel and perhaps redundant pathways that could ensure efficient memory consolidation.

Collectively, our data help to further refine the working model of memory formation by deciphering the interplay between brain regions during the process of systems consolidation of a fear memory, and open several questions for future investigations.

Keywords

Contextual fear conditioning, memory consolidation, prelimbic cortex inputs, engrams, spatiotemporal shift, claustrum, insular cortex.

Résumé

La formation et le stockage des souvenirs ont été largement étudiés depuis plusieurs décennies. Cependant, la contribution précise de chaque région du cerveau impliquée dans ce processus, et leur interaction au cours de la consolidation de la mémoire, restent largement débattues. Bien que l'encodage d'un souvenir nécessite l'hippocampe (HPC), il devient clair que d'autres régions du cerveau sont également importantes dans les premières phases de la formation d'un souvenir : c'est notamment le cas du cortex préfrontal médian (CPM). De plus, des cellules *engrammes* ont été trouvées dans le CPM : ces cellules particulières représentent une approximation de la mémoire biologique, puisqu'elles sont activées pendant l'apprentissage, qu'elles subissent des modifications moléculaires en conséquence de cet apprentissage, et que leur réactivation déclenche ce souvenir. A la différence des cellules engrammes de l'HPC, celles du CPM sont dormantes pendant le rappel d'un souvenir récent et jusqu'à ce que ce souvenir soit complètement consolidé, redevenant donc actives lors du rappel d'un souvenir ancien. Afin d'expliquer les dynamiques de ces cellules engrammes dans le CPM, nous avons émis l'hypothèse que les afférents du CPM pourraient être différenciellement activés au cours de la consolidation d'un souvenir.

Nous avons d'abord déterminé que le cortex prélimbique (CPL), une sous-région du CPM, était spécifiquement activé pendant la phase d'encodage d'un souvenir de peur chez la souris, en mesurant l'expression de la protéine cFos, produite suite à une activation neuronale. Ensuite, nous avons tracé les afférents des cellules du CPL qui étaient actives lors de l'encodage, donc les futures cellules engrammes, afin de présélectionner les connexions pertinentes pour nos investigations à venir. Pour y parvenir, nous avons utilisé une méthode de traçage rétrograde monosynaptique dépendant de l'activité neuronale préalable, basé sur le virus de la rage. Puis, pour déterminer les profils d'activation de ces afférents spécifiques du CPL, nous avons mesuré leur activité pendant la consolidation d'un souvenir de la peur conditionné au contexte, avec un traceur rétrograde couplé à la visualisation de la protéine cFos. Nous avons ensuite testé la fonctionnalité de ces afférents en les inhibant chimogénétiquement pendant les différentes phases de la consolidation du souvenir. Cette approche nous a permis de confirmer le rôle des projections du cortex entorhinal et du noyau basolatéral de l'amygdale pendant la phase d'encodage, pour le rappel d'un souvenir ancien. De plus, nous avons trouvé que la projection du claustrum (CLA) vers le CPL était aussi nécessaire pendant la phase d'encodage, mais uniquement pour le rappel d'un souvenir

récent. En outre, nous avons montré que celle du cortex insulaire (INS) vers le CPL était quant à elle requise spécifiquement pendant le rappel d'un souvenir récent. Finalement, nous avons observé que les manipulations des projections du CLA et du INS conduisaient à une modification de la réactivation des cellules engrammes pendant le rappel d'un souvenir récent.

Dans l'ensemble, nos résultats suggèrent qu'un changement fonctionnel s'opère au niveau des afférents du CPL au cours de la consolidation d'un souvenir, qui impacte aussi la façon dont sont réactivées les cellules engrammes. De plus, nous avons observé que l'activité des afférents du CPM fait partie d'un réseau neuronal plus large, à la fois par la présence d'axones collatéraux qui ciblent d'autres régions du cerveau et par l'existence de voies neuronales parallèles et potentiellement redondantes qui peuvent assurer la consolidation d'un souvenir.

Globalement, nos données permettent de parfaire le modèle actuel de la formation de la mémoire, en déchiffrant l'interaction entre les différentes régions du cerveau pendant la consolidation d'un souvenir, et ouvrent la voie à de futures investigations.

Mots-clés

Conditionnement à la peur lié au contexte, consolidation de la mémoire, afférents du cortex prélimbique, engrammes, changement spatiotemporel, claustrum, cortex insulaire.

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List of abbreviations

ACB	Nucleus accumbens
ACC	Anterior cingulate area
AD	Thalamus, anterodorsal part
AFC	Auditory fear conditioning
AM	Thalamus, anteromedial part
AON	Anterior olfactory nucleus
AUD	Auditory area
BLA	Basolateral amygdalar nucleus
BMA	Basomedial amygdalar nucleus
CA1	Ammon's horn field CA1
CA2-3	Ammon's horn field CA2-3
CFC	Contextual fear conditioning
CLA	Clastrum
CNO	Clozapine-N-oxide
COA	Cortical-amygdalar area
CP	Caudoputamen
CPP	Conditioned place preference
DG	Dentate gyrus
dCA1	CA1, dorsal part
DORpm	Thalamus polymodal association cortex related
DORsm	Thalamus sensory-motor cortex related
Dox	Doxycycline
DP	Dorsal peduncular area
DREADD	Designer receptor exclusively activated by designer drug
EC	Entorhinal area
ECT	Ectorhinal area
EP	Endopiriform nucleus
FRP	Frontal pole
FS	Fundus of striatum
GFP	Green fluorescent protein
GU	Gustatory area
HATA	Hippocampo-amygdalar transition area
HPC	Hippocampal formation
HY	Hypothalamus
IA	Inhibitory avoidance
IEG	Immediate early gene
IL	Infralimbic area
INS	Agranular insular area
IT	Indexing theory
LA	Lateral amygdalar nucleus
LD	Thalamus, laterodorsal part
LHb	Lateral habenula
LSX	Lateral septal complex

LTM	Long-term memory
LTP	Long-term potentiation
MBmot	Midbrain motor related
MBsen	Midbrain sensory related
MBsta	Midbrain behavioral state related
MD	Thalamus, medial dorsal part
MEC	Medial entorhinal cortex
MO	Somatomotor area
MOB	Main olfactory bulb
mPFC	Medial prefrontal cortex
MTT	Multiple trace theory
NRe	Nucleus Reuniens of the Thalamus
ORB	Orbital area
OT	Olfactory tubercle
P	Pons
PA	Posterior amygdalar nucleus
PAA	Piriform amygdalar area
PAG	Periaqueductal grey
PAL	Pallidum
PAR	Parasubiculum
PERI	Perirhinal area
PIR	Piriform area
PL	Prelimbic area
POST	Postsubiculum
PRE	Presubiculum
ProS	Prosubiculum
PTLp	Posterior parietal association area
PVT	Paraventricular thalamus
RSP	Retrosplenial area
RSPag	Retrosplenial cortex, agranular part
RSPg	Retrosplenial cortex, granular part
sAMY	striatum-like amygdalar nuclei
SSp	Primary somatosensory area
SSs	Supplemental somatosensory area
SUB	Subiculum
TEa	Temporal association area
TR	Postpiriform transition area
TT	Taenia tecta
vCA1	CA1, ventral part
VIS	Visual area
VISC	Visceral area
VTA	Ventral tegmental area

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Chapter 1 Introduction

Our brains are ultimately designed to remember. We are constantly learning, and then remembering: how to drive, where is the post office, who won the last soccer world cup, etc. All of our every-day actions are based on integrated previous knowledge, from the simplest action as drinking a glass of water, to complex geopolitical discussions at the dinner table. Eventually, the sum of our experiences, turned into memories, makes us who we are. This thesis aims to be a small stone to help understanding how the brain is able to remember.

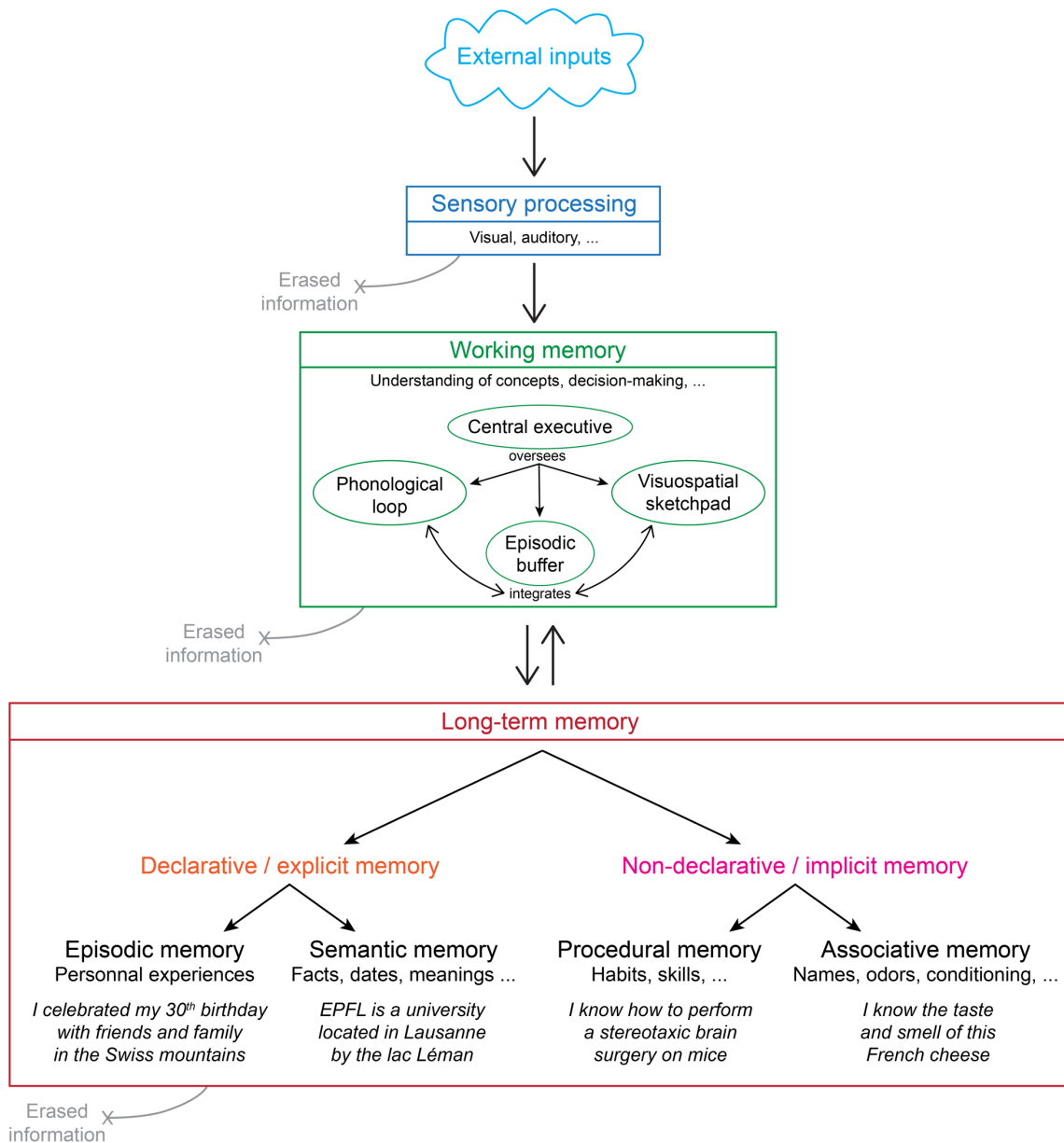
1.1 What is memory?

The brain is carefully wired to encode, integrate, sort, store and retrieve information, most of the time even without our conscious awareness. The term “memory” includes all these pieces of information of diverse nature (sensory feelings, spatial locations, salient events, motor sequences, ...) but also of diverse storage time, from immediate recall of a phone number just spelled out, to the recollection of a childhood event. Memories are therefore classified in different sub-types, each depending on a different set of brain regions (Camina and Güell, 2017). In this introduction, we will first focus on memory in humans, before turning to the use of animal models to investigate the mechanisms involved in memory formation and storage.

1.1.1 The different types of memory

The main distinction between the memory sub-types is the one based on the “age” of this memory, in other words whether if it was only retained in our mind while we needed this information, or if it had been stored in the brain for some time before being recalled. This classical delineation allows to separate working memory (or short-term memory, STM) from long-term memory (LTM). The memory subtypes and their interactions are summarized in **Figure 1.1**. Incoming information from external stimuli is first processed and integrated by the sensory system (Calvert et al., 2004), before being either erased if considered irrelevant (background noise, peripheral vision, ...), or sent to the working memory for further processing.

A Different types of memory



B An example of interaction between the different types of memory

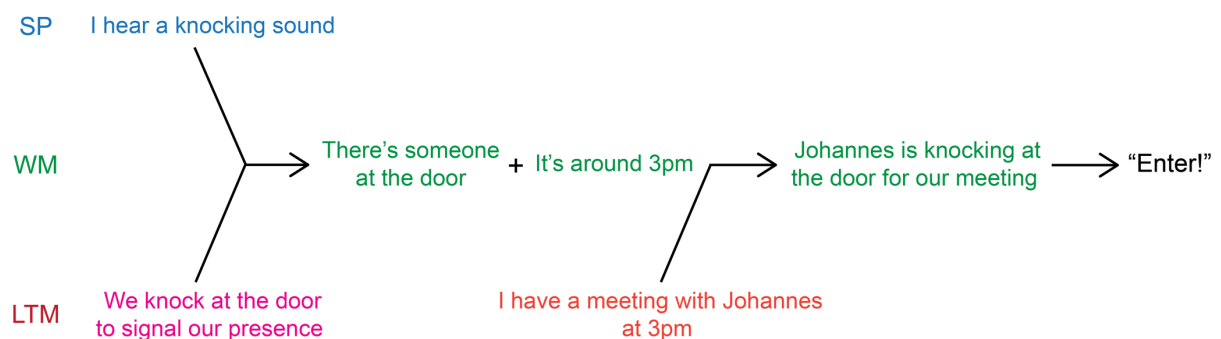


Figure 1.1. The different types of memory and their interactions.

(A) Overview diagram of the different memory systems and the main types of memory. **(B)** Example of the possible interactions between the memory subtypes, sensory processing (SP) in blue, working memory (WM) in green, and long-term memory (LTM) in pink (implicit memory) and orange (explicit memory).

1.1.1.1 Working memory, or short-term memory

Working memory is the memory system we use constantly, as it integrates incoming information from the environment and from relevant previous knowledge, and stores this information for a limited time to allow us to understand an oral explanation, make decisions or read this thesis. As such, it can be conceptualized as the brain system that allows “thinking” in the common sense of the term, and is therefore closely related to consciousness, although it also partially relies on unconscious processes (Velichkovsky, 2017). It is thought to be composed of 4 main components interacting with each other, as initially proposed and further developed by Baddeley (**Figure 1.1A**) (Baddeley, 2007; Baddeley and Hitch, 1974). The **central executive** has a supervision and attention role but does not store information itself, while the 3 other systems can: the **phonological loop** stores verbal and acoustic information, crucial for language acquisition notably; the **visuospatial sketchpad** creates and stores visuospatial representations of our surroundings or of mentally recalled locations; and the **episodic buffer**, which forms an interface between the above sub-systems and LTM, can store those interactions but only for a limited time and with a limited capacity. For a longer storage, the episodic buffer can transfer information to the LTM, in a process generally termed “memory consolidation”.

1.1.1.2 Long-term memory

LTM encompasses all of the information that can be retrieved with some delayed time after it has been originally experienced. It refers to both recent and remote memories that have left the working memory space and can be recalled later on. The nature of the stored memory defines further memory subtypes within the LTM (**Figure 1.1A**) (Camina and Güell, 2017).

Explicit memory

Explicit, or declarative memories, are memories that can be consciously evoked and verbally reported. Two types of explicit memories have been defined (**Figure 1.1A**): **episodic** and **semantic** memories (Tulving, 1972). Episodic memories are unique personal experiences (for example where and with whom I celebrated my 30th birthday) while semantic memories are learned facts (for example what is EPFL and where it is located) or meanings. Tulving described “semantic memory [as] the memory necessary for the use of language”, in the sense that this knowledge is required to choose the words we use based on their learned meaning. While episodic memories are subjected to distortion, interference and bias, semantic memories are thought to be more stable (Woike, 2008).

Implicit memory

Implicit memories, or non-declarative memories, are unconscious memories. They can be further divided in several categories (**Figure 1.1A**), the main ones being **procedural** memories (skills, habits, ... that initially required practice and later became automated, for example knowing how to perform a stereotaxic surgery) and **associative** memories (linking two initially unrelated items, such as a name and a face, or a taste with a cheese). Associative memories typically include classical conditioning, as popularized by Pavlov's dog and its salivary reflex: Pavlov consistently rang a bell before giving food to his dog, later resulting in the dog automatically starting to salivate when he heard the bell, even in absence of food (Pavlov, 1927). The other types of implicit memories include non-associative memory (habituation or sensitization), and priming. Implicit memories are therefore essential in every-day life by decreasing our conscious load while allowing us to perform known tasks, but are also responsible for cognitive biases as they predetermine our thoughts and actions.

1.1.2 Brain areas involved in the different types of memory

As seen with the example given in **Figure 1.1B**, all these types of memories constantly interact so that we can go through the day effortlessly (although some days require a little more effort than others). Observations of patients with specific memory impairments, either due to neurodegenerative diseases or brain lesions, enabled to identify the relationships and interdependence of some memories in their formation, storage or retrieval, thus underlying the brain networks involved in these processes.

The classical example of patient H.M. (Scoville and Milner, 1957) was foundational in the understanding of memory brain processes. This patient suffered from strong epileptic seizures originating from a brain region called the **medial temporal lobe** (MTL), and he underwent a surgery to remove most of this area. The seizures were reduced and his recovery was "uneventful, except from [a] grave memory loss". This memory loss was not homogeneous: while he could remember past events from his childhood, he was unable to recall any event since after his surgery, and even that he had a surgery. However, his overall intelligence was unaffected, as well as language or motor functions, but he could not learn new words. Surprisingly, he could still acquire some new motor skills such as mirror-drawing (Dossani et al., 2015). This fascinating case study lasted for 50 years until H.M. death at 82 years old in 2002, and helped delineating the role of the MTL in memory, together with many following studies of amnesic patients (Cohen and Squire, 1980).

As we can deduce from H.M.'s case, both episodic and semantic memories rely on the MTL overall and the **hippocampus** (HPC) in particular (Graf and Schacter, 1985). Although they share HPC function during memory formation, they differ in the other brain areas involved in their encoding and maintenance. Semantic memories rely on **sensory-motor cortices**, as well as on the **inferior parietal** and **temporal** lobes to bind different modalities together, orchestrated by the **frontal cortex** (Binder and Desai, 2011); alternatively, episodic memories require cortical areas related to the HPC such as the **entorhinal cortex** (EC) and the **perirhinal cortex** (PERI), as well as the **frontal cortex** for long-term storage (Pause et al., 2013).

Working memory relies on a distributed network of brain regions, but not including the HPC: the phonological loop is involved the **inferior parietal** and **superior temporal** cortices in the left hemisphere (Buchsbaum and D'Esposito, 2008), the visuospatial sketchpad implicates the **ventral** and **parietal areas** in the right hemisphere (Baddeley, 2007), and the central executive relies on the **frontal cortex** for most cognitive tasks (Duncan and Owen, 2000).

Interestingly, the frontal cortex appears to be involved in several different types of memories, from working memory to retrieval of explicit memories. This central position in diverse functions makes it of particular interest in understanding the interplay between memory types. We will go more in depth in the role of the frontal cortex and the medial prefrontal cortex in particular during episodic LTM in the third section of this chapter.

1.1.3 Studying memory in animals

The use of animal models to study learning and memory offers the straightforward advantage to allow more invasive experiments and therefore give more precise results about the networks and cells involved in memory. However, it raises the question of the relevance of such animal models. To answer this, it is interesting to look at the evolution of memory systems.

1.1.3.1 Evolution of memory systems

Intuitively, we can suppose that all animals can form memories, as it seems to be an evolutionary interesting trait for an efficient survival. However, this does not mean that animal memories are comparable to human memories, in their content or in the mechanisms of their formation and storage.

Anatomically speaking, there is a high homology of the main structures involved in memory processes. Notably, the HPC is highly conserved across mammals as well as reptiles, but

analog structures are also found in amphibians and fish, suggesting an early apparition and selection of this structure (Allen and Fortin, 2013). Similarly, the parahippocampal region (including notably the entorhinal and perirhinal cortices, entry points in the hippocampal network) and the prefrontal cortex are present in mammals and birds, although less developed than in humans. Theoretically, mammals and birds have the necessary brain structures to support human-like LTM formation.

Functionally, we have to distinguish between different types of memory. Overall, memory is not straightforward to test in animals, as they obviously lack speech, but this is particularly the case with working memory (Carruthers, 2013). If working memory seems to be shared with primates to some extent, it is harder to assess its importance in other mammals in comparison to humans. However, episodic memories have been widely tested in animals (Allen and Fortin, 2013). It was shown that for instance magpies (Zinkivskay et al., 2009), mice (Dere et al., 2005) and great apes (Martin-Ordas et al., 2010) all share the capacity to form episodic-like memories. Interestingly, mammals do not only share a structurally homolog hippocampus, but also functionally. Indeed, rodents and primates share a conserved hippocampal memory coding strategy (Thome et al., 2017). Some forms of LTM were also reported in worms (Tomchik and Davis, 2013) and flies (Ardiel and Rankin, 2010), confirming the presence of memory in most of the animal kingdom.

Genetically as well, there appears to be a large homology in genes related to learning and memory (Bajajfer et al., 2021). For instance, the CREB family (c-AMP Response Element Binding, a family of transcription factors involved notably in the expression of genes important for memory formation, see below section 2.1 (Silva et al., 1998)) can be found in all organisms from humans to fish (Wang et al., 2019b), suggesting that genetically and molecularly as well, memory mechanisms could be evolutionary conserved.

1.1.3.2 Memory tasks in animals

As it is obviously harder to test memories in animals compared to humans, scientists have developed standardized tests and measures to approximate their memories. Here, we will focus on LTM in rodents. We can note that the distinction between explicit and implicit memories loses its relevance.

Conditioning tasks are widely used, and are based on the association of two unrelated pieces of information such as a bell sound and food for Pavlov's dog, where the successful association can be measured by the salivary reflex. Likewise, many types of association tests have been

developed. Typically, a neutral conditioned stimulus (CS, a context, a sound, an odor, ...) is paired with an unconditioned stimulus (US, that can be negative, such as a footshock, or positive, such as a food reward). After an initial learning phase, the animals will modify their reaction to the CS alone, according to the new valence that is associated with it, and it is this specific change of behavior that is measured as a proxy for memory retention. Classically, fear conditioning triggers robust learning in rodents, and is found in two main versions: **contextual fear conditioning** (CFC, where a context is paired with footshocks), or **auditory fear conditioning** (AFC, where a tone is paired with footshocks). Although similar, it appears that these two paradigms are relying on only partially overlapping brain regions and circuits (Onishi and Xavier, 2010). This, added to the fact that these conditioning paradigms exist in a multitude of slightly different protocols (timing, shock intensity, tone frequency, pre-exposure, ...) (Wehner and Radcliffe, 2004), results in a sometimes contradictory literature. The use of more standardized protocols would help in unifying research results, nevertheless it already points towards the complexity of memory processes in the brain.

Spatial memories are widely and easily tested in rodents, and many types of maze-based tests exist (Vorhees and Williams, 2014). Among them, we can cite the **Morris Water Maze** (Morris, 1981), in which animals learn to locate a hidden rescue platform in a pool filled with an opaque liquid, and the **radial arm maze** (Olton and Samuelson, 1976), with arms radiating around a center point, at the end of which animals can find food rewards, whereby testing working spatial memory.

Discrimination and recognition tasks can also be used. Animals can be motivated and trained to discriminate between two odors, sounds or visual cues, or identify a whisker deflection, in order to get a reward (Broadbent et al., 2007). This type of test can require extensive learning time, therefore automated systems have been developed to optimize these protocols and rely on self-initiated learning (Bernhard et al., 2020; Treviño et al., 2018). On the contrary, object recognition tasks can be also used to assess recognition memory but are easily tested and rely on natural curiosity rather than reward motivation. The **novel object recognition** task consists in presenting an animal with two new objects and let it explore them. Later, the animal is presented again with two objects, one old and one new: If the animal recognizes and remembers the old object, it will rather explore the new one (Grayson et al., 2015).

All those standardized tests allow to assess different aspects of memory in rodents, from working memory to LTM. Even if the ethological relevance of some of these tests is not always

straightforward, they can help deciphering the brain regions and mechanisms involved in each type of memory tested.

Together, the close evolution of memory systems and the possibility to test memory in animals support the relevance of animal models in memory research, and notably of mammals for more complex memories. Accordingly, an impressive number of studies on memory have been carried out on animals in the past century, enabling us to better understand how the brain functions. We will now dive into the brain itself, and the cell populations involved in memory encoding and storage.

1.2 How can brain cells encode and store memory?

The understanding of neurons being individual brain cells, as opposed to a continuum within the whole nervous system, originates from Ramon y Cajal's observations using Golgi staining (Ramon y Cajal, 1894). He confirmed the "neuron theory" proposed by Waldeyer (Waldeyer, 1891), and opened up the way for modern neurobiology.

1.2.1 Cellular substrates of memory

Investigations on memory at the scale of cells and neurons was done in a large variety of animal models and behavioral settings, from sea snails (*Aplysia*) to rodents (mainly mice and rats but also rabbits) via fruit flies (*Drosophila*). Pioneer experiments described instances of synaptic plasticity at the cellular level: after an initial neuronal activation step, neurons changed their firing properties, which could potentially give a cellular readout of memory (Kandel, 2001; Lynch, 2004). Again, the distinction between fast and slow processes proves relevant to delineate the different mechanisms at play, with one main feature being that short-term synaptic plasticity is independent of protein synthesis, while long-term synaptic plasticity is not (see **Figure 1.2**).

1.2.1.1 Short-term plasticity

Memory can be studied in *Aplysia* using an innate withdrawal reflex that can be sensitized (and thus increase its amplitude), in a process called long-term facilitation (LTF) that is at first independent of protein synthesis (Schwartz et al., 1971). It was found that initial modulatory neurotransmitter release at the sensory to motor neuron synapse acts on presynaptic receptors to enhance further neurotransmitter release (therefore facilitating the next behavioral response), in a process mediated by cAMP and protein kinases such as PKA. Following the initial Ca^{2+} influx upon stimulation, these phosphorylation cascades happen at the level of the synapse to strengthen specific connections, relying on post-translational modifications and rearrangements of preexisting proteins, and therefore independently of protein synthesis (Kandel, 2001).

Similarly, electrophysiology studies of the rabbit HPC revealed that these synapses can be potentiated as well, in an activity-dependent process called long-term-potentiation (LTP). Following a high frequency stimulation of presynaptic fibers, post-synaptic cells fired stronger and longer than before (Bliss and Lomo, 1973). LTP was also established *in vivo* (Bliss and Gardner-Medwin, 1973), and in several different brain regions (Clugnet and LeDoux, 1990),

suggesting a widespread phenomenon in mammals, and was later linked to spatial learning in the HPC (Morris et al., 1986). The first phase of LTP, early-LTP, does not require protein synthesis, but needs initial NMDA receptor activation to initiate signaling cascades, notably activating CaMKII (Calcium calmodulin kinase II) which mediates postsynaptic rearrangement of preexisting proteins in order to enhance subsequent synaptic transmission (Baltaci et al., 2019; Collingridge et al., 1983). LTP has a negative counterpart, long-term depression (LTD), which is necessary to prevent neuronal networks from getting saturated.

In both of these examples, the initial modifications giving rise to short-term plasticity by modifying the strength of synaptic connections (**Figure 1.2**), and are also necessary to trigger the second enduring phase of plasticity, relying on new protein synthesis.

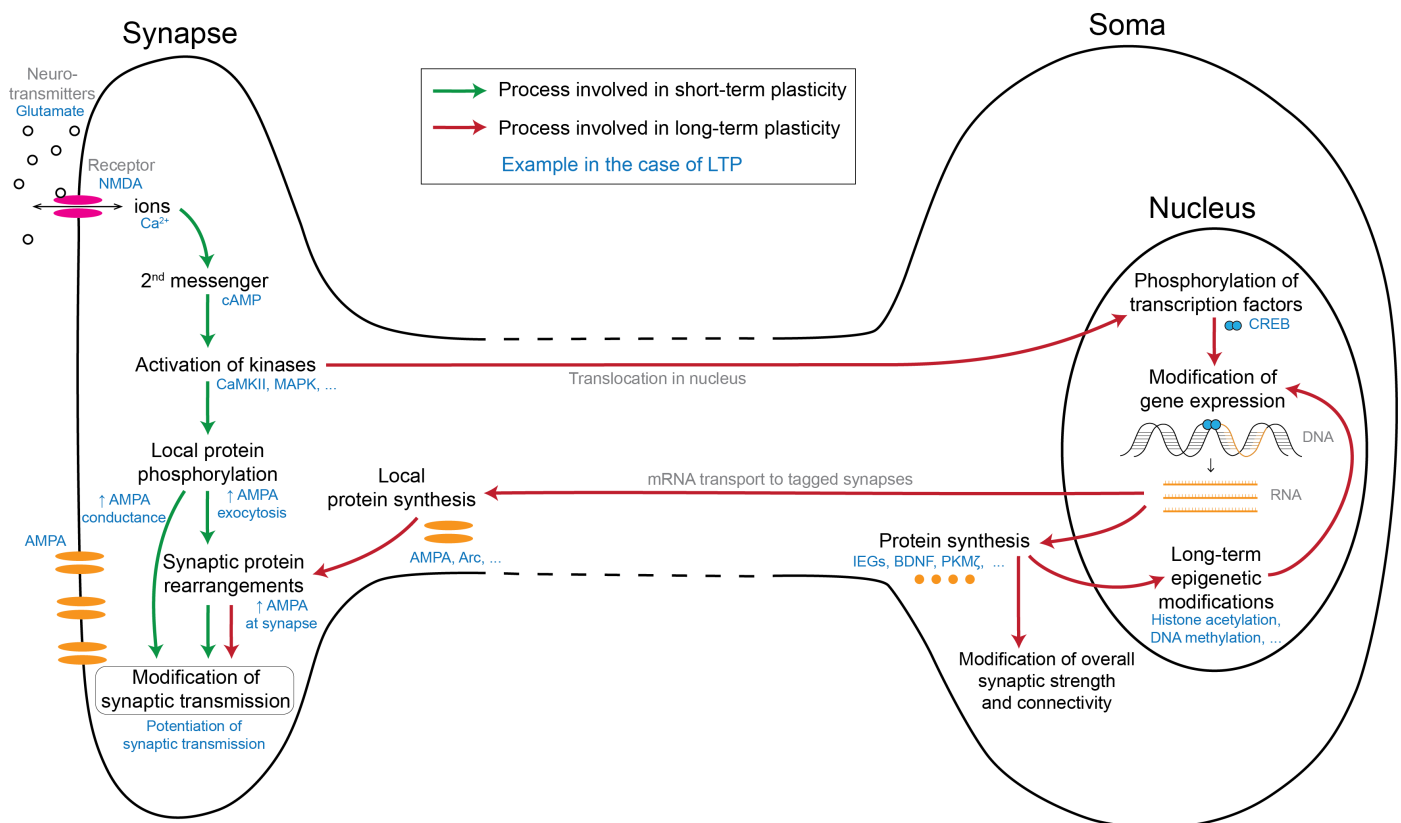


Figure 1.2. General mechanisms involved in synaptic plasticity.

Following transmembrane receptor activation by neurotransmitter binding, signalling cascades are triggered resulting in fast short-term plasticity (green arrows) confined to the synapse, and slow long-term plasticity involving nuclear processes (red arrows). In blue, examples of molecular processes in the specific case of LTP. Note that the general processes apply to other types of synaptic plasticity, whether pre- or post-synaptic, potentiating or depressing synaptic transmission.

1.2.1.2 Long-term plasticity

The fact that long-term plasticity depends on protein synthesis suggests that molecular modifications triggered by short-term plasticity induction reach the nucleus to express specific genes. In the withdrawal reflex in *Aplysia*, it was shown that short-term plasticity-induced kinases (PKA but also MAPK, mitogen-activated protein kinase) are translocated to the nucleus, where they activate the transcription factor CREB-1 (cAMP Response Element Binding protein 1) which then elicits a transcriptional cascade (Kandel, 2012). In parallel, CREB-2 as a transcription repressor has to be inactivated (Abel et al., 1998). The CREB complex therefore acts as an integrator of multiple external stimuli and appears central in the transition from short- to long-term plasticity (Kandel, 2012). Similar observations were made in mammals, where MAPK also triggers CREB activation (**Figure 1.2**) (English and Sweatt, 1997; Impey et al., 1998).

The discovery of genes linked with behavior was pioneered in *Drosophila* by Seymour Benzer and his colleagues (Benzer, 1971), and the very first gene related to memory was part of the cAMP cascade (Dudai et al., 1976). Following that, many genes necessary for long-term plasticity were uncovered, among which several transcription factors including CREB, some synaptic scaffolding proteins such as synaptotagmin in the presynaptic compartment, or PSD-95 in the post-synaptic one (Buchs and Muller, 1996), as well as immediate early genes (Guzowski et al., 1999; Jones et al., 2001; Link et al., 1995). However, the requirement of nuclear transcription during the late phase of LTP suggests that these transcriptional modifications would impact the cell as a whole, and not only the initially potentiated individual synapses. Interestingly, some synaptic tagging mechanisms have been discovered allowing to keep the late phase of LTP (or LTF in *Aplysia*) synapse-specific (Frey and Morris, 1997; Martin et al., 1997), and some local protein synthesis directly at the synapses has been reported (Holt and Schuman, 2013). The correlation between LTP induction and memory has been repeatedly shown (Guzowski et al., 2000; Jones et al., 2001; Morris et al., 1986), giving rise to the synaptic theory of memory (Caroni et al., 2014; Mayford et al., 2012). However the question whether LTP is memory itself is still debated (Eichenbaum, 1996; Gerlai, 2002; Stevens, 1998).

In parallel, epigenetic changes following LTP induction were discovered, providing an interesting long-term storage mechanism for memories (Guan et al., 2002). Indeed, epigenetic modifications are dynamically maintained and are thus more stable than dendritic spines, which have a high turn-over rate (Fornasiero et al., 2018; Pfeiffer et al., 2018). For instance,

DNA methylation, histone methylation and histone acetylation have been linked with the regulation of memory formation (Guan et al., 2009; Jarome et al., 2014; Kim and Kaang, 2017).

Findings of cellular modifications supporting long-term plasticity induction and maintenance have helped deciphering the molecular cascades involved in these mechanisms. However, the exact storage place of cellular memory is still unknown. Interestingly, taking a step back from molecules to look at cell ensembles has recently allowed to get closer to memories.

1.2.2 Engram cells

The concept of *engrams* as biological substrates of memory originally comes from Richard Semon, in his book *The Mneme* (Semon, 1921). His proposed definition of this physical memory substrate and his vastly developed memory theory proved to be surprisingly in agreement with what neuroscientists later discovered, and is still relevant today. Interestingly, his background as an evolutionary zoologist is reflected in the fact that his theory also applied to other organisms such as plants, and encompassed inherited or transgenerational memories as well. Here we will focus on how this theory applies to LTM and helps guiding memory research in mammals.

1.2.2.1 Definition(s)

Semon defined the engram as “the enduring though primarily latent modification of the irritable substance produced by a stimulus” and enunciated two fundamental mnemonic laws which originated from experimental observations: (i) The *law of engraphy* postulates that “All simultaneous excitations [from a given experience] within an organism form a coherent excitation-complex which acts engraphically; that is, it leaves behind it a connected engram-complex, constituting a coherent unity”. (ii) The *law of ecphory* posits that “the partial recurrence of the excitation-complex [from the original experience], which had left behind it an engram-complex, acts ecphorically on the latter”. In between the engraphic action of the stimulus and its later ecphory, the engram enters a latent or dormant state and requires the action of ecphoric influences to be excited again.

In our contemporary words, the law of engraphy governs memory encoding, while the law of ecphory relates to memory retrieval. Our knowledge about neurons as brain cells, their mode of communication and their molecular properties allowed to refine and further develop Semon’s memory theory. Although this concept was largely forgotten until recent years, we now use the term of *engram cells* to define a population of neurons fulfilling 3 criteria (Tonegawa et al.,

2015): (i) they must be activated by learning; (ii) they have to undergo persistent cellular and/or molecular modifications as a consequence of learning; and (iii) their reactivation need to result in the recall of the original memory. These 3 criteria can be used to design experiments in order to identify engram cells.

1.2.2.2 Searching for engram cells

Now that we have defined 3 criteria to consider a cell to be an engram cell, we can look into the techniques available to test them. For a comprehensive review of the possible ways to investigate engrams, see Josselyn et al. (2015) or Josselyn & Tonegawa (2020).

Engram cells are activated during learning

To assess neuronal activity, several techniques are available (summarized in Box 2 in section 3 of this chapter) to either visualize this activity live, or later in time.

Live techniques include electrophysiology, to directly monitor electrical activity of one or a group of neurons (Lynch and Schubert, 1980; Tao et al., 2015), or calcium imaging, using the genetic expression of a fluorescent proxy to monitor intracellular Ca^{2+} variations, known to correlate with neuronal electrical activity (Nakai et al., 2001; Yang and Yuste, 2017). These techniques require invasive measurement systems, but can reach high temporal and spatial resolution.

Delayed techniques mainly rely on the expression of specific genes, called immediate early genes (IEGs). IEGs are transcribed upon neuronal activation, and their expression is limited in time, offering a way to visualize neurons after they have been activated by looking at the RNA or protein expression of those IEGs (Guzowski et al., 1999; Pérez-Cadahía et al., 2011). Using such techniques, temporal resolution is limited, but cellular resolution is maintained. Stemming from this, conditional reporter expression using IEG promoters have being developed, the two main ones being the Tettag system (Reijmers et al., 2007) and the TRAP system (DeNardo et al., 2019a; Guenthner et al., 2013) (see Box 2 section 3 for details). This allows a much-delayed visualization of cells activated during the initial learning phase, and can also be later used to assess the reactivation of these cells or to manipulate their activity (see below section 2.2.3).

Engram cells undergo persistent cellular or molecular changes

An insight on the nature of the persistent changes after learning defining the engram could come from the identification of the precise sub-cellular localization of the memory within

engram cells which is a highly dynamic field within memory research. The non-engram findings of molecular and cellular memory substrates (see above section 2.1 in this chapter) could theoretically be extended to engram cells. Although there is so far no report that LTP occurs specifically in engram cells (Lisman, 2017), enhanced synaptic plasticity at HPC engram to engram synapses has been found using an elegant GRASP-based technique (GFP reconstitution across synaptic partners) (Choi et al., 2018), and plasticity of engram cells was shown to be synapse-specific (Abdou et al., 2018). In addition, CREB has been demonstrated to be required for memory allocation (Han et al., 2009) as well as memory consolidation in engram cells (Matos et al., 2019), further supporting the idea that engram cells could share at least some of the molecular mechanisms of LTP.

To investigate the transcriptional and epigenetic modifications induced by learning in engram cells, engram cell sorting followed by various sequencing methods (RNA-sequencing, ATAC-sequencing (Assay for transposase accessible chromatin), ChIP-sequencing (Chromatin immunoprecipitation), etc.) after learning is now possible (Fernandez-Albert et al., 2019; Marco et al., 2020; Rao-Ruiz et al., 2019). These studies can yield large amounts of data and offer a comprehensive overview of the mechanisms at play during memory formation and retrieval, specifically in engram cells, as well as in targeted cell-types.

Engram cell reactivation triggers memory recall

The third criteria to define whether a cell is an engram cell or not is linked to Semon's law of ecphory, but goes beyond: not only engram cells should be reactivated upon recall, but their reactivation must trigger memory recall. This concept can be tested by two approaches, either by an inhibition of these cells which should prevent natural recall (loss-of-function), or by an activation that should trigger recall (gain-of-function). To restrict such approaches to engram cells, the targeted genetic expression of exogenous modulators of neuronal activity have been widely used. These modulators can be of chemogenetic or optogenetic nature.

The most widely used chemogenetics modulators are GPCR-based (G-protein coupled receptor) engineered receptors called DREADD: Designer receptor exclusively activated by designer drug (Roth, 2016). DREADDs exist in two main variations: an inhibitory DREADD, hM4Di, coupled to a Gi protein that activates inward-rectifying potassium channels (GIRKs) to induce hyperpolarization and inhibits pre-synaptic neurotransmitter release; and an excitatory DREADD, hM3Dq, coupled to a Gq subunit, which induces intracellular Ca²⁺ release and thus potentiates neuronal firing (Alexander et al., 2009; Armbruster et al., 2007). DREADDs can be activated by systemic or local injection of Clozapine-N-oxide (CNO), an inert drug that can

cross the blood-brain barrier (Bender et al., 1994) and bind DREADD receptors to activate them. Their kinetics is therefore relatively slow, and the resulting neuronal inhibition or activation lasts for around an hour (Smith et al., 2016).

The field of optogenetics has rapidly evolved in the past decades, giving rise to a variety of tools, most of which being derived from bacterial opsins, transmembrane ion channels that are activated by light of a specific wavelength (Deisseroth, 2015; Zhang et al., 2010). Classically used, channelrhodopsin is an excitatory opsin activated by a 473nm wavelength, leading to positively charged ions (Na^+ , K^+ , Ca^{2+} , H^+) influx and therefore depolarizing neurons (Boyden et al., 2005); and halorhodopsin is an inhibitory opsin activated by a 589nm wavelength triggering Cl^- influx in neurons and therefore hyperpolarizing them (Gradinaru et al., 2008). Therefore, the use of optogenetic tools require the invasive implantation of an optic fiber above the brain area of interest to manipulate neurons, but can reach millisecond precision (Deisseroth, 2015).

Both these techniques were reported to yield equivalent effects on neuronal firing, however the duration of these effects is much longer and less targeted in time using DREADDs (Roth, 2016).

Triggering memory recall by artificial neuronal activation can be evaluated by measuring the animal's behavioral response to the activation. However, whether an artificial recall is equivalent to a natural or physiological recall is hard to determine: would the animal actively recall its past experience in a conscious way? Or would its brain drive an unconscious and uncontrolled behavioral response?

1.2.2.3 Where have engrams been found?

Semon already extensively dissertated about the localization of engrams in *The Mneme* (Semon, 1921), and from clinical observations concluded that the engrams were located “for Man and higher Vertebrata in the cerebral cortex”. However, he began this observation by stating that “not every cell [...] is in possession of the entire stock of engrams acquired during the individual organic life”, hinting towards the idea of sparse engram distribution. Engram cells have been found in several different brain areas: For each region, we will give a brief overview of some of the main engram findings related to fear memories (**Figure 1.3**; for more details, see Josselyn & Tonegawa, 2020; Tonegawa et al., 2018).

As expected from classical memory experiments, the HPC and notably the **dentate gyrus** (DG) has been at the heart of engram research. DG cells have been shown to be specifically

reactivated between encoding and recent recall (Tayler et al., 2013), and learning triggered an increased excitability in DG activated cells which was crucial for memory recall (Pignatelli et al., 2019). Then, inactivation of cells active during learning impaired recall (Denny et al., 2014), and their activation in a neutral context triggered recall (Liu et al., 2012; Ramirez et al., 2014), even after the extinction of the fear memory (Yoshii et al., 2017). All criteria are then fulfilled, demonstrating that the DG hosts an engram cell population able to carry fear memory.

The amygdala is also part of the MTL, and has been implicated in the processing of emotional components of memories. Notably, the **basolateral amygdala** (BLA) is activated by fear learning and recall (Choi et al., 2018; Silva et al., 2018), cells active at encoding undergo synaptic plasticity modifications (Nonaka et al., 2014) and are highly reactivated at recent recall (Reijmers et al., 2007; Tayler et al., 2013). Reactivation of these cells triggered recall in a neutral context (Kim et al., 2014; Redondo et al., 2014), while their inhibition prevented fear recall (Gore et al., 2015). Taken together, we can conclude that the BLA hosts an engram cell population as well.

The **retrosplenial cortex** (RSP), traditionally implicated in spatial memories, is activated by contextual fear memory encoding (Baumgärtel et al., 2018), shows a high neuronal reactivation between encoding and recall of a CFC (Tayler et al., 2013), and cells active at encoding can trigger recall in a neutral context (Cowansage et al., 2014; Wang et al., 2019a). Activated cells-specific cellular modifications after learning have not been shown yet, but it is clear that the RSP overall undergoes changes in plasticity following learning (Todd and Bucci, 2015), so that we can conclude that the RSP most likely contains engram cells carrying fear memories.

The **medial prefrontal cortex** (mPFC), part of the frontal cortex, has recently been shown to contain engram cells that are activated during learning, reactivated at recall, and which reactivation can drive fear memory (DeNardo et al., 2019a; Matos et al., 2019; Tang et al., 2005; Tonegawa et al., 2018; Zelikowsky et al., 2014). We will go more in depth in the mPFC engram findings in the next section (1.3.5), as they have specific features that makes them of particular interest.

Overall, it is crucial when investigating memory engrams to keep in mind the distributed nature of this engram population, and the likely importance of the dynamic interplay between brain regions, whether they have been reported to contain engram cells or not (**Figure 1.3**). We will now focus in more details on the mPFC and its role in memory consolidation.

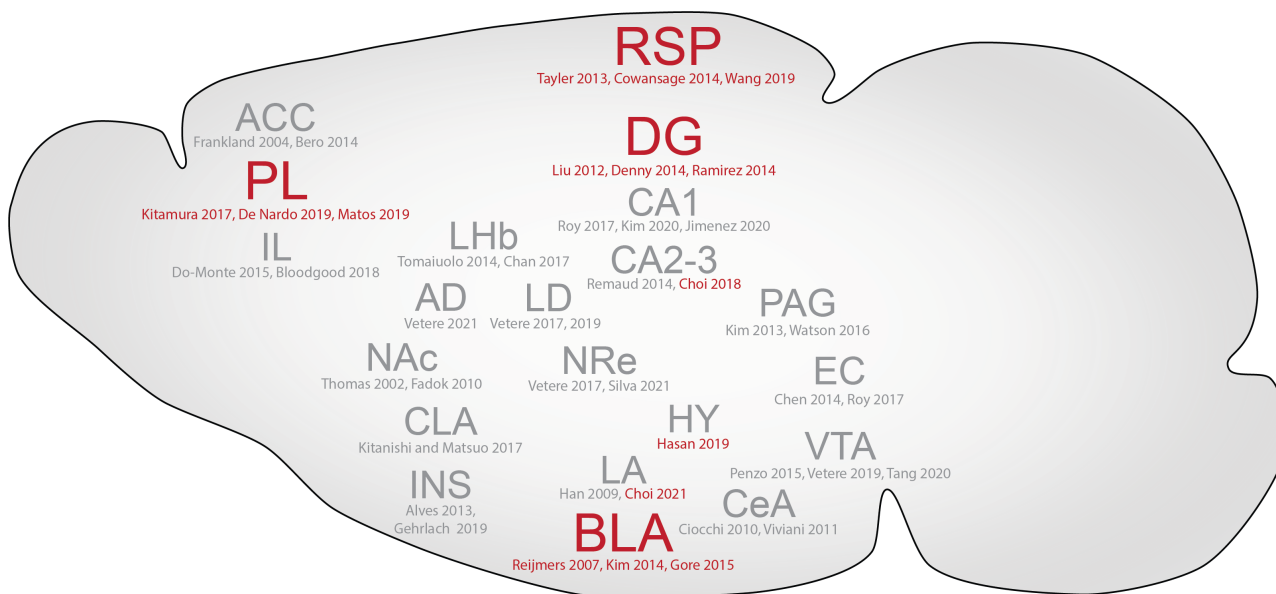


Figure 1.3. Where have engrams been found?

Brain regions involved in fear memory formation and/or retrieval (in grey) in mice, and the ones that have been reported to contain engram cells (in red). Note that for a few regions, some studies have an engram cell resolution (noted in red as well), but they do not provide enough evidence by themselves to confirm the existence of an engram cell population.

ACC: Anterior Cingulate Cortex; AD: Antero-dorsal Thalamus; BLA: Basolateral Amygdala; CeA: Central Amygdala, CLA: Claustrum; DG: Dentate Gyrus; EC: Entorhinal Cortex; LHb: Lateral Habenula; HY: Hypothalamus; IL: Infralimbic Cortex; LA: Lateral Amygdala; LD: Latero-dorsal Thalamus; NAc: Nucleus Accumbens; NRe: Nucleus Reuniens of the Thalamus; PAG: Periaqueductal Gray; PL: Prelimbic Cortex; RSP: Retrosplenial Cortex; VTA: Ventral Tegmental Area.

1.3 The medial prefrontal cortex and fear memory: dynamics, connectivity and engrams

This section represents the following published review article which has been formatted to fit with the style of the thesis:

Dixsaut L, Gräff J. The Medial Prefrontal Cortex and Fear Memory: Dynamics, Connectivity, and Engrams. *International Journal of Molecular Sciences*. 2021; 22(22):12113.

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Author contribution: I researched and wrote this review with discussion and editing provided by Johannes Gräff.

1.3.1 Abstract

It is becoming increasingly apparent that long-term memory formation relies on a distributed network of brain areas. While the hippocampus has been at the center of attention for decades, it is now clear that other regions, in particular the medial prefrontal cortex (mPFC), are taking an active part as well. Recent evidence suggests that the mPFC – traditionally implicated in the long-term storage of memories – is already critical for the early phases of memory formation such as encoding. In this review, we summarize these findings, relate them to the functional importance of the mPFC connectivity, and discuss the role of the mPFC during memory consolidation with respect to the different theories of memory storage. Owing to its high functional connectivity to other brain areas subserving memory formation and storage, the mPFC emerges as a central hub across the lifetime of a memory, although much still remains to be discovered.

1.3.2 Introduction

The understanding of memory processes in the brain has been at the heart of neuroscience research for more than a century, and a lot has been discovered since. We now know about brain regions, circuits, cells, synapses, electrophysiological, and molecular properties that are important for specific aspects of memory formation and storage. However, we are still quite far from understanding how precisely memories are created, and how and where they are stored for years so that we can still, for example, remember the house we grew up in.

The hippocampal formation (HPC) was the first region to be linked with episodic memory formation, with reported clinical cases of anterograde amnesia following HPC damage (Scoville and Milner, 1957). This role was further confirmed in rodent models by observing specific neuronal activation at precise timepoints in the life of a memory, as well as by manipulating subsets of HPC neurons (Josselyn et al., 2015). From decades of research into the mnemonic role of the HPC, it is now firmly established that the HPC is necessary for the encoding of new associative memories, including fear memories, as well as for their early recall. This role was recently supported by the discovery of engram cells in the HPC. These neurons are first activated during the initial learning phase, undergo enduring molecular changes (cellular consolidation), and are reactivated during the recall of that same memory. In addition, the artificial reactivation of this neuronal ensemble triggers recall (Liu et al., 2012; Ramirez et al., 2013; Tonegawa et al., 2015).

At the same time, we now also know that other brain areas are necessary during the process of memory formation and storage. For instance, it has for a long time been established that older memories are no longer stored in the HPC, but rather rely on cortical areas. Specifically, the medial prefrontal cortex (mPFC, see Box 1 for definition) was reported to be necessary for the recall of remote fear memories (Bontempi et al., 1999; Frankland et al., 2004a). These findings gave rise to the classical model of memory formation, which posits that the initial formation and storage relies on the HPC, and that the mPFC is only recruited for longer storage (Frankland and Bontempi, 2005).

Surprisingly, the mPFC was recently identified as crucial already during the early phases of learning using molecular or optogenetic manipulations (Bero et al., 2014; Einarsson and Nader, 2012; Rajasethupathy et al., 2015; Rizzo et al., 2017). Moreover, an mPFC engram population was identified already at learning, which could be reactivated to regulate memory at later times (Kitamura et al., 2017; Matos et al., 2019). These findings urge us to reconsider the classical model of memory formation, and to further investigate the more complex role of the mPFC during fear memory consolidation.

In this review, we will summarize the available experimental data around the involvement of the mPFC in memory processes across consolidation, highlighting its importance during the entire lifetime of a memory. Then, we will consider the mPFC as a hub within a brain-wide network for memory storage and highlight its functional connectivity and engagement during this process. Lastly, we will discuss current theories of memory storage and how they can reconcile the experimental data within a global model of memory formation. Unless otherwise

stated, we will focus on contextual fear memories, the most widely used rodent behavioral model to study memories that last up to several months. These salient memories are particularly interesting to investigate since they are often used in the context of traumatic memories in rodent and human studies alike.

1.3.3 The mPFC in remote memory recall

The importance of the mPFC and its subregions has initially been described during the recall of old, or remote, memories. A memory is classically considered remote in rodents at least 12 days after the initial learning; if tested earlier, the memory is considered recent. In a seminal study using immediate early gene expression (IEG, see Box 2) as a marker of neuronal activity, the anterior cingulate cortex (ACC), the prelimbic cortex (PL), and the infralimbic cortex (IL) were shown to be activated specifically at remote and not recent recall following contextual fear conditioning (CFC) (Frankland et al., 2004a). Moreover, inhibition of ACC, but not PL, with local lidocaine infusion prior to the recall prevented memory retrieval only at remote time points and not recent ones. Together with similar findings on spatial memories (Bontempi et al., 1999; Maviel et al., 2004), these studies cemented an important role for the mPFC at remote times of a memory.

Follow-up studies confirmed the importance of the mPFC for remote memories. Brain-wide cFos mapping following CFC recall not only found an increased activity of mPFC subregions at remote over recent recall, but also identified these cortical regions as central network hubs at remote times (Silva et al., 2018; Wheeler et al., 2013). Correlating with the cFos increase in the ACC as the memory ages, spine density was found to increase specifically after remote recall, whereas in the CA1 region of the hippocampus (HPC), spine density only increased after recent recall (Aceti et al., 2015; Restivo et al., 2009). In addition, precise electrophysiological recordings in ACC together with other brain regions demonstrated a brain-wide reorganization of the brain networks with the age of a memory, highlighting the predominant role of ACC at later time points (Corcoran et al., 2016; Makino et al., 2019). Lastly, establishing the functional involvement of the mPFC, optogenetic inhibition of ACC during recall specifically impaired remote memory (Goshen et al., 2011).

Taken together, these data suggest that the mPFC becomes predominantly important for the expression of old memories. Conjointly with findings showing that the HPC was primarily required at early recall (Beeman et al., 2013; Scoville and Milner, 1957; Varela et al., 2016), this led to and underscored the classical dogma of memory formation, called the standard theory of memory consolidation (Frankland and Bontempi, 2005) (also see discussion below).

Box 1. Nomenclature of the rodent medial prefrontal cortex and its subregions.

Although prefrontal areas have been widely studied and implicated in various brain functions and disorders, there is a surprising lack of commonly accepted nomenclature and delineation of its subdivisions. Rodent stereotaxic atlases, on which experimentalists rely the most, are regularly updated as no consensus is found (Le Merre et al., 2021). In the absence of clear landmarks to define the mPFC, a lot is left to individual appreciation which can lead to apparent incoherencies between studies and overall misinterpretation. Until a unified nomenclature is accepted in the field, it is necessary that authors report precise stereotaxic coordinates and explicitly define the brain region(s) they study.

For simplicity here, we will use the following nomenclature for the 3 major subdivisions of the mPFC:

- the **Anterior Cingulate Cortex (ACC)**, sometimes referred to as Anterior Cingulate Area, dorsal and ventral (dACA and vACA-Allen Brain Atlas) or Cingulate cortex area 1 and 2 (Cg1 and Cg2-Paxinos and Franklin).
- the **Prelimbic Cortex (PL)** or Prelimbic Area
- the **Infralimbic Cortex (IL)** or Infralimbic Area

Although the human PFC evolved to be relatively bigger and more complex than the rodent PFC, notably with more clearly defined subregions, homologies in embryological development, layer organization, cell-type distribution and connectivity patterns advocate for potentially shared functions. For an anatomical definition and a comparison between human and rodent PFC, see Carlén, 2017. For a very detailed description of the cytoarchitecture of the mouse PFC, see Van De Werd et al., 2010, and for a comparison between mouse reference atlases, see Le Merre et al., 2021.

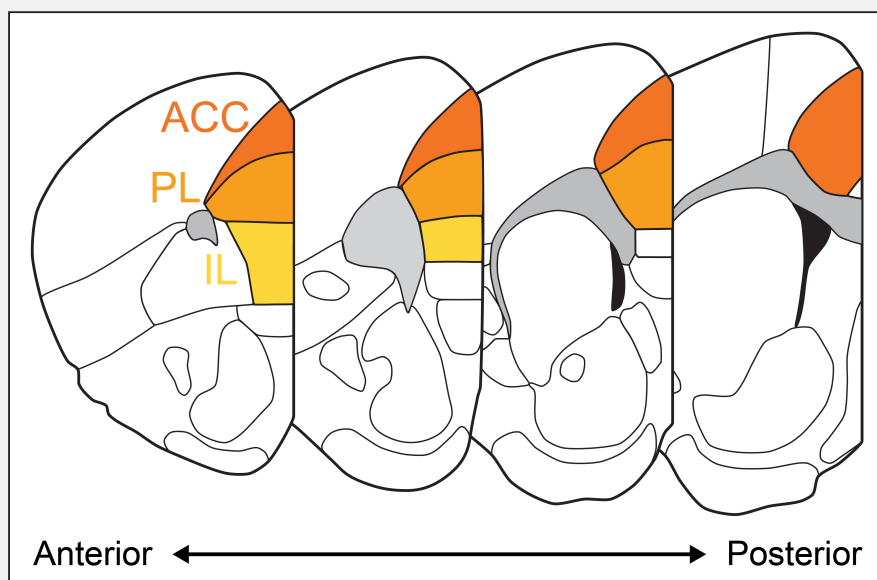


Figure 1.4. The mPFC subdivisions in coronal sections of the mouse brain.

The 3 major subdivisions of the mPFC are highlighted (Anterior cingulate cortex ACC, Prelimbic cortex PL, Infralimbic cortex IL) based on the Allen Brain Atlas, along the antero-posterior axis.

Box 2. Neuronal activity visualization and engram-tagging techniques.

Neuronal activity can be visualized using several techniques with their unique advantages and disadvantages depending on the research question being asked.

Electrophysiology enables direct monitoring of the electrical activity of a single neuron *ex vivo* or *in vivo*, with a high temporal and spatial resolution, and can be used to identify specific neuronal subtypes based on their distinct firing patterns (Lynch & Schubert, 1980; Tao et al., 2015). At a lower spatial resolution, it is also possible to record local field potentials or global electrical oscillation patterns at the scale of an entire brain region (Buzsáki & Draguhn, 2004; Whittington et al., 2019).

Calcium imaging is based on a fluorescent reporter, the activity of which correlates with intracellular calcium concentration, considered as a proxy for neuronal activation (Nakai et al., 2001). It has a lower temporal resolution than electrophysiology, but allows for monitoring of many neurons at once, with a potentially high spatial resolution depending on the type of imaging technique it is paired with it (Yang & Yuste, 2017). In both cases, technological advances are increasing the power of those techniques to monitor live brain activity in freely behaving animals with minimum tissue damage (Hamel et al., 2015; Hong & Lieber, 2019).

Immediate early genes (IEGs), such as cFos, Arc, Npas4, Zif 268, etc., are transcribed upon neuronal activation. Visualizing the corresponding mRNA or protein expressed after behavior enables identification of recently active neurons. Taking advantage of the specific dynamics of each of those IEGs, they can also be combined to disentangle neurons that take part in two successive tasks (Guzowski et al., 1999; Khalaf et al., 2018; Khalaf & Gräff, 2019; Nonaka et al., 2014), but they cannot provide permanent labelling themselves.

To this end, **conditional reporter expression using IEG promoters** have then been developed, allowing for the long-lasting tagging of neurons that were active at a given point in time. Two main strategies have been used: (1) the **Tettag system** uses the Doxycyclin-dependent transcription factor tTA, expressed under the cFos promoter, to restrict the expression of a chosen protein under a Tet promoter to only tag neurons active during the desired tagging time-window (Reijmers et al., 2007); (2) the **TRAP system** uses a Tamoxifen-dependent Cre recombinase Cre-ERT2 under a cFos or Arc promoter to restrict recombination of a chosen gene to activated neurons at the time of Tamoxifen injection (DeNardo et al., 2019; Guenther et al., 2013). In both cases, those systems can be used within transgenic mouse lines or through stereotaxic delivery of viral constructs. They allow restriction in time and, if desired, in space, of the initial tagging, and subsequent manipulation of engram neurons by either chemogenetic (Alexander et al., 2009; Cruz et al., 2013; Roth, 2016) or optogenetic tools (Boyden et al., 2005; Deisseroth, 2015).

More recently, **artificial promoters** have also been designed to be more sensitive to neuronal activation, such as the RAM promoters (Robust Activity Marking), while maintaining their sensitivity to a given transcription factor cascade, for instance, cFos and Npas4 (Sørensen et al., 2016; Sun et al., 2020).

Furthermore, **GRASP techniques** (GFP Reconstitution Across Synaptic Partners) coupled with the Tettag system enable visualization of direct synaptic contact between engram and/or non-engram cells that are located in different brain areas, and specifically manipulate those pair types (Choi et al., 2018).

Lastly, using cell-type specific promoters or viral vectors with restricted tropism or antero- and retrograde transport, the scope of action of these techniques can be even more refined. These methods can also be coupled to enhance their potential. For instance, calcium imaging and Tettag tagging were used together to monitor replay of engram cells during sleep (Ghandour et al., 2019).

1.3.4 The mPFC in early memory phases

The restricted involvement of the mPFC to remote time points was, however, challenged by many studies over the years, and increasingly so as technical advances allow for a more precise dissection of neural circuits.

1.3.4.1 At memory encoding

The first hint suggesting that the mPFC is already involved early on originates from long-term potentiation (LTP) recordings after auditory fear conditioning (AFC) in rats: Doyère and colleagues demonstrated that fear memory encoding elicited LTP in the CA1 to PL projection already 20 min after training (Doyère et al., 1993). A decade later, a somewhat overlooked study observed that reversible ACC inhibition with a GABAA agonist at AFC encoding impaired recent memory recall, and that electrical stimulation paired with tone presentations resulted in the creation of an artificial fear memory to the tone (Tang et al., 2005). These findings already suggested that the ACC is necessary to encode fear memories but was not followed up until later studies started examining the mPFC at encoding again.

Investigating IEG induction, brain-wide cFos quantification after encoding and recall of several conditioning protocols highlighted a significantly increased activity in many cortical areas at early time points, notably in all mPFC subdivisions at CFC encoding (Cho et al., 2017). Furthermore, whole mPFC inhibition prior to encoding using either TetTox (tetanus-toxin light chain blocking synaptic transmission) or synaptotagmin-1 knockdown to reduce synaptic transmission resulted in altered context discrimination at recent recall, and impaired remote recall (Xu et al., 2012), suggesting an overall role of the mPFC at encoding that broadly impacts memory consolidation. In parallel, several studies focusing specifically on the ACC pointed out transcriptional changes at encoding (Bero et al., 2014) as well as necessary new spine development for proper memory consolidation (Vetere et al., 2011). Nevertheless, the requirement of protein synthesis in ACC afterlearning for recent recall is still debated (Einarsson and Nader, 2012; Rizzo et al., 2017). However, most strikingly, optogenetic inhibition of the ACC during encoding prevented memory retrieval at both recent and remote time points (Bero et al., 2014).

In contrast, data on the involvement of the PL and IL at encoding are not as clear and yielded conflicting results. In one study, lesion of the IL using NMDA infusion long before CFC training impaired recent memory recall, but PL lesion did not (Zelikowsky et al., 2013). This result was also observed in an active avoidance paradigm using electrolytic lesions in IL or PL (Moscarello

and LeDoux, 2013). On the contrary, inhibition of global protein synthesis 1h after CFC encoding in PL impaired recent recall (Rizzo et al., 2017). In another study, optogenetic activation of parvalbumin (PV) interneurons PL/IL before AFC training had no effect on recent recall, but, surprisingly, the activation of CaMKII neurons impaired recent memory recall to both context and tone (Yizhar et al., 2011). Different techniques used to inhibit those regions, different stereotaxic coordinates, as well as slightly different behavioral paradigms and timing of the inhibitions could explain those disparities. Nevertheless, these studies highlight the importance of the mPFC and several of its subregions during the encoding phase of a fear memory.

1.3.4.2 At recent memory recall

In addition to the encoding phase, the mPFC was also found to play a role during recent memory recall. For example, optogenetic activation of ACC to dCA1-3 projection led to increased freezing to a neutral context one day after CFC (Rajasethupathy et al., 2015). Inhibiting this projection at recent recall impaired memory, suggesting that the ACC has a top-down control over the HPC at recent recall. In a separate study, optogenetic inhibition of the whole PL during an AFC recall impaired freezing both at 6 h and 7 days post AFC, times at which cFos was already increased in PL (Do-Monte et al., 2015a). Along the same lines, inactivation of PL with muscimol at recent AFC recall impaired freezing to the tone, whereas inactivation of IL specifically prevented extinction learning at recent recall (Sierra-Mercado et al., 2011). Such early importance of the mPFC was also confirmed using electrophysiological recordings during an AFC recall, identifying the necessity of mPFC neuronal assemblies to be functionally organized in a precise oscillation phase in order to drive memory recall (Dejean et al., 2016). The mPFC is therefore also involved in early recall of conditioned fears, most likely with different roles of its subdivisions.

Together with evidence that the HPC is also involved for the recall of remote memories (Goshen et al., 2011; Lehmann et al., 2007), the involvement of the mPFC at early time points provides supporting evidence for the multiple trace theory (MTT, see below).

1.3.5 The mPFC engram

Further evidence regarding the engagement of the mPFC early in the lifetime of a memory surfaced with the development of precise engram technologies (see Box 2). Engram cells are commonly defined as “a population of neurons that are activated by learning, have enduring

cellular changes as a consequence of learning, and whose reactivation by a part of the original stimuli delivered during learning results in memory recall” (Tonegawa et al., 2015).

A first indication that a subset of mPFC neurons are carrying memory-related information at encoding came from a study by Zelikowsky et al. (2014). Although not considered an engram study per se, it is the first to investigate the reactivation of mPFC neurons between encoding and recent recall, using a catFISH technique with Arc mRNA (see Box 2). In both a classical CFC protocol and an immediate shock protocol, PL neurons were found to be strongly reactivated after recent recall. Moreover, a population of PL neurons encoded context information from encoding to recall, and an additional population specifically encoded the context–shock association. This significant reactivation of PL neurons linked to a learning event, but not IL neurons, suggested that PL could contain engram cells already at encoding, but the remaining criteria to define engram cells were still to be fulfilled.

In a seminal study using a Tettag engram-tagging approach (see Box 2), Kitamura and colleagues (Kitamura et al., 2017) showed that PL neurons activated and tagged at CFC encoding could be optogenetically reactivated at later time points and that this reactivation could drive freezing behavior in a neutral context at both a recent and a remote time point. However, using IEG as well as calcium imaging, they observed that PL neurons tagged at encoding were naturally reactivated only during a remote recall and not a recent one. In addition, the inhibition of the CFC-tagged neurons during a recent recall had no effect on freezing but impaired memory recall at a remote time point. This study suggested the idea of a “silent engram” in the mPFC, which is already activated early on but requires consolidation to be naturally recruited in order to drive recall.

These important findings were confirmed by two subsequent studies using different techniques. The first one used chemogenetics in a viral TRAP setting (see Box 2) to demonstrate that the inhibition of CFC-tagged PL neurons specifically impaired remote and not recent recall in a context-dependent manner, and that the activation of this population was able to drive freezing at both recent and remote time points in a neutral context (Matos et al., 2019). In addition, CFC-activated PL neurons were only naturally reactivated at remote and not recent recall. The second study used an AFC paradigm and a transgenic TRAP system, and confirmed the time-dependent reactivation of PL neurons at recall and the ability of those cells to drive recall 1 day after conditioning (DeNardo et al., 2019a).

Taken together, these results demonstrate that engram cells are present in PL from encoding but are kept silent during early recall until the full consolidation of the memory. The existence of engram cells in ACC and IL still needs to be investigated.

1.3.6 Post-learning molecular modifications in the mPFC

Similar to findings in the hippocampus, *de novo* transcription as well as *de novo* protein synthesis are required in the mPFC at CFC encoding for proper memory consolidation (Einarsson and Nader, 2012; Pereira et al., 2019; Rizzo et al., 2017). More precisely, whole-ACC RNA-seq revealed a global shift in the mPFC transcriptome towards biological processes linked with synaptic plasticity, and a decreased expression of genes involved in immunity 1 h after conditioning (Bero et al., 2014). At the scale of a neuron, CFC triggered an increase in spine density as well as in mini excitatory post synaptic potential (mEPSCs) frequency, and the number of docked vesicles in individual synapses increased. Overall, this suggested that 1h after CFC, ACC neurons are globally primed for plasticity and activity. In addition, epigenetic modifications could also be important molecular changes resulting from learning as cortical areas, such as the orbitofrontal cortex, elicited histone H3 acetylation after learning for remote memory consolidation (Lesburguères et al., 2011).

As parts of the whole region, we can hypothesize that engram cells would also share those molecular modifications after learning, but whether those are specific features of engram cells still remains unknown. Using a viral TRAP approach, PL engram cells were found to require functional CREB (cAMP response element binding) for proper consolidation. Indeed, expressing a repressor of CREB-dependent transcription in engram cells at encoding specifically impaired remote memory recall (Matos et al., 2019). More precisely, single cell RNA-seq of mPFC engram cells tagged at remote recall identified specific transcriptional programs induced by consolidation, and dependent on neuronal subtypes (Chen et al., 2020). Those specific transcriptional programs share some common features, such as upregulation of genes involved in vesicle exocytosis, suggesting an enhanced neurotransmitter release in engram cells.

Overall, these studies indicate early mPFC molecular and transcriptional changes, with potentially important roles in long-term memory consolidation.

1.3.7 The mPFC functional connectivity

The mPFC is a densely connected structure receiving and sending projections from all major areas in the brain. As such, it is not surprising that it was identified as a central hub by brain-wide cFos mapping during CFC learning and recall (Silva et al., 2018; Wheeler et al., 2013). However, which subsets of cells within the mPFC were responsible for this hubness, as well as their direct functional connectivity to other brain areas during memory formation and recall was not investigated in these studies. In the following section, we will describe the anatomical projections to and from the mPFC that have been functionally implicated in memory formation and retrieval (**Figure 1.5 and 1.6**). For a detailed review on connectivity and subregions specificities, see Le Merre et al. (Le Merre et al., 2021).

1.3.7.1 mPFC functional inputs

Only few of the mPFC inputs were functionally investigated in the context of fear memory formation and retrieval, mainly from regions that were also implicated independently of those processes, namely the entorhinal cortex (EC), the BLA, and the HPC. More precisely, at the time of memory encoding, three inputs to the mPFC were demonstrated to be required for long-term learning (represented in blue in **Figure 1.5**): BLA to PL, MEC (medial part of the EC) to PL, and CA1 to ACC. In all cases, inhibition of those projections at the time of encoding (the BLA and MEC inputs to PL were inhibited using optogenetics (Kitamura et al., 2017), while the CA1 to ACC was inhibited using chemogenetics (Kol et al., 2020)) led to impaired remote memory recall, while recent recall remained unchanged. This suggests that CA1, BLA, and MEC all send crucial information to the mPFC at the time of encoding that is necessary to establish and consolidate a functional cortical engram.

At recent recall, two additional inputs were functionally characterized (represented in green in **Figure 1.5**): dCA1 to PL and IL, as well as BLA to mPFC. The dCA1 input was studied chemogenetically in an inhibitory avoidance (IA) paradigm. The results highlighted a differential effect on these two subregions, as the inhibition of the dCA1 to PL projection impaired memory expression, while inhibition of the dCA1 to IL pathway prevented extinction (Ye et al., 2017). A similar dichotomy between fear recall and extinction was observed in the context of an AFC paradigm investigating the electrophysiological properties of BLA neurons projecting to mPFC but without distinction between mPFC subregions (Herry et al., 2008). Two neuronal subpopulations encoding either fear or safety after extinction were identified, both projecting to the mPFC but part of distinct neuronal circuits: BLA fear neurons received input mainly from the ventral HPC and extinction neurons from the mPFC. Subsequent studies confirmed that

fear and extinction neurons project to the PL or the IL, respectively (Bayer and Bertoglio, 2020; Do-Monte et al., 2015b; Giustino and Maren, 2015).

Surprisingly, a functional input has yet to be identified at the time of remote recall, although the storage role of the mPFC after consolidation has long been established. However, an interesting experiment could give a hint on where to start looking. Kitamura et al. (2017) investigated the inputs of mPFC engram cells tagged at CFC encoding using a Tettag system and monosynaptic retrograde tracing with a modified rabies virus. With this technique, PL engram cells were found to receive direct input mainly from anteromedial (AM) and medial dorsal (MD) thalamic nuclei, MEC (layer V), BLA, retrosplenial cortex (RSP), and vHPC, but also from the lateral orbital cortex, insular cortex, medial septum, subiculum, and posterior ACC. Future research on the functional inputs of mPFC engram cells should therefore focus on those specific long-range projections having a monosynaptic connection with engram cells.

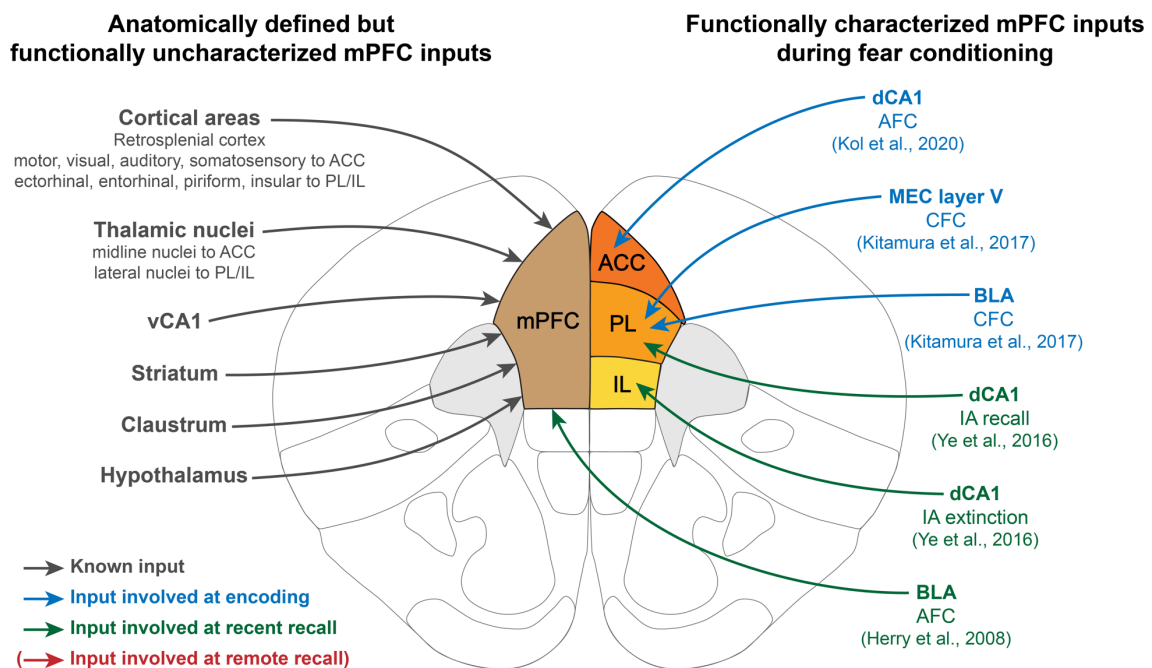


Figure 1.5. mPFC inputs are involved in encoding and recent recall of fear memories. Left: Anatomically defined inputs to the mPFC. Right: mPFC inputs functionally involved during fear memory consolidation, color coded by the phase of the memory (blue: encoding, green: recent recall, red: remote recall). Note the absence of functionally characterized mPFC inputs at the time of remote recall. AFC: Auditory fear conditioning; CFC: Contextual fear conditioning; IA: Inhibitory avoidance; MEC: Medial entorhinal cortex; BLA: Basolateral amygdala.

1.3.7.2 mPFC functional outputs

The outputs of the mPFC involved in fear learning were mainly investigated in a correlative manner (i.e., a projection exists, and the target area is more or less activated at given times), and only few research articles have reported manipulations to assess the functional role of these projections. It is important to note that overall, engram cells as a population seem to project to the same brain regions as the mPFC as a whole. Indeed, in an experiment using TRAP transgenic mice, PL engram cells were tagged at the time of recent or remote recall and their axonal projections were traced in a brain-wide manner, highlighting that engram cells project to all the classical target regions of the mPFC .

At recent recall, three projections from the mPFC were studied at relatively early time points, to the HPC and the amygdala, as well as to the lateral habenula (LHb) (green in **Figure 1.6**). A direct mPFC to HPC projection was identified between the ACC and the dCA1-3, and was found necessary for a recent CFC recall by optogenetic manipulation (Rajasethupathy et al., 2015). In addition, the ACC to dCA1-3 projection recruited a subpopulation of HPC neurons that encodes contextual information, suggesting that the mPFC exerts top-down control over the HPC at the time of recent recall.

In parallel, the dense projections between the mPFC and the amygdala have also been studied at recent time points but using AFC protocols. Specifically, optogenetic inhibition of PL to BLA projection prevented memory expression 6 hours after AFC training but not 7 days later, suggesting that this projection is necessary for a recent recall (Do-Monte et al., 2015a). The link between the mPFC and the BLA at this time point is also supported by oscillations studies, which, although independent of direct connectivity, confirm that the mPFC drives BLA neurons firing during a recent AFC recall via 4 Hz oscillations that synchronize the two regions (Karalis et al., 2016). Investigating the subregion connectivity in more depth, Knapska et al. (Knapska et al., 2012) correlated lateral amygdala (LA) neuron activity in high or low fear states after AFC recent extinction with different inputs from the mPFC. In the non-extinguished context where rats showed high fear, activated LA neurons received input preferentially from the PL, and in the extinguished context where the animals were less afraid, they had more IL inputs. These findings were later expanded with chemogenetic inhibition of the IL to BLA projection, resulting in an impaired extinction (Bloodgood et al., 2018). This further confirms the dichotomy between PL and IL in fear and extinction, as well as the importance of mPFC input to the amygdala in recent AFC recall.

Finally, the LHb also receives specific input from the mPFC, and notably projections from a PL engram tagged in a recent recall of a shock-based conditioned place preference paradigm (Ye et al., 2016).

At more remote points, projections from the PL to the paraventricular thalamus (PVT, represented in red in **Figure 1.6**) were found to be necessary for a 7-day-old recall of a CFC, although not at a 6 h recall, suggesting a progressive recruitment of this cortico-thalamic connection during memory consolidation (Do-Monte et al., 2015a). At a later remote recall, PL engram cells tagged at CFC encoding projecting to the BLA are also progressively recruited (represented in red in **Figure 1.6**). Indeed, optogenetic terminal inhibition during recall only impairs memory after 22 days but not before (Kitamura et al., 2017). In parallel, electrophysiological recordings between ACC and CA1 highlighted the progressive synchronization of the two regions during the consolidation of a CFC memory, resulting in ACC coordinating CA1 neuronal activity via phase locking of the firing to the ACC theta rhythm at remote time points only (Makino et al., 2019). Interestingly, the ability of ACC to drive CA1 activity was linked to successful memory recall, suggesting that at remote recall also, the mPFC could exert top-down control over the HPC.

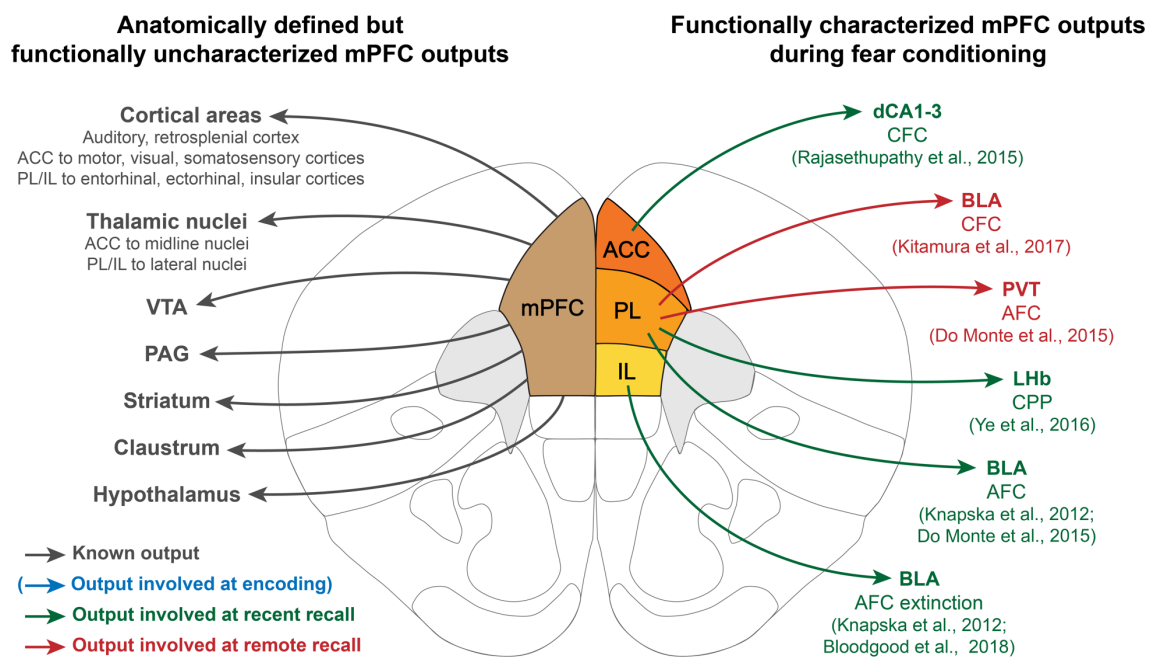


Figure 1.6. mPFC outputs are involved in recalling fear memories.

Left: Anatomically defined outputs from the mPFC. Right: mPFC outputs functionally involved during fear memory consolidation, color coded by the phase of the memory (blue: encoding, green: recent recall, red: remote recall). Note the absence of functionally characterized mPFC outputs at the time of encoding. AFC: Auditory fear conditioning; CFC: Contextual fear conditioning; CPP: Conditioned place preference; BLA: Basolateral amygdala; LHb: Lateral habenula; PAG: Periaqueductal gray; PVT: Paraventricular thalamus; VTA: Ventral tegmental area.

Globally, the mPFC functional connectivity has only been partially investigated, and we still lack a comprehensive understanding of the neuronal network involved during fear memory consolidation. Surprisingly, the projections to and from the thalamic nuclei have been little studied, although some of these regions are becoming of high interest in the regulation of the consolidation and the extinction of both recent (Taylor et al., 2021) and remote fear memories (Silva et al., 2021; Vetere et al., 2021). This, in addition to the possibility of tracing connections from and to engram cells specifically (DeNardo et al., 2019a; Kitamura et al., 2017), opens up many areas of investigation that will be of high interest in the future.

1.3.8 Models of memory formation

Several models of memory formation, storage, and retrieval have been brought forward over the past decades that encompassed all the experimental results that were available at the time of writing. As techniques evolved and became more refined, these models were continuously updated and complexified. Here, we will summarize the main theories that emerged and try to reconcile them with the abovementioned experimental findings of the mPFC during the different phases of memory consolidation.

1.3.8.1 The Standard Model of Memory Formation and Its Limitations

The first model of memory formation, known as the standard or the classical model, relies on the early findings suggesting that the HPC was only necessary for the initial storage and early retrieval phase, while cortical areas and in particular the mPFC were later progressively recruited for the long-term storage and retrieval of old memories (Beeman et al., 2013; Bontempi et al., 1999; Frankland et al., 2004a; Restivo et al., 2009; Varela et al., 2016; Wheeler et al., 2013). This model was proposed by Squire (Squire, 1992; Squire et al., 1984) based on extensive data gathered from humans, primates, and rodents, and was further refined by Frankland and Bontempi (Frankland and Bontempi, 2005). It was initially based on purely observational studies, then on broad inhibition studies using lesions or pharmacological inhibition, and more recently on IEG expression studies.

Several findings have in the meantime challenged this standard model. On the one side, cortical areas were shown to be involved in early phases of memory formation as well (Bayer and Bertoglio, 2020; Bero et al., 2014; Cho et al., 2017; Einarsson and Nader, 2012; Tang et al., 2005; Vetere et al., 2011; Xu et al., 2012), and on the other side, the HPC was also found to be necessary for the recall of remote memories, and not only recent ones, by means of pharmacological (Lehmann et al., 2007) as well as optogenetic inhibition (Goshen et al., 2011).

Precisely, an acute optogenetic inhibition of CA1 during remote recall impaired memory, but a prolonged inhibition that started before the recall itself did not impair memory and resulted in increased cFos expression in ACC, which suggested the recruitment of alternative cortical circuits when the HPC is inhibited for an extended period of time (Goshen et al., 2011). Conversely, opto- and chemogenetic reactivation of DG engram cells could alter freezing even at remote timepoints, indicating that the hippocampal engram stays functional even after consolidation (Khalaf et al., 2018; Kitamura et al., 2017). Taken together, these results advocate for the existence of parallel memory traces distributed in different brains areas.

1.3.8.2 The Indexing Theory (IT)

The IT, initially developed by Teyler and DiScenna (Teyler and DiScenna, 1986), theorized how the HPC would exchange information with cortical areas, notably the mPFC. The HPC is presented as an ideal storage place for maps, or indexes, of specific cortical locations, enabling a link to be made between individual cortical modules that were co-activated during the initial learning event. Upon recall, which would reactivate some of the cortical modules involved in the original memory trace, the HPC index would reactivate the full panel of cortical modules in order to recreate the complete original memory. No memory content would be stored in the HPC, only the location of cortical modules that as a whole would form the memory trace. Initially, this theory was developed as an explanation of the first phase of the standard theory model, when memories are still HPC dependent, and suggested that older consolidated memories would not necessarily rely on an HPC index anymore. More recently, the IT was revisited and expanded, including our current knowledge of engrams: DG engram reactivation experiments driving freezing point to engram cells serving as indexes themselves (Kitamura et al., 2017; Liu et al., 2012; Ramirez et al., 2013). However, it still remains unclear if indexes are still present in the HPC after remote consolidation or if they disappear, as cortical connections between modules appear to be strengthened enough to not require HPC indexes anymore (Goode et al., 2020; Teyler and Rudy, 2007). Interestingly, the presence of engram cells in the mPFC (DeNardo et al., 2019a; Kitamura et al., 2017; Matos et al., 2019) could also be explained if their activation triggers the HPC indexes that in turn would drive memory recall.

1.3.8.3 The Multiple Trace Theory (MTT)

The MTT (sometimes referred to as the trace transformation theory) was proposed by Nadel and Moscovitch (Nadel and Moscovitch, 1997) in order to take into account the experimental data challenging the standard model, and was further developed more recently (Moscovitch et al., 2006). It incorporates the IT to a broader model, which also extends into remote memory

consolidation. The MTT postulates that the hippocampal formation is the first to encode the initial information upon learning, in a sparse and distributed manner, and that, similarly as in the IT, this hippocampal ensemble acts as an index to link the cortical neurons that are also representing the incoming information. Both neuronal ensembles, cortical and hippocampal, form the full memory trace, or engram as we would call it nowadays. The hippocampus provides the contextual information to link the individual pieces of information stored in the cortex from the start. However, in contrast to the IT, a new hippocampal trace will be created at each recall, indexing the same, or part of the same, cortical information, resulting in the creation of parallel and multiple traces distributed in the brain, making them harder to disrupt or erase. This theory would explain how new schemas can be integrated faster into an existing framework that was previously learned (Tse et al., 2011). It is supported by studies highlighting the role of the HPC at late times post-encoding (Goshen et al., 2011; Lehmann et al., 2007), of the mPFC at early times (Bayer and Bertoglio, 2020; Bero et al., 2014; Cho et al., 2017; Einarsson and Nader, 2012; Tang et al., 2005; Vetere et al., 2011; Xu et al., 2012), as well as by reports of distributed engram cells throughout the brain (Cowansage et al., 2014; Rashid et al., 2016; Redondo et al., 2014; Reijmers et al., 2007; Ryan et al., 2015; Sun et al., 2020; Todd et al., 2016).

1.3.9 Memories are Dynamic: Discussion and Outlook

Taken together, reconciling all experimental results in one unified theoretical model is difficult. A possible explanation of the apparent complexity of memory processes is their high flexibility and the fast dynamics of brain circuits. Indeed, many brain areas aside from the mPFC have been involved in learning and/or retrieval, which could potentially take over if one important brain circuit is damaged or impaired. For example, engram cells have been found in the HPC (Liu et al., 2012; Redondo et al., 2014; Ryan et al., 2015; Sun et al., 2020) and amygdala (Rashid et al., 2016; Reijmers et al., 2007) but also in other cortical areas, such as the RSP. Interestingly, the RSP has been involved in the retrieval of remote fear memories (Todd et al., 2016), and early engram cells formed at the time of learning were found to drive fear recall (Cowansage et al., 2014) and correlate with spatial memory retention (Milczarek et al., 2018). This suggests that cortical regions other than the mPFC can also store memories or at least parts of it, complicating the experimental deciphering of brain networks involved. Brain-wide (engram) studies and multiple-site recordings would enable taking into account the various regions implicated in these processes.

A consequence of this distribution of the memory network is that brain circuits can be redundant and therefore compensate for any natural or artificial perturbation of the system, allowing for the great stability of salient memories. Several experiments indeed reported such a reorganization of brain circuitries, notably of mPFC taking over from the HPC in case of lesion before CFC encoding (Zelikowsky et al., 2013) or prolonged optogenetic inhibition at the time of recall (Goshen et al., 2011). In addition, the dynamics of consolidation could be modulated bidirectionally by either adding a novel learning event between the encoding and the recall of a CFC (Haubrich et al., 2016; Pedraza et al., 2017) or during a spatial learning task (Wartman and Holahan, 2013), which resulted in a faster memory dependance on cortical areas, or conversely by adding external factors, such as odors, to prolong the HPC dependency of a memory (Grella et al., 2020). Moreover, the phenomenon of co-allocation of memories that are experienced close in time underlines the importance of the brain state at the time of learning (Cai et al., 2016; Rashid et al., 2016).

From a technical viewpoint, neuronal activity-based mapping by means of IEGs is also not without caveats, as the wide use of IEGs in engram-tagging strategies biases studies towards a specific neuronal population, e.g., those expressing cFos, while leaving aside other neurons in which activity would trigger a different transcriptional pathway (Sun et al., 2020). Additionally, although neurons are thought to be the central elements in memory consolidation, it is now apparent that other cell types should also be taken into account as they can greatly influence memory formation, such as astrocytes in CA1 to ACC communication at encoding (Kol et al., 2020), HPC microglia in forgetting (Wang et al., 2020), and myelinating oligodendrocytes in the mPFC for remote memory consolidation (Pan et al., 2020). Indeed, some of these cell types in the mPFC are showing important transcriptional modifications following remote recall (Chen et al., 2020), suggesting that they play a specific role in this process.

These limitations and open questions notwithstanding, it is clear from experimental data and the diverse theoretical models alike that the mPFC plays a critical role in all phases of the lifetime of a memory, the current state of the knowledge we have summarized in this review.

1.4 Aim of the thesis

This introduction highlighted the many open questions in the field of memory consolidation, which makes it a highly dynamic and fascinating area of research. When asked to define my PhD research project, I was naturally drawn to the brain circuits aspect of memory, allowing me to touch upon the dynamic interplay between brain regions during the lifetime of a memory (and also giving me the opportunity to spend hours looking at captivating tracing images). The focus on the mPFC was evident as I wanted to investigate the consolidation of old memories, but also because around that time engram cells had just been discovered in the PL. Overall, my PhD project aimed at deciphering the functional role of individual PL inputs, in order to understand from where PL neurons were getting the necessary information to first become engram cells and later recall the encoded memory when needed, or in other words answer the question: *where does the engram come from?*

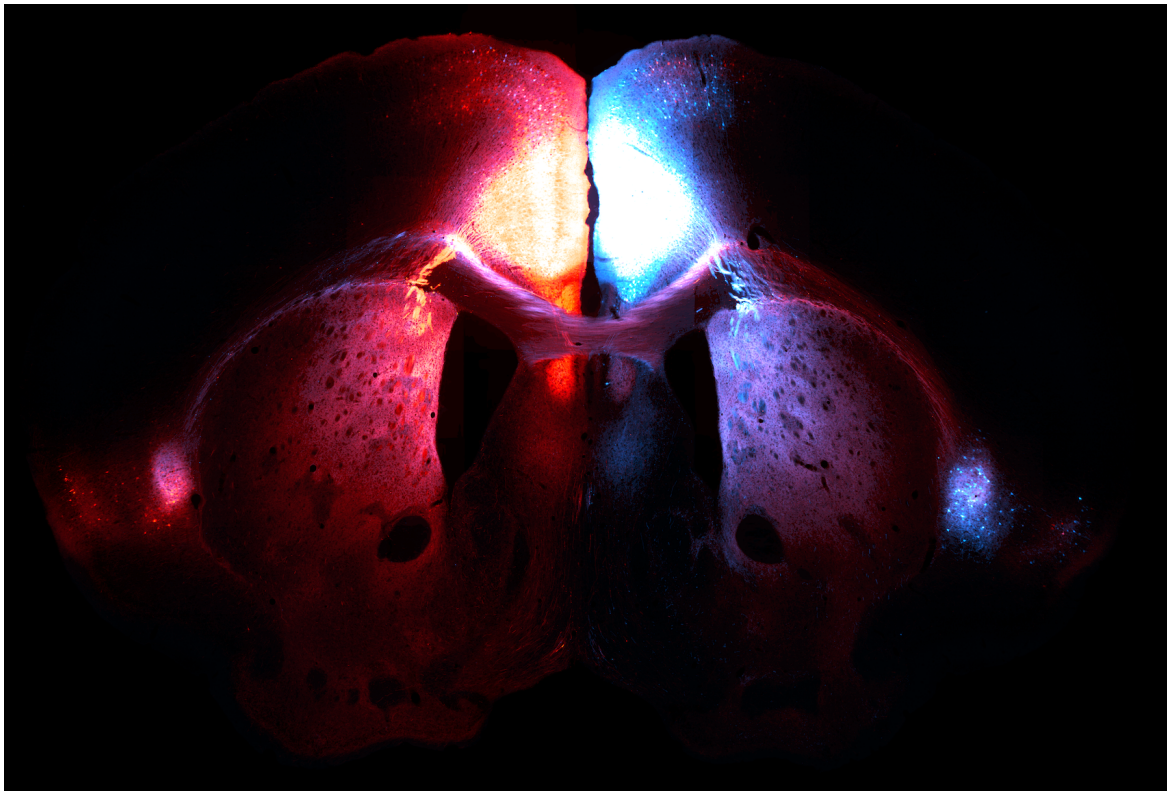


Figure 1.7. The Memory Ghost.

Image created for the Figure 1A scientific art exhibition in 2020, where it received an award from the public. The animal was injected symmetrically with two retrograde AAVs in the mPFC, to trace its input regions throughout the brain. Note the large but not complete hemispheric segregation of inputs, and the importance of projections from the Claustrum.

Chapter 2 Results and methods

2.1 Brain-wide screen of prelimbic cortex inputs reveals a functional shift during early fear memory consolidation

This section represents the following submitted research article which has been formatted to fit with the style of the thesis:

Dixsaut L, Gräff J. Brain-wide screen of prelimbic cortex inputs reveals a functional shift during early fear memory consolidation.

Under revision in eLife (as of May 16th 2022).

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Author contribution: I conceptualized the project and wrote the article together with Johannes Gräff, and I performed all the experiments.

2.1.1 Abstract

Memory formation and storage rely on multiple interconnected brain areas, the contribution of which varies during memory consolidation. The medial prefrontal cortex, in particular the prelimbic cortex (PL), was traditionally found to be involved in remote memory storage, but recent evidence points towards its implication in early consolidation as well. Nevertheless, the inputs to the PL governing these dynamics remain unknown. Here, we first performed a brain-wide, rabies-based retrograde tracing screen of PL engram cells activated during contextual fear memory formation to identify relevant PL input regions. Next, we assessed the specific activity pattern of these inputs across different phases of memory consolidation, from fear memory encoding to recent and remote memory recall. Using projection-specific chemogenetic inhibition, we then tested their functional role in memory consolidation, which revealed a hitherto unknown contribution of claustrum to PL inputs at encoding, and of insular cortex to PL inputs at recent memory recall. Both of these inputs further impacted how PL engram cells were reactivated at memory recall, testifying to their relevance for establishing a memory trace in the PL. Collectively, this data identify a spatiotemporal shift in PL inputs important for early memory consolidation, and thereby help to refine the working model of memory formation.

2.1.2 Introduction

The brain's ability to form enduring memories is essential for an individual's survival. Memories first need to be encoded and subsequently stored in the brain, a process that is termed memory consolidation. Memory consolidation occurs both at the scale of individual cells (cellular consolidation), which happens in the order of seconds to hours, and at the scale of brain networks (systems consolidation), which takes place in the days to weeks after learning (Dudai, 2004, 2012). Systems consolidation across several brain areas is thought to be essential for the establishment of enduring memories (Nadel and Hardt, 2011).

Traditionally, the hippocampus (HPC) was demonstrated to be necessary during the early stages of memory formation and the retrieval of recent memories (which in the mouse are typically studied one day after encoding), while the medial prefrontal cortex (mPFC) was found to be rather responsible for the consolidation and retrieval of remote memories (which are studied at least 14 days after encoding) (Albo and Gräff, 2018; Frankland and Bontempi, 2005). Indeed, multiple studies using immediate early gene mapping (IEGs, that are expressed specifically upon neuronal activation), whole brain region inhibition or cell type specific optogenetic manipulations (Aceti et al., 2015; Frankland et al., 2004b, 2006; Goshen et al., 2011; Makino et al., 2019; Silva et al., 2018; Wheeler et al., 2013) showed that the mPFC was predominantly important at later times as opposed to the HPC. However, recent evidence has challenged this view by highlighting a role for the mPFC also during fear memory encoding (Bero et al., 2014; Cho et al., 2017; Cummings and Clem, 2020; Tang et al., 2005; Zelikowsky et al., 2013) as well as for fear memory recall at recent times (Do-Monte et al., 2015a; Rajasethupathy et al., 2015). The rich connectivity of the mPFC indeed places it as a potential hub region for memory consolidation, as it receives not only inputs from other cortical areas, including sensory ones, but also from various subcortical areas such as the hippocampal formation, amygdala and thalamus (Dixsaut and Gräff, 2021; Le Merre et al., 2021), which are all implicated in memory formation (Cho et al., 2017; Nonaka et al., 2014; Ramirez et al., 2013; Reijmers et al., 2007; Taylor et al., 2021).

At the cellular level, mounting evidence suggests that memories are encoded and stored in *engram* cells, which, by definition (Tonegawa et al., 2015), are cells that are activated during the initial learning, undergo molecular and/or cellular modifications following learning, and the reactivation of which correlates with and can trigger memory recall. Engram cells have been discovered not only in the HPC (Josselyn et al., 2015; Liu et al., 2012; Ramirez et al., 2013), but more recently also in the mPFC (DeNardo et al., 2019a; Kitamura et al., 2017; Matos et al., 2019). Interestingly, engram cells in the mPFC were reported to have the particular feature

of staying silent until the memory is fully consolidated, although they are formed during the original learning phase (DeNardo et al., 2019a; Kitamura et al., 2017; Matos et al., 2019). This implies that mPFC engram cells are first active during encoding, stay silent during a recent recall, and are reactivated at remote recall, although they are functionally able to trigger memory retrieval at any time.

Based on these grounds, we hypothesized that the functional contribution of mPFC inputs may change over the course of memory consolidation to govern how the mPFC engram is formed and subsequently reactivated. For this reason, we first sought out to establish a comprehensive functional map of mPFC inputs across time during fear memory consolidation, and second to analyze the downstream effect of these inputs on memory retention and mPFC engram reactivation.

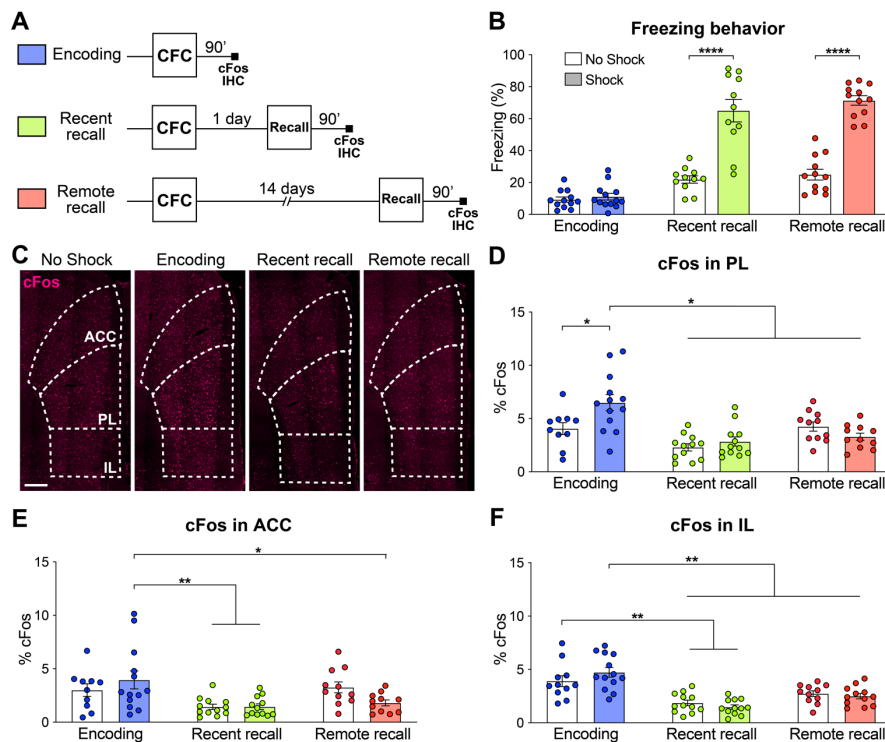


Figure 2.1. The prelimbic cortex (PL) is activated by the encoding of a contextual fear memory.

(A) Experimental design. For encoding, mice were perfused 90 minutes after contextual fear conditioning (CFC). For recent and remote recalls, mice were perfused 90 minutes after a 1-day and a 14-day recall, respectively. **(B)** Percentage freezing measured during the 3 minutes of habituation before the shocks (Encoding, in blue), at recent (in green) or remote (in red) memory recalls, for the animals undergoing CFC (shock, filled) and the control groups that were exposed to the CFC chamber without the shock (no Shock, clear). Two-tailed unpaired t-tests, ****: $p < 0.0001$. **(C)** Representative images of cFos immunostainings in the mPFC. Scale: $250\mu\text{m}$. **(D-F)** Percentage of cFos over DAPI in **(D)** PL (one-way-ANOVA, $F(5, 63) = 9.172$, $p < 0.0001$), **(E)** ACC (one way ANOVA, $F(5, 63) = 4.394$, $p = 0.0017$) and **(F)** IL (one way ANOVA, $F(5, 65) = 13.34$, $p < 0.0001$). (D-F) Stars represent least significant p-values of Tukey's multiple comparisons tests: *: $0.01 < p < 0.05$; **: $0.001 < p < 0.01$. $n = 11-13$ animals per group.

2.1.3 Results

2.1.3.1 The prelimbic cortex is specifically active during the encoding of a fear memory

The mPFC is composed of the three following major areas: The anterior cingulate (ACC), the prelimbic (PL) and the infralimbic (IL) cortices (Carlén, 2017; Le Merre et al., 2021). In order to evaluate the relative activity of these subregions during the different phases of fear memory consolidation, we used a contextual fear conditioning (CFC) paradigm in combination with cFos immunohistochemistry (IHC), an IEG marker of neuronal activity. We measured the freezing percentage of wild-type (WT) mice at CFC encoding before the footshocks occurred, as well as at recent recall 1 day post encoding and at remote recall 14 days post-encoding (**Figure 2.1A**). Each group was controlled for by a no shock group. We observed that both at recent and remote recall, mice display a significantly higher freezing percentage than the no shock control groups (**Figure 2.1B**), indicating successful memory formation.

We then quantified cFos expression in the three subregions of the mPFC (**Figure 2.1C**) 90min after the corresponding behavioral session. We found that while all regions were more active at encoding than during the memory recalls, only in the PL did we observe a significant increase in cFos compared to the no shock control groups (**Figure 2.1C-F**). These results indicate that the mPFC as a whole, and the PL in particular, are activated by the encoding of a contextual fear memory. In turn, this finding suggests an important role of the PL already during this early phase of memory consolidation, which is coherent with the formation of engram cells in the PL at the time of encoding (DeNardo et al., 2019a; Kitamura et al., 2017; Matos et al., 2019).

2.1.3.2 Brain-wide screen of PL engram inputs

Next, we aimed to identify the PL inputs that could be responsible for this peaked PL activity at encoding and for the establishment of its engram during memory consolidation (DeNardo et al., 2019a; Kitamura et al., 2017; Matos et al., 2019). To this end, we employed an activity-dependent monosynaptic retrograde tracing technique (**Figure 2.2A,B**). Specifically, we used the TRAP2 mouse line (DeNardo et al., 2019a), in which the cFos promoter drives the expression of the tamoxifen-dependent CreERT2 recombinase. These mice were first injected in the PL with helper AAVs expressing Cre-dependent nuclear GFP, the TVA

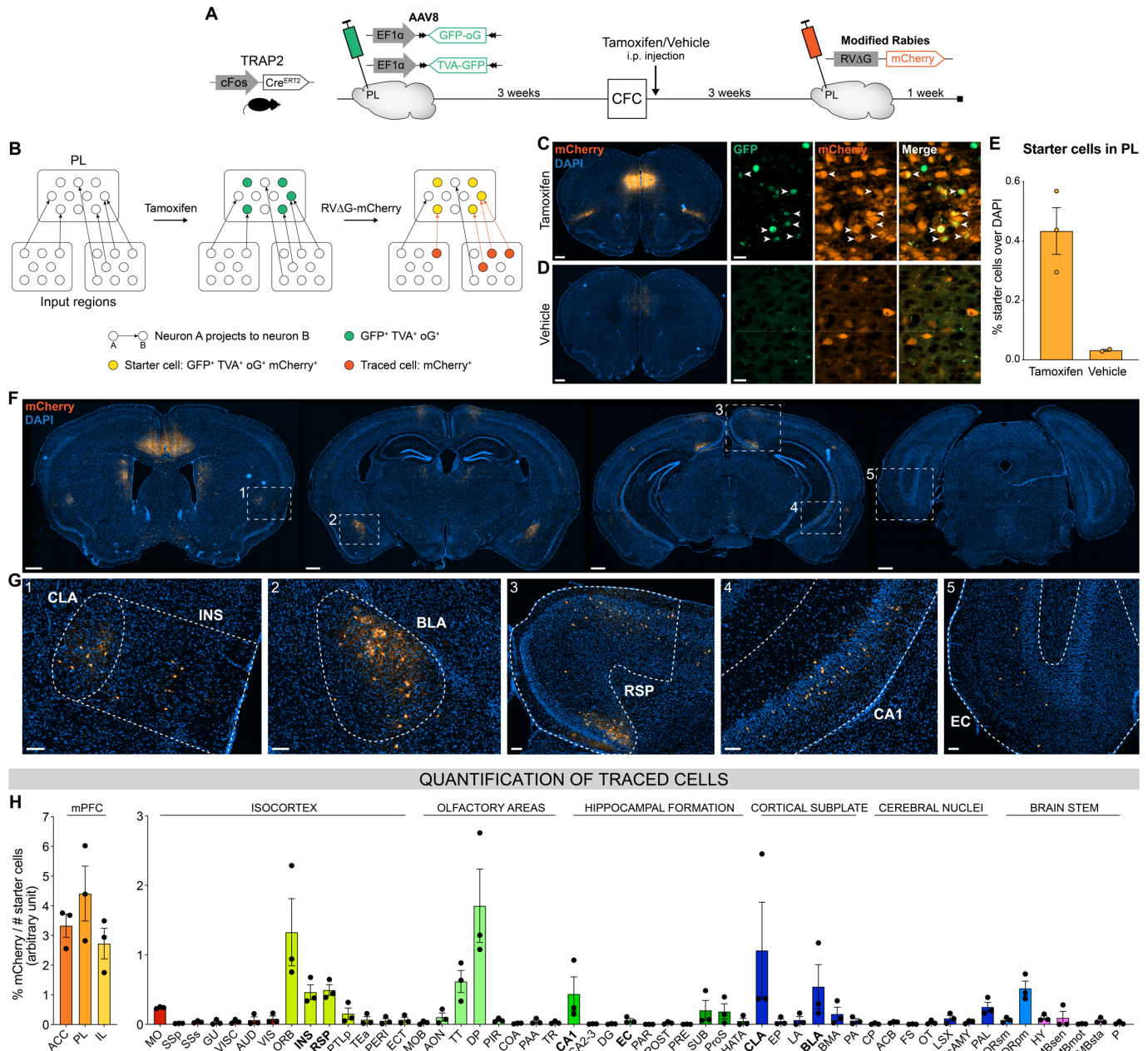


Figure 2.2. Brain-wide retrograde tracing identifies monosynaptic inputs of PL engram cells.

(A-B) Experimental design and timeline. cFos-Cre^{ERT2} animals were first injected in the PL with helper AAVs expressing GFP, TVA receptor and oG (rabies optimized glycoprotein) in a Cre-dependent manner. Tamoxifen (or vehicle for control) was injected right after CFC to trigger recombination in cFos-Cre⁺ cells. 3 weeks later, a modified rabies virus (RVΔG-mCherry with EnvA coating) was injected in PL where it infected TVA-expressing cells, replicated in oG expressing-cells, and was retrogradely transsynaptically transported. A week later, brains were collected to quantify monosynaptic inputs of PL engram cells labelled with mCherry. **(C, D)** Representative images of the PL injection site (scale 400μm) and magnified view of starter cells (scale 20μm) with tamoxifen **(C)** or vehicle **(D)** injection. **(E)** Percentage of starter cells over DAPI in PL. **(F)** Representative images of traced cells throughout the brain (scale 500μm). **(G)** Magnified views of traced cells (scale 100μm) in CLA (inset 1), INS (1), BLA (2), RSP (3), vCA1 (4) and EC (5). **(H)** Brain-wide quantification of traced cells, normalized by the number of starter cells for each animal, in the mPFC subregions (left) and the rest of the brain (right). Tamoxifen: n = 3 animals; Vehicle: n = 2 animals.

receptor and optimized rabies glycoprotein oG. Thus, the expression of these proteins in PL engram cells could be triggered with tamoxifen injection at the time of encoding. 3 weeks post-encoding, we injected a modified rabies vector RVΔG-mCherry (Wickersham et al., 2007) that can only infect and replicate in TVA- and oG-expressing cells, respectively, allowing us to transsynaptically label all monosynaptic inputs of PL engram cells (**Figure 2.2B**). As expected, we found that tamoxifen injection increased the number of starter cells expressing both GFP and mCherry in PL compared to vehicle (**Figure 2.2C-E**), confirming the specificity of these tools to restrict tracing to PL engram cells.

We then quantified the percentage of traced cells (mCherry+ only) throughout the brain (**Figure 2.2F-H**). Although most traced cells were found in the mPFC itself and its neighboring areas (orbitofrontal cortex OFC and dorsal peduncular DP, **Figure 2.2H**), we observed traced inputs in several other brain regions, notably the claustrum (CLA, **Figure 2.2G** inset 1), insular cortex (INS, inset 1), basolateral amygdala (BLA, inset 2), retrosplenial cortex (RSP, inset 3), CA1 field of the HPC (mostly the ventral part, inset 4), taenia tecta (TT), thalamus polymodal association cortex related areas (DORpm), subiculum (SUB), and to a lesser extent the entorhinal cortex (EC, inset 5). Without tamoxifen injection, traced cells were negligible (**Supplementary figure S2.1**), which further confirms the rabies tracing specificity.

With this approach, we identified relevant PL inputs that might be responsible for the development of the PL engram cell population, but we still lack information on whether, when and the extent to which these inputs are activated across memory consolidation.

2.1.3.3 PL inputs are differentially activated across memory consolidation

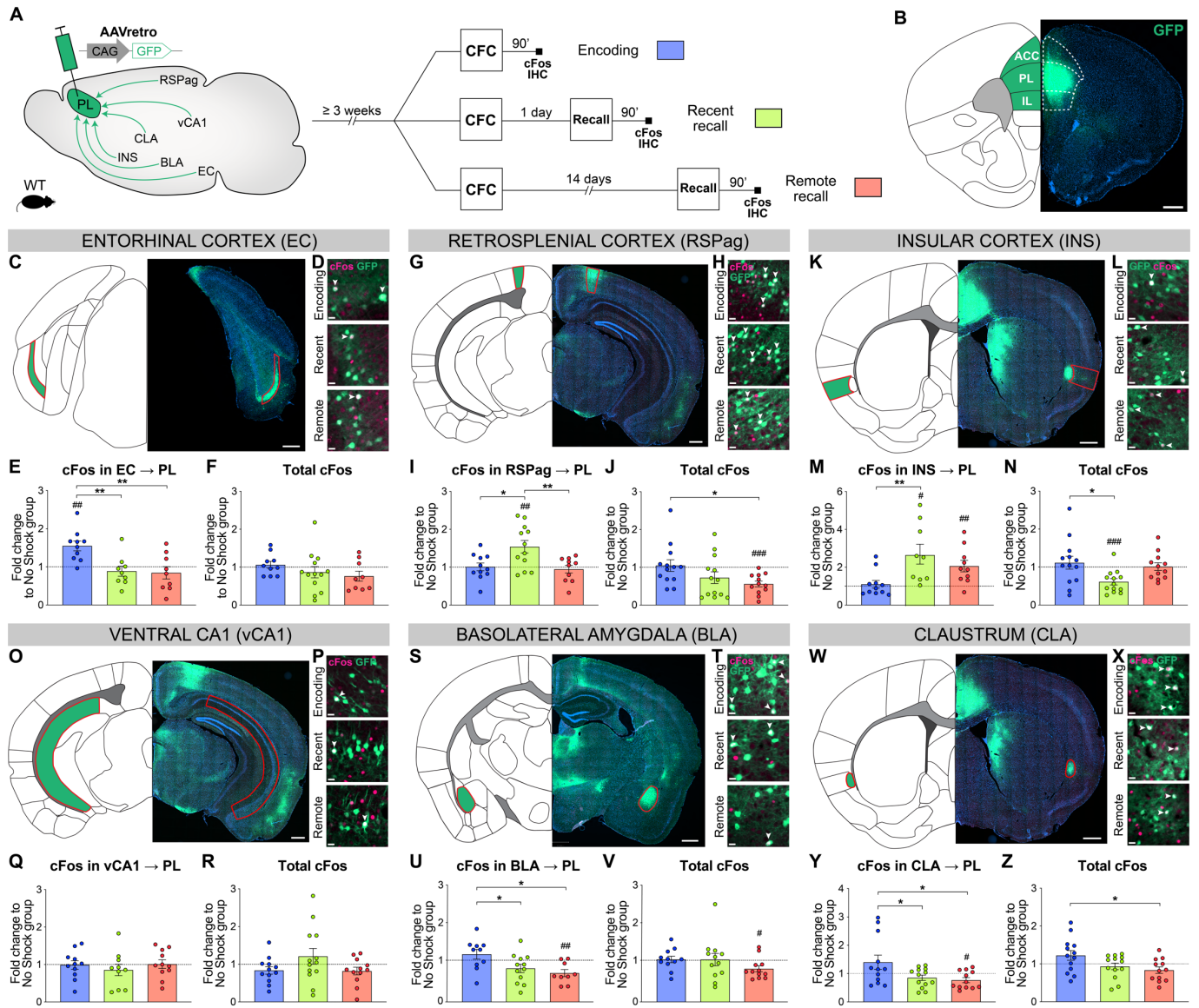
Out of the regions projecting to PL engrams, we selected six brain areas with consistent input tracing for further investigation, because of their previously documented implication in various aspects of fear memory: The EC, for its role in memory formation (Roy et al., 2017) and its known projection to the PL necessary at encoding (Kitamura et al., 2017) and retrieval (Pilkiv et al., 2022); the RSP for its necessity for recent (Cowansage et al., 2014) and remote fear memory recall (Todd et al., 2016); the INS for its requirement during the consolidation and expression of contextual fear memories (Alves et al., 2013), as well as for its regulation of fear expression (Gehrlach et al., 2019; Klein et al., 2021); vCA1 for its importance for CFC encoding (Kim and Cho, 2020) and recent recall (Jimenez et al., 2020); the BLA for the role of BLA to PL projections in memory encoding (Kitamura et al., 2017; Klavir et al., 2017) and PL to BLA projections in memory recall (Do-Monte et al., 2015a; Kitamura et al., 2017); and the CLA as

CLA to EC projections are necessary during memory encoding (Kitanishi and Matsuo, 2017) and for its involvement in attention (Atlan et al., 2018).

To assess the relative activity of these PL inputs during fear memory consolidation, we needed a tracing technique that could be coupled with neuronal activity measurements from encoding to remote recall, which cannot be achieved with rabies tracing from engram cells. Therefore, we combined conventional retrograde tracing with neuronal activity-dependent cFos staining: Injection of AAVretro-GFP in PL prior to any behavioral test allowed to trace all anatomical projections to the PL (**Figure 2.3A,B**), while cFos IHC 90min after CFC encoding, recent and remote memory recall allowed to assess the activation of these projections (**Figure 2.3A**). In each region we measured cFos as well as GFP traced inputs (**Supplementary figures S2.2 and S2.3**), thus controlling for homogenous tracing across behavioral groups. Next, we compared the pattern of activation between PL projectors only and the region as a whole to highlight the specific recruitment of PL projectors, and we focused on the associative information conveyed in this activity by normalizing it to the no shock control groups (**Figure 2.3**, see **Supplementary figures S2.2 and S2.3** for quantifications only normalized to chance level).

We first investigated cortical areas projecting to PL: EC (specifically layer 5, comprising most of EC traced cells, **Figure 2.3C**), RSPag (which contained most of RSP traced cells, **Figure 3G**), and INS (**Figure 2.3K**). In the EC, we observed a significant activation of PL projections at encoding compared to both recent and remote recalls (**Figure 2.3E**), which was not the case in total cFos quantifications (**Figure 2.3F**). This suggests a specific recruitment of EC neurons projecting to PL (EC → PL) at encoding. In contrast, RSPag and INS displayed a different pattern of activation, as there was no activation in RSPag → PL and INS → PL projections at encoding, but during recent memory recall (**Figure 2.3I and M**, respectively). Compared to total cFos in both regions, this activity was again specific to PL projectors (**Figure 2.3J and N**, respectively).

Second, we investigated PL inputs in subcortical areas: vCA1 (**Figure 2.3O**), BLA (**Figure 2.3S**) and CLA (**Figure 2.3W**). In vCA1, we did not observe a differential recruitment of vCA1 → PL projections between different times of memory consolidation (**Figure 2.3Q, R; Supplementary figure S2.3D**). However, the elevated cFos expression in the vCA1 as a whole at encoding as well as in the no shock group supports the role of HPC in context



exploration (**Supplementary figure S2.3B**) (Schiller et al., 2015). For the BLA, we observed a significant activation of BLA → PL projections at encoding compared to the recalls (**Figure 2.3U**), which is not the case for total cFos in BLA (**Figure 2.3V**). The recruitment of BLA → PL projection at encoding is in agreement with its importance during fear memory formation (Kitamura et al., 2017; Klavir et al., 2017). Interestingly, we observed the same pattern of activation in CLA → PL projections (**Figure 2.3N**), together with an overall higher activation of the whole CLA region at encoding compared to remote recall (**Figure 2.3M**).

Taken together, we found that PL inputs from the EC, BLA and CLA were active only at encoding, while RSPag and INS projections were recruited during recent memory recall.

2.1.3.4 PL inputs are functionally relevant at different stages of memory consolidation

Next, in order to establish whether the differential activity in PL inputs across memory consolidation is also functionally relevant, we selectively inhibited each projection at the time(s) when they were most active and tested subsequent memory retention. We used the Designer Receptor Exclusively Activated by Designer Drug (DREADD) receptor hM4Di, which upon Clozapine-N-Oxide (CNO, the DREADD agonist) administration inhibits neuronal activity (Roth, 2016). We targeted hM4Di expression to specific PL inputs by injecting AAVretro-Cre into the PL, and AAV-DIO-hM4Di-mCherry (or AAV-DIO-mCherry for controls) in the desired input region (**Figure 2.4A-F**).

First, we assessed the functionality of projections that were active at encoding, namely the CLA, BLA, and EC. For the CLA → PL inhibition, we observed an impaired memory at recent recall (**Figure 2.4G**). To confirm that CNO indeed inhibits hM4Di-expressing neurons, we expressed it in CLA → PL neurons (**Supplementary figure S2.4A**) and perfused the animals 90 minutes after CFC to stain for cFos (**Supplementary figure S2.4B,C**). While the percentages of hM4Di+ and cFos+ cells were equivalent in both groups (**Supplementary figure S2.4D,E**), the amount of double positive hM4Di+cFos+ cells was significantly decreased with CNO injection, confirming the inhibition of projection neurons during behavior (**Supplementary figure S2.4F**). Furthermore, this behavioral result was not due to a delayed effect of CNO injection, as inhibiting CLA → PL projections right after encoding did not result in impaired memory at any time point (**Supplementary figure S2.5A,B**). Likewise, the effect of CLA → PL inhibition was not due to an unspecific effect on locomotion and exploratory behavior as tested in an open-field arena (**Supplementary figure S2.5C-E**). In contrast to the

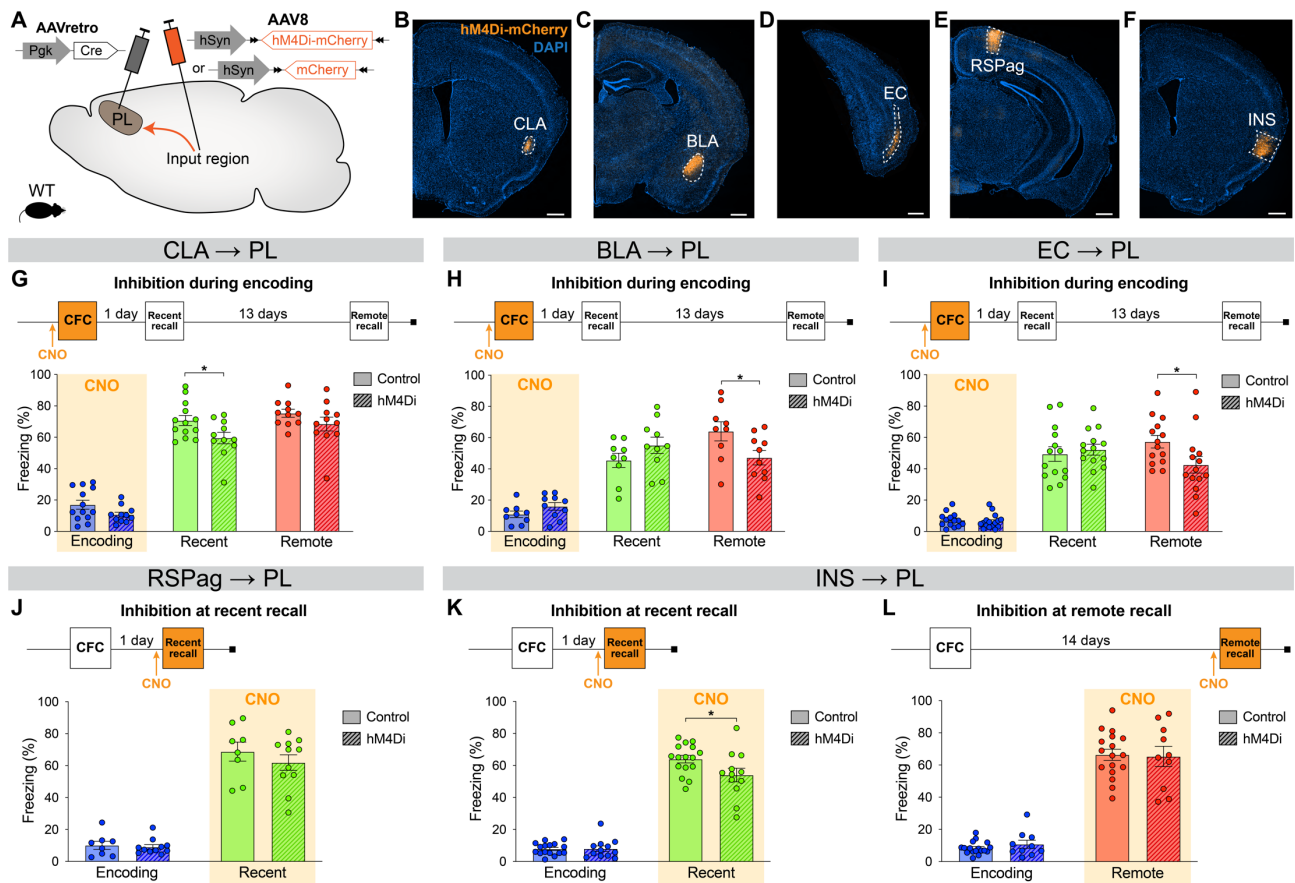


Figure 2.4. Chemogenetic manipulation of PL inputs reveals the functional importance of CLA projections at encoding and INS projections at recent memory recall.

(A) Experimental design. AAVretro-Cre was injected in the PL, and AAV-DIO-hM4Di-mCherry (or AAV-DIO-mCherry for controls) in the desired input region in order to specifically inhibit the projections to the PL upon CNO injection. Representative images of the injection site in the input region for CLA **(B)**, BLA **(C)**, EC **(D)**, RSPag **(E)** and INS **(F)**, all scales 500 μ m. Experimental timeline and freezing percentage of **(G)** CLA → PL inhibition during encoding, **(H)** BLA → PL inhibition during encoding, **(I)** EC → PL inhibition during encoding, **(J)** RSPag → PL inhibition during recent recall, **(K-L)** INS → PL inhibition during recent **(K)** and remote **(L)** recall. Stars represent p-values of two-tailed unpaired t-tests between hM4Di and control groups (*: $p \leq 0.05$). $n = 8-17$ animals per group.

effect of CLA → PL inhibition, when BLA → PL and EC → PL projections were inhibited during encoding, we observed an impairment of remote memory recall for both, while recent recall was unaffected (**Figure 2.4H and I**, respectively). These results indicate that while the BLA → PL and EC → PL projections are necessary at encoding for the consolidation of remote memories, as shown previously (Kitamura et al., 2017), the CLA → PL projection is necessary at encoding for recalling recent memories.

Next, we tested the functionality of projections that were most active during fear memory recall, namely the RSPag and INS to PL projections. We found that although the RSPag → PL projection was specifically active at recent recall (**Figure 2.3I**), its inhibition during this time did

not affect memory retrieval (**Figure 2.4J**). Interestingly, it was recently reported that although the whole RSP is necessary for recent and remote recall, it is rather the granular subregion of the RSP and not the RSPag that is responsible for this effect, suggesting a dissociated role of the two RSP subregions which could explain our observations (Tsai et al., 2022). In contrast, INS → PL projection inhibition during recent recall resulted in decreased freezing (**Figure 2.4K**). Lastly, consistent with no significant activation of the INS → PL projection at remote memory recall, the inhibition of this projection did not result in any behavioral effect (**Figure 2.4L**).

Taken together, these findings indicate that CLA, BLA and EC projections to the PL are necessary at encoding, but with different time implications. While the BLA and EC connections are important for recalling remote memories, the CLA projection is specifically important for recalling recent ones. In addition, recent memory recall is also under the influence of the INS → PL projection, since its inhibition at this time led to significant memory impairment. This suggests a progressive functional shift in PL projections regulating memory consolidation.

2.1.3.5 PL engram reactivation correlates with memory retrieval when CLA or INS inputs are inhibited

Lastly, we decided to further investigate the effect of CLA and INS input inhibition on engram reactivation in the PL. We hypothesized that if the inhibition of a specific PL input results in memory impairment, then the reactivation of the original PL engram, established at the time of memory encoding, may also be altered. Indeed, engram reactivation has been correlated with memory retention in BLA (Reijmers et al., 2007), and artificial engram reactivation in the PL (Kitamura et al., 2017) or HPC (Liu et al., 2012) has been found to trigger memory recall. In order to measure engram reactivation, we used the cFos::tTA mouse line (Reijmers et al., 2007), expressing the Doxycycline (Dox)-dependent tTA transcription factor under the cFos promoter, which we injected with AAV-TRE-GFP into the PL 3 weeks before CFC (**Figure 2.5A**). As tTA specifically binds the TRE (tetracycline responsive element) promoter in absence of Dox, this approach allows for inducible long-term expression of GFP in PL engram cells during a desired time-window (**Figure 2.5B,C**). In combination with chemogenetic inhibition of projection neurons as previously described (**Figure 2.4**), we then silenced selective PL inputs and assessed the degree of engram reactivation between CFC encoding and recall, by measuring cFos and GFP overlap in PL (**Figure 2.5C**).

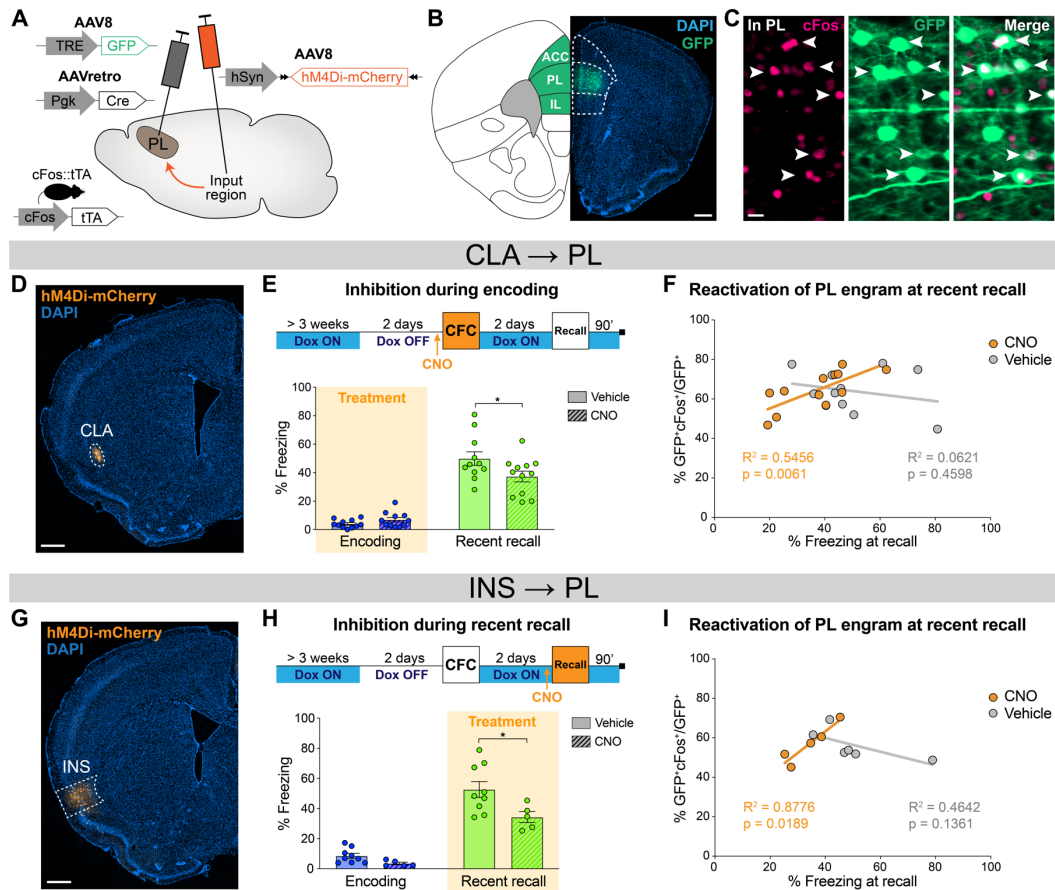


Figure 2.5. PL engram correlates with freezing when CLA or INS inputs are inhibited.

(A) Experimental design. 3 weeks before behavior started, cFos::tTA mice were injected with AAVretro-Cre in PL and AAV-DIO-hM4Di-mCherry in the desired input region, as well as AAV-TRE-GFP in PL, so that GFP was only expressed in cFos⁺ cells in absence of Doxycycline (Dox). (B) GFP expression at the PL injection site (scale 400 μ m). (C) Magnified view in PL (scale 20 μ m) of reactivated engram cells, indicated by white arrows. (D) Representative image of the CLA input region. (E) Experimental timeline (top) and freezing percentage (bottom) during recent memory recall when CLA → PL projections were inhibited during encoding. (F) Reactivation of PL engram cells (%GFP+cFos⁺/GFP⁺) at recent recall for CLA → PL inhibition, correlated with freezing percentage at recent recall for CNO (orange) and vehicle (grey) groups. (G) Representative image of the INS input region. (H) Experimental timeline (top) and freezing percentage (bottom) during recent memory recall when INS → PL projections were inhibited during recent recall. (I) Reactivation of PL engram cells (%GFP+cFos⁺/GFP⁺) at recent recall for INS → PL inhibition, correlated with freezing percentage at recent recall for CNO (orange) and vehicle (grey) groups. (E, H) Stars represent p-values of two-tailed unpaired t-tests between CNO and vehicle groups (*: $p \leq 0.05$). (F, I) Correlations assessed with linear regressions, R^2 and p-values are reported on the graphs. $n = 11-12$ (CLA) or $5-9$ (INS) per group.

First, we focused on the CLA → PL inhibition at memory encoding (**Figure 2.5D**). Behaviorally, this approach confirmed the impaired memory at recent memory recall as observed in wild-type mice (**Figure 2.5E**, see also **Figure 2.4G**). Furthermore, we found a significant correlation between PL engram cells reactivation, measured as double positive GFP⁺cFos⁺ cells normalized to the total number of GFP⁺ cells, and freezing at recent recall, which was observed only in the CNO group (**Figure 2.5F**). No differences were observed in overall GFP⁺, cFos⁺,

double positive GFP⁺cFos⁺ and total reactivation percentages in PL between CNO- and vehicle-treated animals. (**Supplementary figure S2.6A-D**). In contrast, when we inhibited the CLA → PL projection during encoding and tested remote recall, memory was not impaired (**Supplementary figure S2.7B**, see also **Figure 2.4G**) and we observed no effect on PL engram reactivation (**Supplementary figure S2.7C-G**). This result indicates that CLA → PL inhibition during encoding modifies PL engram reactivation at recent recall only, and that following this inhibition, the less animals reactivate the original PL engram, the less they recall the fear memory.

Second, we inhibited the INS → PL projection at recent recall (**Figure 2.5G**), which also confirmed the behavioral effect on memory retrieval (**Figure 2.5H**) in wild type mice (**Figure 2.4K**). Similar to the CLA results, there was no difference between the CNO and vehicle groups in the percentage of GFP⁺, cFos⁺, double positive GFP⁺/cFos⁺ cells or total reactivation (**Supplementary figure S2.6E-H**). However, we again observed a significant correlation in the CNO group between PL engram reactivation and freezing at recent recall (**Figure 2.5I**), indicating that INS → PL inhibition at recent recall impairs recent memory retrieval and modifies PL engram reactivation.

These findings suggest that the spatiotemporal shift in the activity and functionality of PL projections during memory consolidation also occurs at the level of PL engram cells.

2.1.4 Discussion

In this study, we investigated the role of specific PL inputs during the course of fear memory consolidation. Using an unbiased tracing approach combined with pathway-specific chemogenetic inhibition experiments, we discovered a novel functional implication of two PL inputs, namely from the CLA and INS, and confirmed the role of two others, from the BLA and EC. More precisely, we found that the BLA → PL and EC → PL projections are necessary at encoding for remote memory recall, confirming previous results (Kitamura et al., 2017), while the CLA → PL projection is necessary also at encoding, but specifically for recent memory recall. In addition, we found the INS → PL pathway to be necessary for memory expression during recent recall.

These results expand the existing literature on memory consolidation and refine the working model of memory formation and retrieval. Importantly, our data add to the growing evidence on the importance of the mPFC during early phases of memory consolidation (Bero et al., 2014; Cho et al., 2017; Rajasethupathy et al., 2015; Takehara-Nishiuchi et al., 2020;

Zelikowsky et al., 2013). Thereby it further challenges the standard theory of memory consolidation, which posits that the HPC is necessary for encoding and recent recall, while the mPFC would take over from the HPC only at remote recall (Frankland and Bontempi, 2005). Indeed, we observe a significant activation of the PL during memory encoding already, as well as the necessity of several of its inputs at encoding and recent recall, which advances the temporal engagement of the mPFC to earlier than remote recall only.

At remote memory recall, in contrast, we did not observe any PL input that was engaged or behaviorally relevant. To our knowledge, such an input has never been reported, although the importance of mPFC as a whole (Bero et al., 2014; Frankland et al., 2004b; Goshen et al., 2011), and of its functional outputs at remote recall is well established (Do-Monte et al., 2015a; Kitamura et al., 2017). This apparent gap, or the impossibility to trace back the flow of information upstream of the mPFC, could be explained if the inputs are distributed across a vast network after memory consolidation. In that case, they could potentially be redundant and therefore harder to functionally identify. As such, these results are in agreement with the multiple trace theory (Moscovitch et al., 2006; Nadel and Moscovitch, 1997), which posits that the HPC first encodes memory upon learning, but does not store the memory per se. Rather, it is a distributed cortical network of neurons – with inputs from the HPC – that represents pieces for long-term information storage. With time, this leads to the creation of multiple traces in the brain for a given memory, making it more stable and less likely to be disrupted, as we have observed here.

Corroborating the implication of the PL during the early phases of memory consolidation, we found that the CLA → PL is necessary at encoding, which is the first report that this PL input is functionally important during fear memory formation. The CLA has known roles in attention (Atlan et al., 2018) and context exploration (Kitanishi and Matsuo, 2017), which are likely to support its role in memory formation. Interestingly, we observed that the CLA → PL projection was necessary at encoding only for recent recall, and not for a later remote recall. This result suggests that this projection has a time-limited effect on memory consolidation, and that other brain areas allow for a proper remote recall and thereby compensate in case of the CLA → PL inhibition at encoding. Of note, a CLA → EC projection has also been reported to be necessary at CFC encoding for recent recall (Kitanishi and Matsuo, 2017), while here we have observed that the EC → PL projection is functional at encoding for remote recall. This opens the possibility of an indirect circuit from the CLA to the PL via the EC, which could compensate in case the CLA → PL direct connection is impaired, but this remains to be experimentally determined. Nevertheless, these results imply a shift in PL circuits underlying contextual fear

memory encoding, reminiscent of findings that reported a shift in PL circuits when an auditory fear memory is retrieved (Do-Monte et al., 2015a).

In line with the notion of an overall shift in PL circuits during early memory consolidation, we report that the INS → PL projection is necessary at recent, but not at remote memory recall. The INS as a whole has classically been involved in taste learning (Yiannakas and Rosenblum, 2017) and the encoding of conditioned taste aversion (Sano et al., 2014), but has also been implicated in recent CFC recall (Alves et al., 2013) as well as in auditory fear memory extinction (Klein et al., 2021). The INS to mPFC reciprocal connectivity has only been investigated in the context of taste learning, where it was recently found necessary for the expression of novel taste aversion (Kayyal et al., 2021). However, a direct role of INS input to PL during fear memory consolidation has not been described before. This finding therefore supports a broader role for the INS in learning beyond taste-related tasks (Boughter and Fletcher, 2021; Shi et al., 2020).

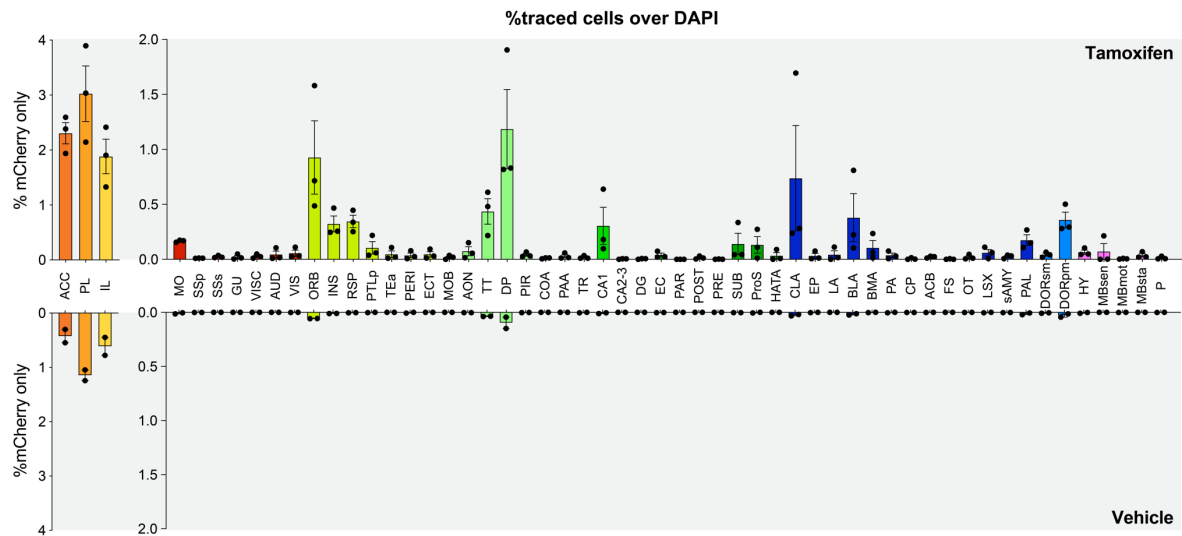
Since the CLA and INS manipulations both resulted in impaired recent memory recall, we decided to assess PL engram cells for their reactivation, a characteristic of engram cells that is linked with memory performance during recall (Kitamura et al., 2017; Liu et al., 2012; Reijmers et al., 2007). We found that the reactivation of PL engram cells significantly correlated with freezing behavior, but only when the CLA and INS inputs were inhibited, and not in controls. This finding raises the question of the functionality of PL engram cells at recent recall in normal conditions. Recently, a concept of “silent” engram cells in the PL has been developed, which postulates that silent PL engram cells have the particular feature of not being activated by recent recall – although their artificial reactivation can trigger recall at this time –, but of becoming active only at remote recall (Kitamura et al., 2017; Matos et al., 2019). Our findings are thus in line with this concept: When the CLA → PL projection is inhibited at encoding, or when the INS → PL projection is inhibited at recent recall, overall engram reactivation is left unchanged, but recent memory expression is impaired, which would suggest that the PL engram population itself is not required for recent memory recall. Since upon inhibition we observe the emergence of a correlation between PL engram cells reactivation and memory retention, it is possible that CLA and INS inputs target inhibitory neurons in the PL, which normally prevent engram reactivation during recent recall, thus allowing the PL engram cells to stay functionally silent. Releasing this inhibition could perturb normal memory expression, explaining the observed memory impairment at recent recall. Indeed, it has been reported that CLA → mPFC targets inhibitory neurons (Jackson et al., 2018), and that PL

interneurons are necessary for memory encoding (Cummings and Clem, 2020), but this hypothesis remains to be tested at the level of PL engram cells.

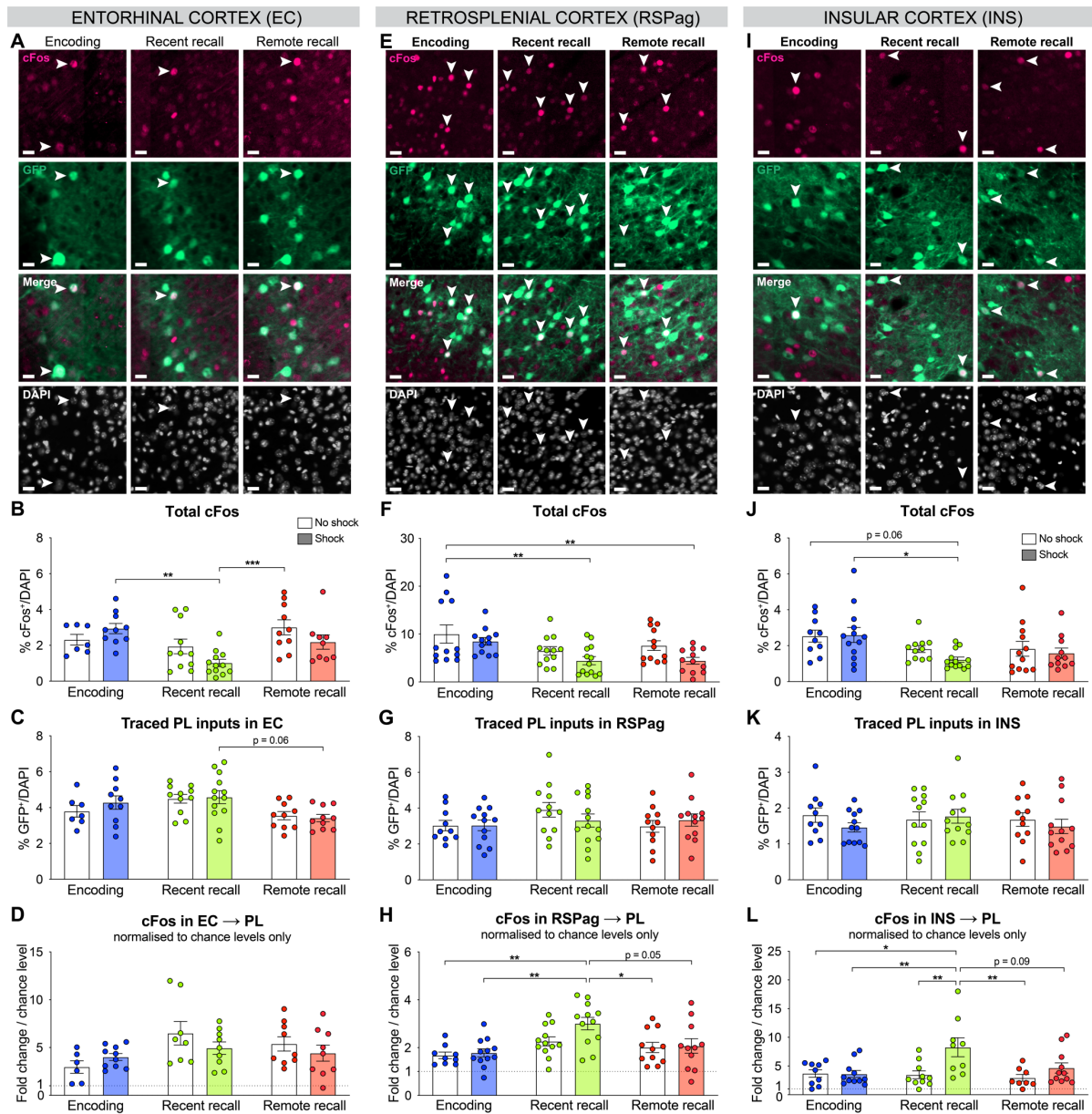
There are several experimental limitations that accompany these findings. First, the use of two different engram-tagging mouse lines, TRAP2 for the rabies tracing and cFos::tTA for the engram reactivation experiments, was dictated by technical constraints. A TRE-dependent rabies tracing system was not readily available at the start of this study, and the use of a Cre-dependent system for chemogenetic inhibition precluded the use of the TRAP2 line again for the engram reactivation experiments. However, as these two mouse lines are both cFos promoter-based (DeNardo et al., 2019a; Reijmers et al., 2007), we would not expect major differences with the use of one or the other lines. Indeed, rabies tracing from PL engram cells using the cFos::tTA line has been published since, and the reported input areas are all also found in our brain-wide screen (Kitamura et al., 2017). Second, the use of two different retrograde tracing viruses raises the question of tropism. Indeed, RVΔG and AAVretro have been reported to not trace the exact same set of input regions, notably with AAVretro being biased towards cortical inputs (Sun et al., 2019). We could have therefore missed some regions due to preferential input tracing. A third limitation are the relatively slow kinetics of chemogenetic inhibition. As CNO is injected 30 minutes before behavior, we cannot exclude that compensation mechanisms may take over, especially in the case of remote recall inhibition, which would prevent a functional isolation of the targeted projection during behavior as reported previously (Goshen et al., 2011). By restricting the inhibition to the smallest possible period, the use of optogenetics could allow to visualize the consequences of this inhibition in real time in future experiments. Lastly, differences in timing and strength of behavioral protocols could explain discrepancies with other studies. For example, using cFos IHC, we did not observe an increased activity at remote recall in mPFC regions as opposed to previous findings (Frankland et al., 2004b). Unified conditioning protocols could help to clarify these.

These limitations notwithstanding, here we have shown that PL circuits undergo a spatiotemporal shift during contextual fear memory consolidation, with claustral inputs being critical at encoding, and insular cortical inputs at recent memory recall. Our results therefore support a dynamic and distributed nature of memory formation and storage.

2.1.5 Supplementary figures

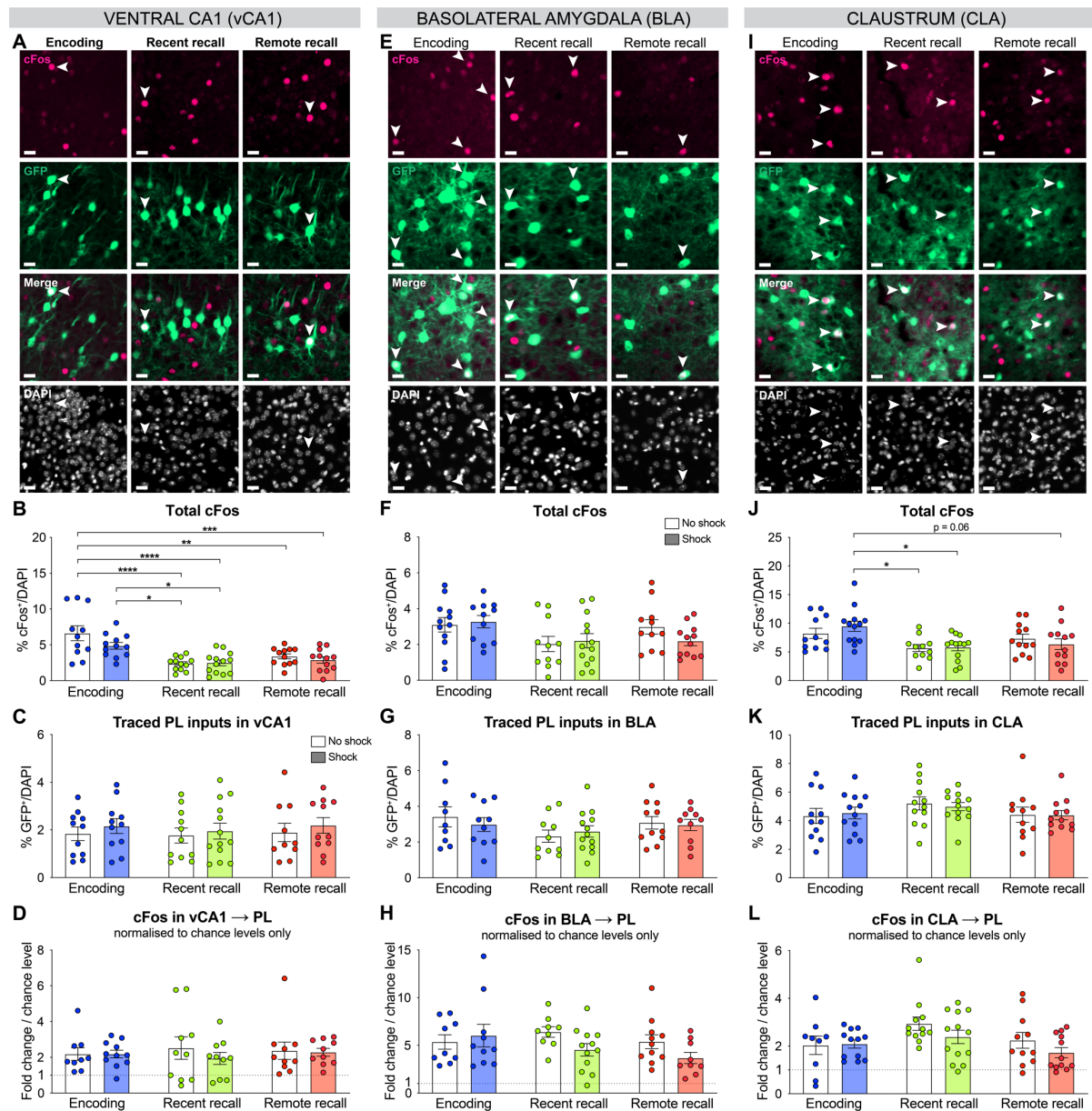


Supplementary Figure S2.1. Raw quantifications of the rabies tracing experiment. Percentage of traced cells (mCherry⁺ only) in all regions with tamoxifen (top, n = 3 animals) or vehicle (bottom, n = 2 animals).



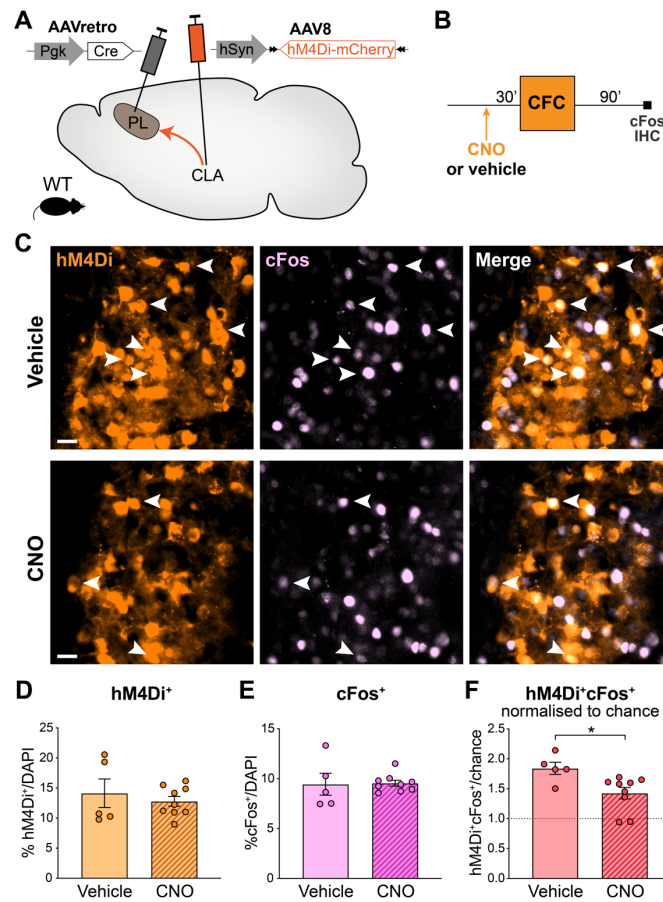
Supplementary Figure S2.2. Complementary quantifications for the activation of PL inputs in EC, RSPag and INS.

All representative images (from Figure 2.2) for EC (**A**), RSPag (**E**) and INS (**I**), scale 20 μ m. Total cFos percentage in EC (**B**, one-way ANOVA, $F(5, 53) = 5.075$, $p = 0.0007$), RSPag (**F**, one-way ANOVA, $F(5, 67) = 4.156$, $p = 0.0024$) and INS (**J**, one-way ANOVA, $F(5, 64) = 2.971$, $p = 0.0179$). Distribution of traced PL inputs across behavioral groups in EC (**C**, one-way ANOVA, $F(5, 55) = 2.794$, $p = 0.0255$), RSPag (**G**) and INS (**K**). Double positives (cFos+GFP+) normalized to chance levels in EC (**D**), RSPag (**H**, one-way ANOVA, $F(5, 61) = 4.618$, $p = 0.0012$) and INS (**L**, one-way ANOVA, $F(5, 52) = 4.312$, $p = 0.0023$). Stars represent p-values of Tukey's multiple comparisons tests (*: $p \leq 0.05$; **: $0.001 < p \leq 0.01$, ***: $0.0001 < p \leq 0.001$, ****: $p \leq 0.0001$). $n = 9-13$ animals per group.

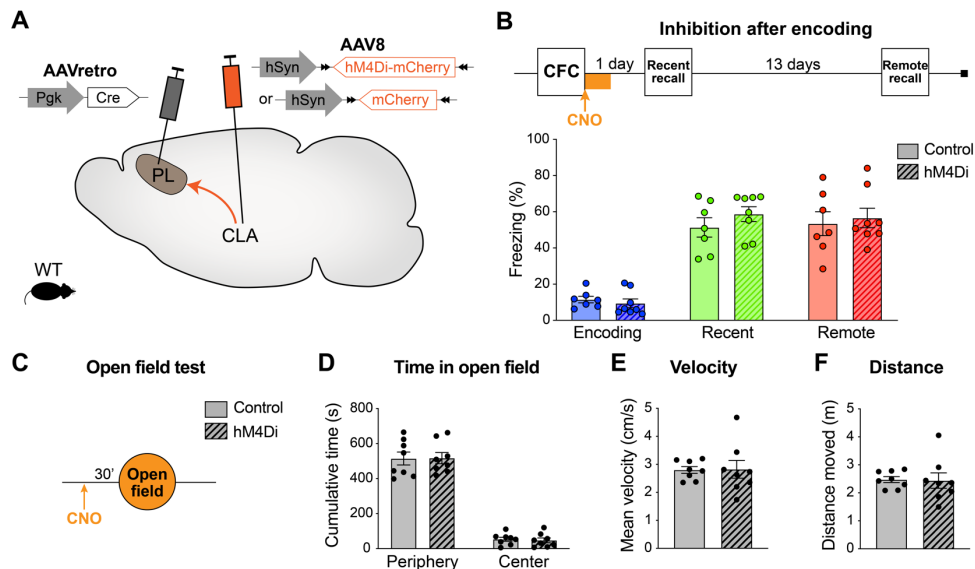


Supplementary Figure S2.3. Complementary quantifications for the activation of PL inputs in vCA1, BLA and CLA.

All representative images (from Figure 2.2) for vCA1 (**A**), BLA (**E**) and CLA (**I**), scale $20\mu\text{m}$. Total cFos percentage in vCA1 (**B**, one-way ANOVA, $F(5, 66) = 9.298$, $p < 0.0001$), BLA (**F**) and CLA (**J**, one-way ANOVA, $F(5, 66) = 3.593$, $p = 0.0062$). Distribution of traced PL inputs across behavioral groups in vCA1 (**C**), BLA (**G**) and CLA (**K**). Double positives (cFos+GFP+) normalized to chance levels only in vCA1 (**L**), BLA (**H**) and CLA (**L**, one-way ANOVA, $F(5, 63) = 2.282$, $p = 0.0570$). Stars represent p-values of Tukey's multiple comparisons tests (*: $p \leq 0.05$; **: $0.001 < p \leq 0.01$; ***: $0.0001 < p \leq 0.001$; ****: $p \leq 0.0001$). $n = 9-13$ animals per group.

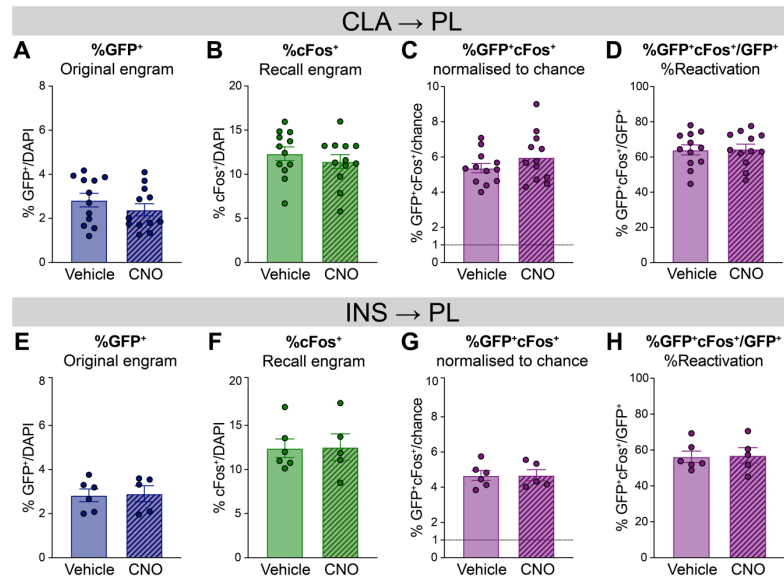


Supplementary Figure S2.4. Quantification of the chemogenetic inactivation of CLA → PL projections. **(A)** Experimental design: AAVretro-Cre was injected in the PL and AAV-DIO-hM4Di-mCherry in the CLA, to express hM4Di specifically in CLA → PL projections. **(B)** Timeline of the experiment. CNO was injected 30 minutes before CFC, and mice were perfused 90 minutes later for cFos IHC. **(C)** Representative images of CNO and vehicle-injected groups, in the CLA. hM4Di in orange, cFos in light pink. Arrows indicate double positive hM4Di+cFos+ cells. Scale 20μm. **(D)** Quantification of hM4Di+ in CLA. **(E)** Quantification of cFos+ in CLA. **(F)** Quantification of double positive hM4Di+cFos+ cells in CLA, normalized to chance. Star represents p-value of two-tailed unpaired t-test between CNO and vehicle groups (*: $p \leq 0.05$). Vehicle: n = 5 animals; CNO: n = 9 animals.

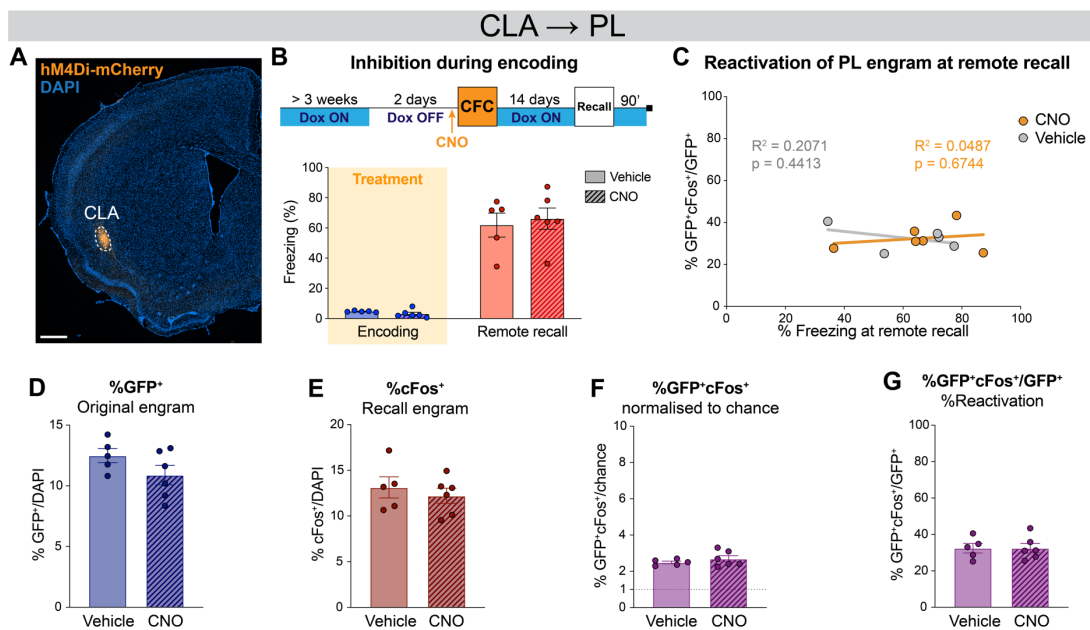


Supplementary Figure S2.5. CLA → PL inhibition after encoding does not impair memory recall and does not alter locomotion and exploration behavior.

(A) Experimental design: AAVretro-Cre was injected in the PL and AAV-DIO-hM4Di-mCherry in the CLA (or AAV-DIO-mCherry for controls), to express hM4Di (or mCherry) specifically in CLA → PL projection. **(B)** Experimental timeline and freezing percentage for CLA → PL inhibition after encoding. CNO was injected i.p. after CFC, and every 2 h, for a total of 4 injections. n = 7-8 per group. **(C)** Experimental timeline of the open field test, 30 minutes after a single CNO injection, measuring **(D)** time spent in the periphery or center of the arena, **(E)** velocity and **(F)** total distance moved. n = 8 per group.



Supplementary Figure S2.6. Complementary quantifications for the engram reactivation analysis in the PL. **(A-D)** For CLA → PL inhibition during encoding, percentage of **(A)** GFP⁺ cells, **(B)** cFos⁺ cells, **(C)** double positive GFP⁺cFos⁺ cells normalized to chance and **(D)** reactivation expressed as %GFP⁺cFos⁺/GFP⁺; **(E-H)** For INS → PL inhibition during recent recall, percentage of **(E)** GFP⁺ cells, **(F)** cFos⁺ cells, **(G)** double positive GFP⁺/cFos⁺ cells normalized to chance and **(H)** reactivation.



Supplementary Figure S2.7. CLA → PL inhibition during encoding does not affect remote recall and engram reactivation in PL.

(A) Representative image of the CLA input region. **(B)** Timeline and freezing percentage of CLA → PL inhibition during encoding and tested at remote recall. **(C)** Reactivation of PL engram cells (%GFP⁺cFos⁺/GFP⁺) at remote recall, correlated with freezing percentage for CNO (orange) and vehicle (grey) groups. Percentage of **(D)** GFP⁺, **(E)** cFos⁺, **(F)** double positives GFP⁺cFos⁺ normalized to chance and **(G)** reactivation as %GFP⁺cFos⁺/GFP⁺. Correlation assessed with linear regression, R² and p-value are reported on the graphs. n = 5-6.

2.1.6 Materials and methods

2.1.6.1 Animals

All animals and procedures used in this study were approved by the Veterinary Office of the Federal Council of Switzerland under the animal experimentation licenses VD2808.1 and VD2808.2. C57Bl/6JR wild-type male mice were purchased from Janvier Labs (France) around 6-7 weeks old and left for at least one week before the beginning of the experiments. cFos::CreERT2 (TRAP2) animals were bred in house from the original Jax strain #030323 on a C57Bl/6JR background. cFos::tTA male mice were bred in house from the original JAX strain #018306 on a C57Bl/6JR background. Animals were housed in a 12h light/dark cycle with water and food available ad libitum. All animals were group-housed except for the input tracing experiment where they were single caged 2 days before the end of the experiment. They were all handled by the experimenter for at least 3 days before the first behavioral procedure to reduce stress levels.

All behavioral procedures were performed between 1pm and 5pm local time and animals were randomly assigned to experimental groups.

2.1.6.2 Viral stereotaxic injections

Procedure: At 7-8 weeks, animals were anesthetized with a mix of Fentanyl (0.05 mg/kg), Midazolam (5 mg/kg) and Metomedin (0.5 mg/kg), i.p. After shaving and subcutaneous injection of a local anaesthetic mix (Lidocaine 6 mg/kg and Bupivacaine 2.5 mg/kg), the animal was placed on a stereotaxic frame. The skin was disinfected with Betadine and opened with a scalpel. The skull was thoroughly cleaned, the orientation of the head was adjusted, and holes were drilled at the desired coordinates with a 0.5mm drill bit. The virus was loaded into pulled glass capillaries (intraMARK, Blaubrand, tip diameter 10-20 μm), and injected to the target area at a speed of 100 nL/min. The needle was left in place for 5 min, and slowly pulled up to limit backflow. After all injections were done, the skin was sutured (5/0 Prolene, Ethicon), the animal was injected i.p. with Atipamezol (2.5 mg/kg) to reverse the anaesthesia, and placed back in a heated cage. After surgery, the animals were administered paracetamol in the drinking water for a week (Dafalgan, 1mg/mL).

Viruses: The following viruses were used in this study:

- AAV-DIO-TVA-2A-oG (Salk Institute Vector Core, serotype 8), here referred to as AAV-DIO-TVA-oG. Titer: 8.78×10^{12} GC/mL, mixed 1:1 with AAV-FLEX-GFP-oG (see below).
- AAV-EF1a-FLEX-H2B-GFP-P2A-oG (Salk Institute Vector Core, serotype 8), here referred to as AAV-FLEX-GFP-oG. Titer: 3.93×10^{12} GC/mL, mixed 1:1 with AAV-DIO-TVA-oG; total injection volume in PL: 400nL.
- Modified rabies virus RVΔG-mCherry, EnvA pseudotyped (Salk Institute Vector Core, SADB19 strain). Titer: 3.5×10^8 ifu/mL. Injection volume in PL: 300nL.
- AAV-CAG-GFP (Addgene 37825, retrograde serotype), here referred to as AAVretro-GFP. Titer: 7×10^{12} GC/mL; injection volume in PL: 200nL.
- AAV-pgk-Cre (Addgene 24593, retrograde serotype), here referred to as AAVretro-Cre. Titer: 1.7×10^{13} GC/mL; injection volume in PL: 200nL.
- AAV-hSyn-DIO-hM4D(Gi)-mCherry (Addgene 44362 or Zürich VVF v84, serotype 8), here referred to as AAV-DIO-hM4Di-mCherry. Titer: 1.8×10^{13} GC/mL (Addgene – diluted $\frac{1}{2}$) or 4.5×10^{12} GC/mL (VVF); injection volume: 150-200nL depending on the regions.
- AAV-hSyn-DIO-mCherry (Addgene 50459, serotype 8), here referred to as AAV-DIO-mCherry. Titer: 2.3×10^{13} GC/mL, diluted $\frac{1}{2}$; injection volume: 150-200nL depending on the regions.
- AAV-TRE3G-GFP (UNC Vector Core, serotype 8), here referred to as AAV-TRE-GFP. Titer: 4.1×10^{12} GC/mL, mixed 1:1 with AAVretro-Cre; injection volume in PL: 250nL.

Injection coordinates from bregma:

- PL: AP +2.0; ML ± 0.35 ; DV -2.2.
- EC: AP -4.15; ML ± 3.55 ; DV -4.3.
- RSPag: AP -2.6; ML ± 1.1 ; DV -0.6.
- INS: AP +1.0; ML ± 3.85 ; DV -4.0 in WT mice, or AP +1.0; ML ± 3.9 ; DV -4.0 in cFos::tTA mice.
- BLA: AP -1.0; ML ± 3.15 ; DV -4.55.
- CLA: AP +1.0; ML ± 3.2 ; DV -4.0 in WT mice, or AP +1.0; ML ± 3.1 ; DV -4.0 in cFos::tTA mice.

For input tracing experiment with AAVretro-GFP, animals were injected unilaterally. For all other experiments, animals were injected bilaterally.

2.1.6.3 Behavioral procedures

Contextual fear conditioning (CFC): CFC encoding and recall were performed in the same chamber (TSE Systems). CFC encoding consisted in a first 3min exploration phase, followed by three 2s long 0.8mA foot-shocks spaced by 28s. After the last shock, the animal was left in the chamber for an additional 15s and brought back to its home cage. The recall consisted in a 3min exposure to the same context, without any shock. For all experiments except the engram reactivation, recent recall took place 1 day after the encoding and remote recall 14 days later. For the engram reactivation experiment, recent recall took place 2 days after the encoding, to leave enough time for GFP expression. The movement of the animals was automatically measured using an infrared beam cut detection system (TSE Systems). Freezing detection threshold was set at 1s of immobility. No shock control animals underwent the same procedure but did not receive any shocks. Animals without any chemogenetic manipulation were excluded if they froze less than 20% of the time during the recall (in total 2 animals were excluded in all experiments).

Tamoxifen injection: In the rabies tracing experiment, TRAP2 mice were injected i.p. with Tamoxifen (4-hydroxytamoxifen, Sigma-Aldrich, CAS 68392-35-8, 50 mg/kg) immediately after CFC. Tamoxifen was prepared as follows: powdered tamoxifen was dissolved in Ethanol 100% at a concentration of 20 mg/mL and stored at -20°C. On the day of the experiment, tamoxifen was re-dissolved by shaking at 37°C, 2 volumes of corn oil were added and ethanol was evaporated shaking at 37°C, for a final concentration at 10 mg/mL. Tamoxifen was kept at 37°C until injection to prevent precipitation.

Open field test: For CNO control experiments, 30min after CNO injection the animals were placed in a large circular arena and left to freely explore for 15min. Video-tracking of the animals and locomotion quantification was automatically performed using the EthoVision software (Noldus).

Engram reactivation: cFos::tTA mice were administered Doxycycline (Dox, Sigma-Aldrich, CAS 24390-14-5) in the drinking water at 0.2 mg/mL. Dox was prepared as follows: Powdered Dox was resuspended in water from the animal facility at 50mg/mL, aliquoted and frozen at -20°C until further use. It was then diluted in water bottles to reach a concentration of 0.2 mg/mL. Dox was administered at least 3 weeks before the behavioral protocol started, and was refreshed every week. In order to open the tagging-window, Dox was removed 2 days before encoding, and administered back right thereafter for the remaining time of the protocol.

Sample size and behavioral replicates: No statistical method was used to predetermine sample size. The number of animals used in each experiment was the minimum required to obtain statistical significance, based on our experience with this behavioral paradigm and in agreement with standard literature. Data from the input tracing experiment was pooled from 3 independent batches (**Figure 2.1 and 2.3** and supplements). Data from the rabies tracing experiment comes from one batch (**Figure 2.2** and supplements). Data from the chemogenetic manipulation in WT was pooled from at least 2 independent batches for each manipulation (**Figure 2.4**). Data from the CNO controls comes from one batch each (**Supplementary figure S2.4 and S2.5C-F**). Data from the engram reactivation was pooled from 1-2 batches (**Figure 2.5** and supplement).

2.1.6.4 Histology

90min after the last behavioral test, animals were anesthetized with pentobarbital (150 mg/kg, Streuli Pharma) and transcardially perfused with first 1X PBS and then 4% paraformaldehyde (PFA) in 1X PBS. Brains were extracted, post-fixed overnight in 4% PFA, transferred in cryoprotectant (30% sucrose in 1X PBS) for at least 48h, and frozen at -80°C. Sections of 20 μ m were cut using a cryostat and kept free-floating in an antifreeze solution (30% ethylene glycol, 15% sucrose, 0.02% azide in 1X PBS) until staining.

For cFos immunostaining, sections were incubated in blocking buffer (1% BSA, 0.3% Triton-X in 1X PBS) for 90min at room temperature, followed by primary antibody incubation for 2 nights at 4°C in antibody dilution buffer (1% BSA, 0.1% Triton-X in 1X PBS). After 4 washes in 1X PBS + 0.1% Triton-X, they were incubated with secondary antibody in antibody dilution buffer for 2h at room temperature, stained with Hoechst (Life Technologies, 1:10.000 in 1X PBS) for 5min and washed 3 times before mounting on glass slides and covered with Fluoromount-G mounting medium (Southern Biotech). Images were acquired on an Olympus slide scanner VS120 L100 with a 20X objective.

For the input tracing experiment, a primary antibody goat anti-cFos (1:1000, Santa Cruz #sc-52-G, RRID: AB_2629503) with a secondary antibody donkey anti-goat AF-647 (1:1000, Thermo Fisher Scientific #A21447, RRID: AB_2535864) was used. For all other experiments, a primary antibody rabbit anti-cFos (1:1000, Synaptic Systems #226003, RRID: AB_2231974) with a secondary antibody donkey anti-rabbit AF-647 (1:1000, Thermo Fisher Scientific #A31573, RRID: AB_2536183) was used. GFP and mCherry signals were not amplified.

For verification of the injection sites, 6 sections per animal were taken spanning the injection site, stained with Hoechst (Invitrogen, 1:10.000) and mounted. Images were acquired on an Olympus slide scanner VS120 L100 with a 10X objective.

For the rabies tracing experiment, 1 every 4 sections of 20 μ m were mounted on Superfrost slides (Fisher scientific) and stained with Hoechst, before imaging on an Olympus slide scanner VS120 L100 with a 10X objective.

For simplicity and clarity in the text, we used “DAPI” to refer to nuclei stained with Hoechst.

2.1.6.5 Image analysis

Images were analysed using QuPath (v0.1.4 to v0.3.1) (Bankhead et al., 2017), by an experimenter blinded to the groups.

For the rabies tracing experiment (**Figure 2.2**), every section was aligned to the reference Allen Brain Atlas using a Fiji plugin developed by the bioimaging platform at EPFL (Chiaruttini et al., 2022), before using a QuPath custom-built script for cell detection and classification (see supplementary material). It used multiple rounds of the built-in “Cell Detection” plug-in (once for each channel, plus one for DAPI). After detection, cells are given a classification based on the overlap of their coordinates to the DAPI channel detections.

For the input tracing experiment (**Figure 2.3**), 2-3 sections per brain region per animal were manually annotated based on the Allen Brain Reference Atlas, and identification of the detected GFP⁺ and cFos⁺ cells within each annotated region was established using the custom-made QuPath script. An animal was excluded from further analysis if the percentage of traced inputs in a given area was below a region-specific threshold, as the amount of traced cells was region-dependent (thresholds were EC: 2%; RSPag: 1%; INS: 0.5%; vCA1: 0.5%; BLA: 0.6%; CLA: 1%). The chance ratio was calculated as (GFP⁺cFos⁺/DAPI)/chance level, where chance level was calculated as (GFP⁺/DAPI)x(cFos⁺/DAPI). Then, chance ratios were further normalized by the averaged chance ratio of the matching No Shock control groups (**Figure 2.3**). cFos⁺ cells in mPFC (**Figure 2.1**) were quantified in the non-injected contralateral mPFC using 3-4 sections per animal.

For the chemogenetic manipulation experiments (**Figure 2.4 and 2.5**), animals were excluded if the hM4Di-mCherry signal was leaking outside of the target region or if the amount of infected cells was too low. cFos⁺ and hM4Di⁺ in CLA (**Supplementary figure S2.4**) were quantified using the QuPath custom-built script, in 3-4 sections per animal.

For engram reactivation experiments (**Figure 2.5**), cFos⁺ and GFP⁺ cells were quantified in PL using the QuPath custom-built script, in 3-4 sections per animal. Animals were excluded from further analysis if the percentage of GFP was below 1%.

2.1.6.6 Statistics

All statistics and graphical representations were done with GraphPad Prism 9. All data are represented in mean \pm SEM, with one dot representing one animal in all graphs. Data from the input tracing screen were analyzed using ordinary one-way ANOVAs, and further comparisons were performed with Tukey's multiple comparisons tests between all groups ($\alpha = 0.05$). In case of normalizations, difference to 1 was analyzed using two-tailed one sample t-tests ($\alpha = 0.05$). Data from the chemogenetic manipulation and engram reactivation experiments were analyzed using two-tailed unpaired t-tests between the two groups ($\alpha = 0.05$), and correlations were assessed with simple linear regressions.

2.2 PL inputs are part of a broad fear memory network

This section presents additional results that were not included in the submitted article above. They put PL inputs in a wider context and therefore bring interesting elements for the discussion.

So far, we have considered specific PL projections individually. However, we know that other brain regions are involved in fear memory processing and are connected to each other independently of the PL, such as the HPC, the EC, or the BLA (Cho et al., 2017; Herry and Johansen, 2014; Vetere et al., 2017; Zelikowsky et al., 2014). Therefore, we need to consider that these PL inputs are likely incorporated in a broader fear memory network, which links several areas together via multiple pathways. The complexity of these connections, direct or indirect, excitatory or inhibitory, renders the task of unravelling the whole functional network very arduous. In addition, some individual neurons are able to send axons to several brain areas: This has been observed in vCA1 neurons, targeting both BLA and mPFC, which are involved in contextual fear memory expression (Kim and Cho, 2017). Perhaps even more strikingly, this feature has also been observed in CLA neurons with the report from Christof Koch (Allen Institute of Brain Sciences) of a “giant neuron” from the CLA sending processes all around the brain (Reardon, 2017). The existence of axon collaterals from CLA neurons has since then been confirmed in various pathways (Jackson et al., 2018; Kitanishi and Matsuo, 2017; Marriott et al., 2021; Zingg et al., 2018). Therefore, we aimed to first better characterize the PL projections that we were previously chemogenetically manipulating, in particular from the CLA, but also from the INS and EC, in order to decipher the perhaps invisible interplay between (and within) brain regions in fear memory consolidation; and second, to compare PL projections activity patterns to projections to another region also largely involved in fear consolidation, the EC, to address the specificity of the observed neuronal activity.

2.2.1 Results

2.2.1.1 PL-specific inputs also innervate other brain regions

To visualize the axon collaterals sent by PL projecting-neurons, we injected unilaterally AAVretro-Cre in PL as before, as well as AAV-FLEX-tdTomato-synaptoGFP in the input region, which express in a Cre-dependent manner GFP fused with the pre-synaptic protein synaptophysin (**Figure 2.6A**). This allowed us to visualize all the axonal targets of neurons projecting from a given input region to the PL. We started by investigating specifically CLA →

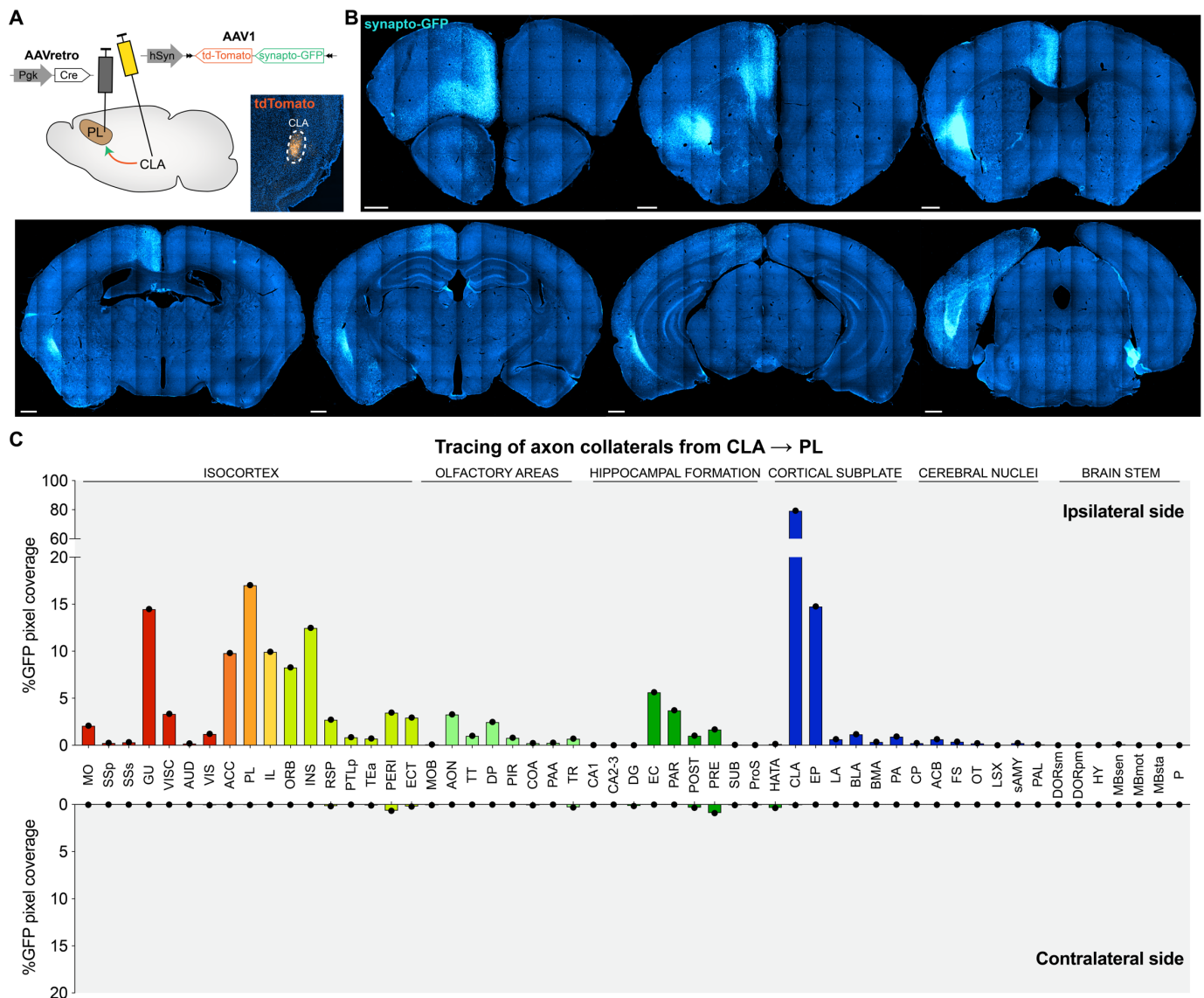


Figure 2.6. CLA to PL projections send axon collaterals in various brain regions.

(A) Experimental design: Mice were injected unilaterally with AAVretro-Cre in PL and AAV-FLEX-tdTomato-synaptoGFP. Representative image of CLA injection site in inset. **(B)** Representative images of synaptoGFP expression across the brain (scale 500 μ m). **(C)** Brain-wide quantification of GFP pixel coverage to measure the density of axonal projections in each region in the ipsilateral (top) or contralateral side (bottom) of the injection site. $n = 1$.

PL projections as CLA was previously reported to send a high number of axon collaterals throughout the brain (Jackson et al., 2018; Reardon, 2017). Indeed, we observed dense projections in many brain regions (**Figure 2.6B,C**). The highest GFP density was found, as expected, in the CLA itself and then in the PL. Neighboring regions of the CLA also expressed high GFP, such as the INS, GU and EP, as well as neighboring regions of the PL: ACC, IL and ORB. Interestingly, more distant regions also showed a high GFP expression, notably the EC, PERI, ECT, RSP and MO cortices, as well as the amygdala subregions. However, the HPC, thalamic and midbrain regions were not targeted by these axon collaterals. Interestingly, we

observed a high hemispheric segregation of these projections that only innervated the ipsilateral side of the injections (**Figure 2.6C**). These observations, although limited to one animal only, confirm the existence and relative importance of axon collaterals in CLA projecting neurons, and we report for the first time a brain-wide screen of collaterals of specific CLA → PL projections. This anatomical feature most likely underlies an important integrative role of the CLA, as well as a top-down influence on many different brain areas.

In addition, we investigated axon collaterals from two other projections, EC → PL and INS → PL (**Supplementary figure S2.8**). Although not quantified in a brain-wide fashion, we also observe the presence of axon collaterals: INS → PL also targets CLA, ACC, amygdala (BMA, LA and BLA) and amygdala-associated cortices, NAc as well as EC and the midline thalamic nuclei, with some projections reaching the contralateral side of the injection (**Supplementary figure S2.8A**); EC → PL also targets CLA and BLA, as well as INS, NAc, vCA1 and lateral EC, but only on the ipsilateral side (**Supplementary figure S2.8B**).

Interestingly, this technique also enabled us to visualize the distribution within the PL of CLA, INS and EC projections (**Supplementary figure S2.9**). We observed a different layer targeting for each input: The very dense CLA projections end primarily in layer 6, but also in layer 2/3 and 5 (**Supplementary figure S2.9A**), while INS projections target mainly layer 2/3 and to a lesser extent layer 6 (**Supplementary figure S2.9B**). In contrast, EC projections are less dense and terminate mainly in layer 5 and layer 2/3 (**Supplementary figure S2.9C**). These differences could underlie the different functional roles of each projection and would be worth investigating further.

Overall, we found that PL-specific projections also innervate other brain regions than the PL via axon collaterals, with a repartition that is specific for each input region. Strikingly, CLA and INS projections to PL also targeted the EC, anatomically located opposite to the PL and also involved, as we have seen here, in fear memory consolidation.

2.2.1.2 EC inputs differential activation across fear memory consolidation

As a fraction of CLA and INS projections to the PL also target the EC, we wanted to assess if the dynamic patterns of activation we observed in PL inputs were specific to this pathway, or could be also observed in EC inputs. We traced in parallel PL and EC inputs using a second retrograde tracer (AAVretro-tdTomato, **Figure 2.7A, B**) injected in the EC, and we could likewise measure the activity of EC inputs in 4 of the regions previously studied to compare

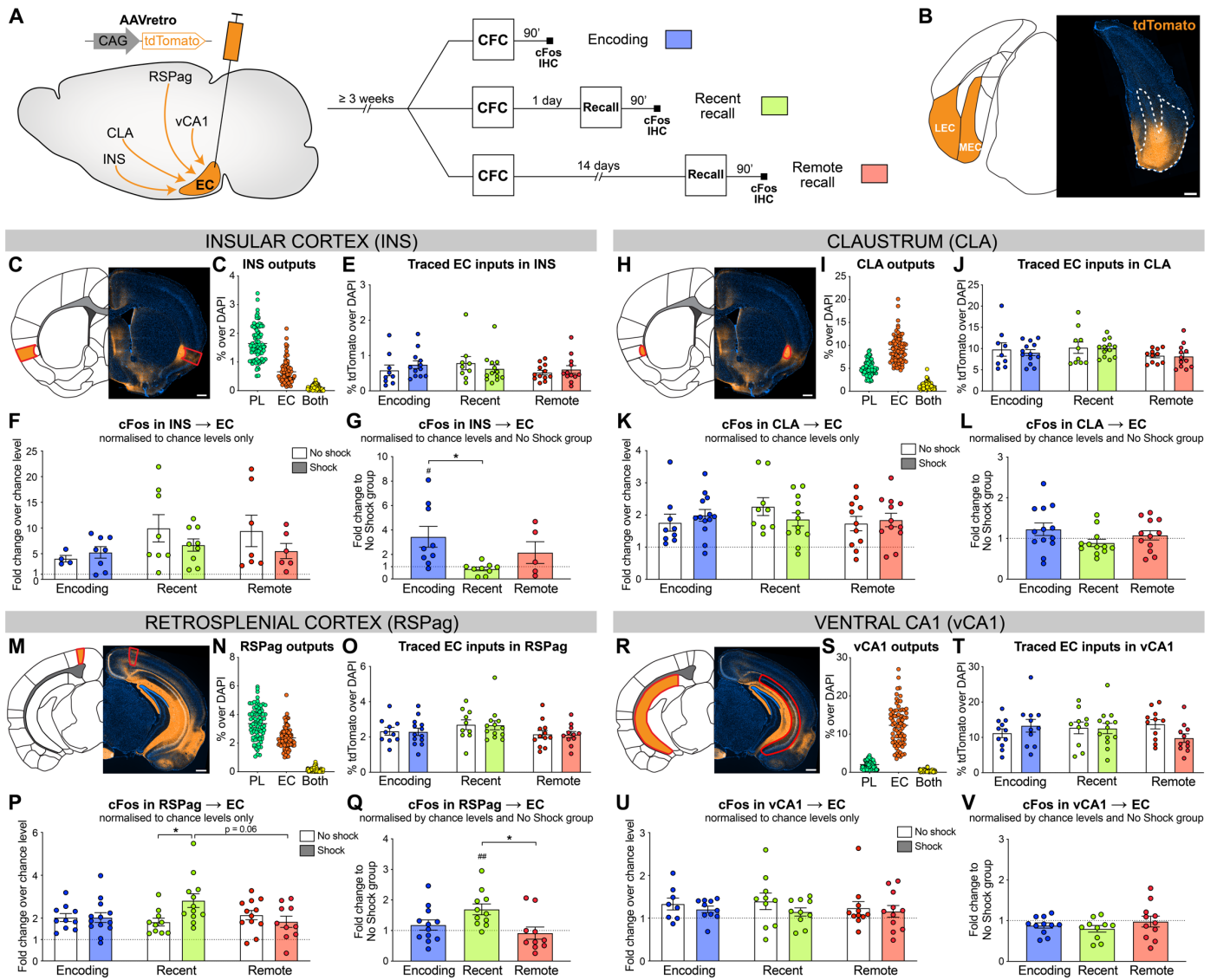
the activity of the projections to PL and EC: in INS, CLA, RSPag and vCA1. BLA projections to EC were too sparse to investigate their precise dynamics.

Unfortunately, the number of double labelled cells projecting to both EC and PL was very low and variable, which prevented us from quantifying with precision their own specific activity ($0.1\% \pm 0.08$ in INS; $1.2\% \pm 0.7$ in CLA; $0.14\% \pm 0.13$ in RSPag; $0.38\% \pm 0.27$ in vCA1, **Figure 2.7C, I, N and S**, respectively). Therefore, in order to examine the specific contribution of EC inputs not projecting to PL, they were excluded from further quantifications.

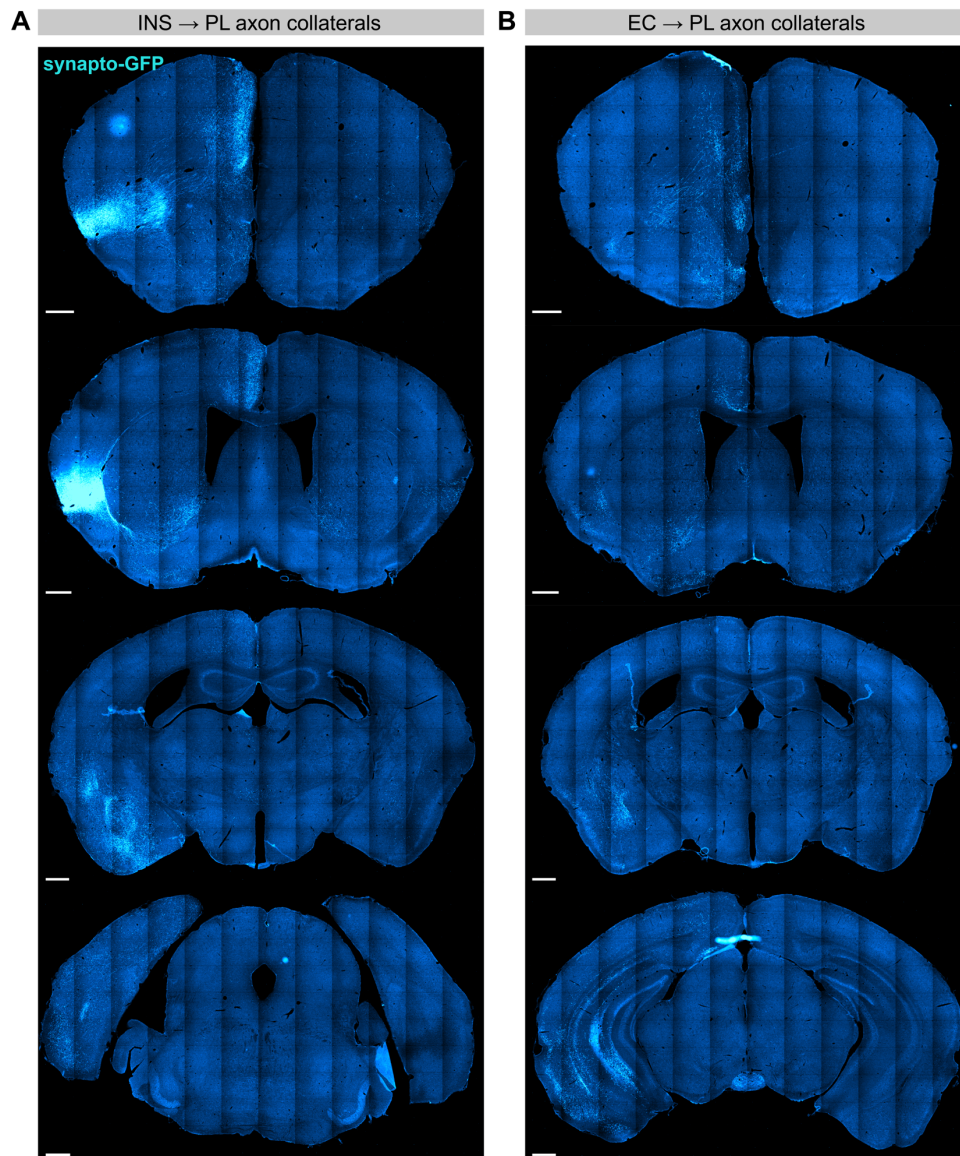
First, in the INS and the CLA (**Figure 2.7C-G** and **Figure 2.7H-L**, respectively), we observed different patterns activation between the PL and EC projections. Indeed, the INS \rightarrow EC projection is rather recruited at encoding (**Figure 2.7G**), whereas INS \rightarrow PL is recruited at a recent recall (**Figure 2.3M**). In addition, CLA \rightarrow EC projection is not differentially activated across consolidation (**Figure 2.7L**), contrary to CLA \rightarrow PL projection which was active at encoding (**Figure 2.3Y**). As the pattern of activation is projection-specific in INS and CLA, these projections most likely convey different information to their target structure across memory consolidation.

However, for the RSPag and vCA1 (**Figure 2.7M-Q** and **Figure 2.7R-V**, respectively) we observed the same pattern of activation in PL and EC projections. Indeed, RSPag projections to both PL and EC are specifically recruited at the recent recall time point (**Figure 2.7Q** and **Figure 2.3I**), while the activity of vCA1 projections does not differ between the different phases of memory formation (**Figure 2.7V** and **Figure 2.3Q**). These findings might suggest that these projections are redundant, or at least convey similar information to their downstream targets.

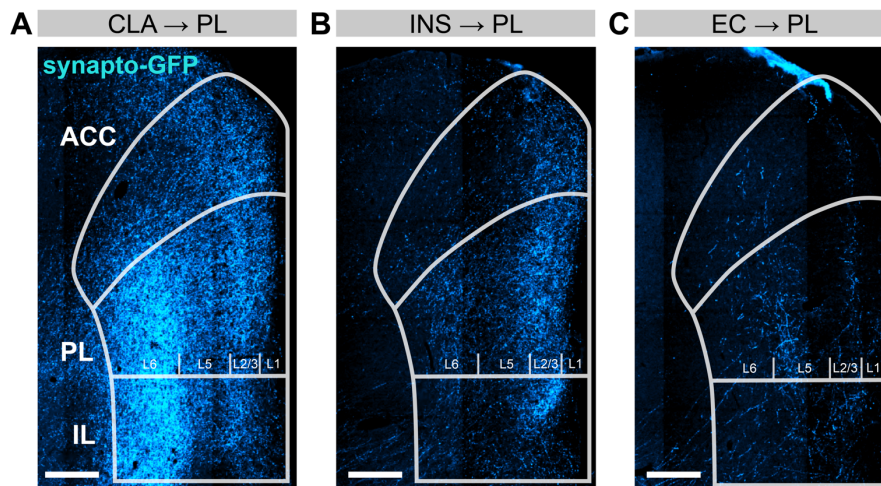
Overall, we have put in perspective the importance of PL inputs during fear memory consolidation with regard to the fact that these projections also innervate other brain regions known to be necessary in fear memory. However, the patterns of activation observed in PL inputs are specific to these projections in the CLA and INS, when compared to the dynamics of EC inputs. Taken together, these results strengthen the importance of CLA and INS and of the functional shift of their projections to PL during fear memory consolidation, and further support the existence of a shift during fear memory consolidation at the scale of these input regions as well.



2.2.2 Supplementary figures



Supplementary Figure S2.8. Axon collaterals of INS to PL and EC to PL projections. Representative images of synaptoGFP expression across the brain (scale 500 μ m) of **(A)** INS to PL and **(B)** EC to PL targeted expression.



Supplementary Figure S2.9. PL inputs target specific layers. Representative images of synapo-GFP expression in mPFC and layer distribution of (A) CLA to PL, (B) INS to PL and (C) EC to PL projections.

2.2.3 Additional material and methods

Axon collaterals tracing

Animals were injected with AAVretro-Cre in PL and AAV-FLEX-tdTomato-synapoGFP in the desired input region. 3 weeks later, brains were perfused, sectioned and stained like previously described (section 2.1.6). GFP was amplified using a goat anti-GFP antibody (1:2000, Abcam #ab6673) and a secondary Donkey anti-goat AF647 (Invitrogen 1:1000). Images were taken on an Olympus slide scanner VS120 L100 with a 10X objective. Sections were aligned to the Allen Brain reference Atlas as previously described (section 2.1.6).

EC input tracing

Animals used for PL input tracing were also injected unilaterally in EC with AAVretro-tdTomato. Behavior, staining and quantifications were done exactly as previously described (section 2.1.6).

Extra viruses used:

- AAV-hSyn-FLEX-tdTomato-synaptophysinGFP (Addgene 51509, serotype 1), here referred to as AAV-FLEX-tdTomato-synapoGFP. Titer: 1.6×10^{13} GC/mL, diluted $\frac{1}{2}$; injection volume (unilateral): 150nL.
- AAV-CAG-tdTomato (Addgene 59462, retrograde serotype), here referred to as AAVretro-tdTomato. Titer: 7.2×10^{12} GC/mL; injection volume in EC (unilateral): 350nL.

Chapter 3 Discussion

3.1 On the importance of mPFC during early fear memory consolidation

Traditionally, the mPFC was thought to be involved in the recall of remote memories, in opposition to the HPC which was required at encoding and recent recall (Frankland and Bontempi, 2005). This standard model of memory (see section 1.3.8) has been largely challenged notably by studies demonstrating the implication of the mPFC already during the encoding phase of the memory, and also during a recent recall. These observations supported the multiple trace theory of memory (see section 1.3.8) as they highlighted the importance of several brain areas at the same time, suggesting the existence of a distributed and dynamic memory network (Moscovitch et al., 2006; Nadel and Moscovitch, 1997). Here, my results are in line with this theory: first, because I observe the engagement of the mPFC (and in particular of the PL) already at the time of encoding, both in increased neuronal activity with cFos staining (**Figure 2.1D-F**) and functionally through the effect of the inhibition of some of its inputs (**Figure 2.4G-I**); second, as I describe the importance of a distributed network of brain regions that each contributes to the proper consolidation of the memory (**Figure 2.4**).

To my knowledge, I report the first direct comparison of mPFC activity using cFos IEG staining at 3 key time points in CFC memory consolidation (encoding, recent and remote recalls), and I found that the mPFC is most active at encoding (**Figure 2.1D-F**). A similar quantification was done in AFC, which described equivalent dynamics of the mPFC throughout consolidation (Cho et al., 2017). Several studies have compared IEG expression between different CFC recall times, and found increased cFos at remote over recent recall when normalized to no shock groups (Frankland et al., 2004b; Wheeler et al., 2013). In my results, I did not observe a differential engagement between the two recall time points; however, these discrepancies could be explained by differences in strength of conditioning protocols (number of shocks, test time, ...) but also of recall timing. As a matter of fact, I chose to study remote recall at 14 days post-encoding (as others have done (Kitamura et al., 2017)), while the aforementioned studies investigated a 36-day remote recall. It is therefore possible that memory consolidation continues after the 14-day time point, which could keep strengthening the importance of mPFC

during recall as time goes by. Indeed, another study from my laboratory using the same CFC protocol as I did showed that at a 34-day remote recall, cFos is increased in ACC and PL compared to animals that have not been shocked but re-exposed to the context several times (Silva et al., 2018), which I did not observe at 14 days. However, a longitudinal study on cFos expression in the ACC reported an increase of activity from a 5-day recall that kept increasing for a month (Aceti et al., 2015). Consequently, to formally conclude on the long-term progression of memory consolidation, a time-course of cFos expression after recall at different post-encoding times with our protocol would be required.

Nevertheless, my results add to the growing literature on the importance of prefrontal cortical structures already at the time of memory encoding (Bero et al., 2014; Cho et al., 2017; Cummings and Clem, 2020; Kitamura et al., 2017; Lesburguères et al., 2011; Tang et al., 2005; Zelikowsky et al., 2014). In the mPFC, both the ACC and PL subregions have been reported to be necessary for memory encoding, while IL seems to be rather recruited for the extinction learning of a fear memory (Bayer and Bertoglio, 2020; Do-Monte et al., 2015b; Knapska et al., 2012; Ye et al., 2016). I chose to pursue my investigations on the PL first as my cFos data highlighted a significantly increased activity at encoding compared to the no shock control group, suggesting a specific recruitment beyond contextual processing; and second because some of the PL inputs had already been functionally implicated at the time of encoding, giving an interesting point of comparison for my upcoming investigations (Kitamura et al., 2017; Klavir et al., 2017; Kol et al., 2020).

3.2 On the role of specific PL inputs during fear memory consolidation

I hypothesized that the activity of direct PL inputs could explain its dynamics during memory consolidation, and could help deciphering its functional role at the different stages of memory formation and recall. However, the PL is known to have a large array of input regions, from several other cortical areas but also many sub-cortical areas, such as the HPC, amygdala, thalamus or striatum (Carlén, 2017; Le Merre et al., 2021). Screening the activity of PL inputs in all these regions at all time points would have been quite challenging, so I decided to first pre-screen for relevant input brain regions by restricting retrograde tracing from specific PL targets: the PL engram cells.

3.2.1 Screening for PL engram inputs

In order to identify input regions to the PL that could be important in memory consolidation, I screened the inputs of PL cells that were active at CFC encoding. Indeed, as these cells are supposed to become functional PL engram cells (DeNardo et al., 2019a; Kitamura et al., 2017; Matos et al., 2019), their direct inputs should be the ones crucial for the dynamics of PL during memory consolidation. I restricted input tracing to PL engram cells using the TRAP2 mouse line coupled with monosynaptic rabies tracing, and I report for the first time a complete brain-wide screen of inputs targeting PL engram cells at encoding (**Figure 2.2**). I identified multiple brain regions of interest, some whose projections to PL were already identified in the context of fear memory consolidation (EC, BLA, vCA1), and some others that were not (CLA, INS, RSP, but also thalamic areas, the orbitofrontal and motor cortices, the pallidum and taenia tecta). These results are in line with a previously reported similar experiment using the Tettag system to restrict tracing to PL engram cells, although they measured tracing in a preselected set of brain areas (Kitamura et al., 2017). These results highlight the high number and variety of PL engram cells inputs throughout the brain, and suggests that these cells can receive a large amount of incoming information of diverse nature and processing type. The PL overall and the PL engram cells specifically can then be considered as an anatomical hub, which supports the observation of the mPFC being a functional hub region for memory retrieval (Silva et al., 2018; Vetere et al., 2017).

Following up on this experiment, I took a step back from engram cells to evaluate the activity pattern of general projections to the PL in 6 of these identified brain regions. I used cFos staining coupled with a non-engram specific retrograde tracer, and further tested the functionality of each projection if it was significantly activated during a specific stage of memory consolidation. In summary, I have found that CLA → PL is necessary at encoding for a recent recall, BLA → PL and EC → PL are necessary at encoding for remote recall, and that INS → PL is required to recall recent memories (**Figure 2.4 and 3.1**). I will now discuss those results in more details for each input brain region, and put them in perspective with the existing literature.

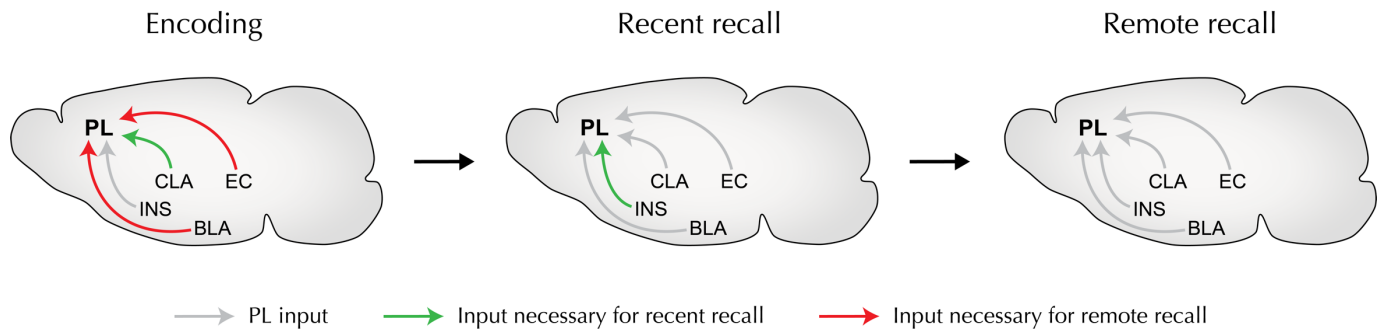


Figure 3.1. Summary of the experimental results on the functionality of PL inputs during fear memory consolidation.

Inputs to PL (in grey) which activity is necessary for recent recall (in green) or remote recall (in red).

3.2.2 The CLA and its projection to PL

The CLA is a thin structure located medial to the insular cortex and lateral to the striatum, anatomically characterized by a remarkable reciprocal connectivity with most brain structures, and notably cortical areas (Atlan et al., 2016; Marriott et al., 2021; Narikiyo et al., 2020; Wang et al., 2016; Zingg et al., 2018). It contains several types of interneurons as well as excitatory projection neurons (Graf et al., 2020; Kim et al., 2016) which are topographically organized in largely overlapping territories based on their projection targets as well as their input regions, and that send axon collaterals in multiple brain regions (Atlan et al., 2016; Jackson et al., 2018; Marriott et al., 2021; Zingg et al., 2018). Functionally, the CLA has gained a lot of attention since the bold suggestion by Francis Crick and Christof Koch that it could be the center of consciousness in the brain (Crick and Koch, 2005), and has since being implicated in various high order cerebral functions. Briefly, the CLA was shown to be necessary for the encoding of several associative paradigms such as CFC (Kitanishi and Matsuo, 2017) and drug reward contextual association (Terem et al., 2020), focus or attention during complex tasks (Atlan et al., 2018; White et al., 2020), novel context exploration (Kitanishi and Matsuo, 2017) and paradoxical sleep (Luppi et al., 2017). Interestingly, it was recently reported that the activation of CLA to ACC projection inversely modulates engagement, from sleep to focused attention, which could gather in one general mechanism all previous observations mentioned before (Atlan et al., 2021).

Here, I have observed that the CLA is most activated at CFC encoding, and the CLA → PL projection is specifically recruited at this time (**Figure 2.3Y,Z**). Functionally, I found that the CLA → PL projection is necessary during the encoding phase, but not after the encoding, for a recent recall only (**Figure 2.4G, S2.5B, 2.5E**). This time-limited consequence of CLA → PL

inhibition during encoding suggests that either different brain areas are responsible for the consolidation of recent and remote memories, or that compensation mechanisms come into play to still permit the consolidation of remote memories despite the CLA → PL inhibition. Interestingly, it has been reported that the CLA → EC projection is necessary at CFC encoding for a recent recall as well, but remote recall was not assessed (Kitanishi and Matsuo, 2017). As I and others have observed that EC to PL is necessary at encoding for remote recall only (**Figure 2.4I**) (Kitamura et al., 2017), it is therefore possible that the CLA could send information to the PL via an indirect pathway through the EC, which would maintain the consolidation of remote memories intact. In my hands, CLA → EC only displayed a slight trend towards a specific activation at encoding (**Figure 2.7L**), but a dual projection inhibition experiment could precisely assess whether blocking CLA → PL and CLA → EC together at encoding would prevent both recent and remote recall (experimental design proposed in **Figure 3.3A**). However, as I have observed that CLA → PL projections also send collaterals to the EC (**Figure 2.6**), confirming previous observations (Kitanishi and Matsuo, 2017), it is possible that the CLA → EC pathway is already partially disrupted in my CLA → PL manipulations, and that the dual inhibition would still yield the same result as CLA → PL inhibition alone. In that case, the hypothesis that different PL input regions are independently responsible for the consolidation of recent and remote memories would be favored, with the CLA being involved only in the consolidation of recent memories.

It has been demonstrated that CLA → PFC projections target mostly parvalbumin (PV) interneurons, resulting in a global inhibition of PFC neuronal activity (Jackson et al., 2018). Interestingly, I have observed that CLA → PL projections tend to be inhibited during remote recall (**Figure 2.3Y**), which could then promote an activation of downstream PL targets. Therefore, it would be interesting to test whether the activation of CLA → PL projections at remote recall impairs memory retrieval, potentially by inhibiting PL neurons. Extrapolating in the context of CLA activity inversely modulating engagement with the external world (Atlan et al., 2021), such a result would support that the CLA influences memory retrieval by allowing the animal to properly engage in recalling a past event. In that perspective, inhibiting the CLA → PL projection at encoding could have provoked a hyper-engagement, preventing rapid and efficient memory formation, as low CLA → ACC activity was linked with impulsive and uncontrolled reactions (Atlan et al., 2021). The same process could have been at play in the two other studies that reported an impaired association encoding with the inhibition of CLA → EC projection during CFC (Kitanishi and Matsuo, 2017) or CLA → frontal cortex projection during reward-associated place preference (Terem et al., 2020).

The notion of engagement is perhaps the one that could narrow down and ground the general concept of consciousness that was suggested by Crick and Koch, which is very hard to define and anchor to something tangible, and therefore to test. Indeed, the CLA is ideally located to integrate multiple sensory information as well as dynamic internal states to regulate the degree of cognitive commitment to a task or the level of attention dedicated to the external world, which is also crucial to trigger memory mechanisms. The CLA will most likely be the subject of intense research in the upcoming decades in various neuroscience fields as the therapeutic potential of such a region could be substantial, from treatment of sleep disorders, hyperactivity or trauma, to apathy or lethargic symptoms observed in some neurodegenerative diseases.

3.2.3 The INS and its projection to PL

The INS is an elongated and layered cortical structure lateral to the CLA, receiving inputs from gustatory, visceral and piriform sensory cortices as well as from thalamic areas and amygdala, and sending projections back to the sensory cortices, thalamus and amygdala, as well as massively to the striatum. It is also reciprocally connected to the PFC (for a very detailed connectivity report see Gehrlach et al., 2020). The INS was traditionally implicated in taste-related tasks (Yiannakas and Rosenblum, 2017), notably in taste association or conditioning learning (Kayyal et al., 2021; Sano et al., 2014), but it was also found necessary in fear memory consolidation and retrieval (Alves et al., 2013; Casanova et al., 2018; Gehrlach et al., 2019; Klein et al., 2021; Shi et al., 2020), broadening its role to more general associative learning. Interestingly, a recent study reported that INS integrates internal states to modulate fear memory retrieval, positively or negatively, in order to maintain an adequate behavioral response: if INS activity is high, then the animals can display fear (Klein et al., 2021).

Overall, my results are in line with previous reports of the INS being implicated the expression of fear memory (Alves et al., 2013; Casanova et al., 2018; Shi et al., 2020) as I found that inhibiting INS → PL connection during a recent CFC recall impaired memory expression (**Figure 2.4K** and **2.5H**). Particularly, the INS → mPFC projection has been recently investigated in the context of novel taste learning, demonstrating that this projection is necessary for the expression of the innate aversion for novelty (Kayyal et al., 2021). In other words, they suggest that the INS → mPFC connection is necessary for the animal to understand that something is new, or more generally for his past experience to matter in the present. This is indeed what I observed upon INS → PL inhibition: the animals seem to not remember their past fearful experience as they do not express fear. Further elaborating on the Klein et al. results, it is also possible that by lowering INS activity, I prevented memory recall

by high-jacking the system responsible for the integration of internal states necessary to allow normal fear expression (Klein et al., 2021). In that case, the INS, and the INS → PL pathway in particular, would not be responsible for the storage of the memory itself, but rather for a top-down control over the memory retrieval process, which is dependent on the internal state of the animal.

In addition, I observed memory impairment only when inhibiting INS → PL at recent recall, but not at remote recall (**Figure 2.4K,L**). This result further confirms the existence of a spatiotemporal shift in PL inputs during fear memory consolidation, but also suggests that consolidated networks are harder to disrupt, and could therefore be less sensitive to the influence of internal states. I will discuss this specific aspect of remote memories below in section 3.3.

Overall, these results confirm the importance of the INS in recent fear memory retrieval, and can be incorporated in the proposed top-down control by the INS of fear expression depending on the current internal state of the animal.

3.2.4 The EC and its projection to PL

The EC is a large cortical structure located at the very posterior end of the telencephalon, and part of the hippocampal formation. It is viewed as the gatekeeper of the HPC, as most of the HPC incoming and exiting information transits through the EC. Outside of the hippocampal formation, the EC is reciprocally connected to various cortical areas including the mPFC, as well as to the CLA, amygdala, thalamus and striatum (Canto et al., 2008; Schultz and Engelhardt, 2014). Functionally, the EC has been repeatedly implicated in spatial navigation and learning. Notably, it contains grid cells, border cells and head direction cells that convey precise information on the animal's position in space (Buzsáki and Moser, 2013; Diehl et al., 2019). As such, it was expected that the EC would be involved in associative memories related to specific contexts, during the formation of such memories or their retrieval (Hales et al., 2018; Kitamura et al., 2017; Kitanishi and Matsuo, 2017; Roy et al., 2017; Wahlstrom et al., 2018). Interestingly, EC inputs originating from CA1 and CLA were implicated in CFC encoding (Kitanishi and Matsuo, 2017; Roy et al., 2017), as well as output EC → PL projections (Kitamura et al., 2017), and subiculum inputs to EC were necessary for memory retrieval (Roy et al., 2017). Overall, this places the EC at the intersection of multiple circuits involved in memory consolidation (Takehara-Nishiuchi, 2014).

Using chemogenetic inhibition of the EC → PL projection, I confirmed the importance of this connection at the time of encoding for the consolidation of remote memories specifically (**Figure 2.4I**), which had been already demonstrated using optogenetics (Kitamura et al., 2017). Interestingly, EC → PL was always active during all phases of consolidation even in no shock groups (**Figure S2.2D**), but it is only at encoding that it was more active than in the controls (**Figure 2.3E**). This suggests that the EC most likely conveys contextual information at all times, but that during encoding it transfers extra information to the PL. From the literature, we can suppose that this additional information could come from CA1 or CLA, as these two projections were implicated in CFC encoding as well (Kitanishi and Matsuo, 2017; Roy et al., 2017), but I did not observe any non-contextual-specific activity in CLA → EC or vCA1 → EC at encoding (**Figure 2.7L,V**). However, I have found that the INS → EC connection is specifically recruited at encoding (**Figure 2.7S**), but the functional relevance of this projection remains to be tested.

Overall, it is clear that the EC is implicated in the processing of contextual information at CFC encoding, but is likely to convey more refined information integrated from potentially its HPC, CLA and INS inputs to promote learning in the PL. Interestingly, EC → PL projection is necessary at encoding but specifically for a remote memory recall, not a recent one, suggesting that it could elicit long-term consolidation mechanisms that are only relevant for remote memory expression.

3.2.5 The BLA and its projection to PL

The BLA is a nucleus of the amygdala located in the medial temporal lobe. It receives direct input from various sensory areas, both cortical and thalamic, as well as from the PFC, the EC and the HPC. It sends projections back to all these areas, and to the NAc and the CeA. The BLA has been associated with emotional processing of both fearful and rewarding experiences (Janak and Tye, 2015). In that regard, it has been widely implicated in fear conditioning, although mainly in AFC which I will not extensively discuss here as it appears to involve different pathways in the amygdala compared to CFC, notably related to sound processing. This being said, the BLA has still been implicated in the encoding (Kitamura et al., 2017; Nonaka et al., 2014; Zelikowsky et al., 2014), retrieval (Kitamura et al., 2017; Liu et al., 2022) and extinction (Bloodgood et al., 2018; Herry et al., 2008; Silva et al., 2021) of contextual fear memories, and contains engram cells (see section 1.2.2.3). Specifically, the reciprocal connection between BLA and mPFC has been functionally implicated in memory encoding (BLA to PL) and retrieval (PL to BLA) (Kitamura et al., 2017). Interestingly, oscillatory coupling

between the BLA and the mPFC has been implicated in memory retrieval as well, with the mPFC driving BLA firing (Karalis et al., 2016), which could be synchronized by double projecting vCA1 neurons (Kim and Cho, 2017).

My results therefore confirm previous findings that the BLA → PL projection is necessary at CFC encoding, specifically for a remote memory recall (**Figure 2.4H**). Given that the BLA receives pain information via parallel pathways (from the PAG, parabrachial nucleus and sensory thalamus notably, Almeida et al., 2004), and that it was shown that activation of neuronal subsets in the BLA could replace US presentation during associative learning (Gore et al., 2015), a possibility is that the BLA conveys information about the footshocks to the PL. In addition, the fact that BLA is as active during recalls as during encoding (**Figure S2.3F**) suggests that it can also play a role during memory retrieval as was previously reported.

3.2.6 The RSPag and its projection to PL

The RSP is a large cortical area reciprocally connected with the mPFC, visual and parietal cortices, CLA, HPC, and thalamic nuclei (Vann et al., 2009). It can be further divided in the granular RSP (RSPg, medial) and the agranular (or sometimes termed dysgranular) RSP (RSPag, lateral) with slightly different connectivity features, the hippocampal formation being rather connected with RSPg and the mPFC rather with RSPag (Aggleton et al., 2021). Overall, the RSP has been implicated in spatial navigation and contextual processing, but also in episodic memory (Vann et al., 2009). In particular, the RSPg has been implicated in CFC encoding (Yamawaki et al., 2019), recent (Cowansage et al., 2014) and remote (Fournier et al., 2021; Todd et al., 2016) recalls. However, little is known about the role of RSPag during fear memory consolidation. A recent study reported that the role of RSP in mediating remote CFC recall was supported only by RSPg and not RSPag (Tsai et al., 2022). Here, I report that the RSPag tends to be rather inactivated at recent and remote recalls (**Figure 2.3J, S2.2F**), but that the RSPag → PL and RSPag → EC projections are specifically recruited during recent recall (**Figure 2.3I and 2.7Q**, respectively). However, inhibition of RSPag → PL at recent recall did not impair memory retrieval (**Figure 2.4J**). It is possible that the RSPag → EC projection can compensate for this inhibition as both projections share an equivalent pattern of activation. A whole region or a dual-projection inhibition (in the spirit of **Figure 3.3A**) could help establishing the role of RSPag at recent recall. Eventually, it is very likely that the subdivisions of the RSP are playing different roles in memory retrieval as time goes by, further highlighting the functional shift occurring during memory consolidation.

In addition, it is also possible that the observed activity of the RSPag → PL projection at recent recall is not necessary for the online expression of fear memory itself, but rather for the initiation of reconsolidation or extinction processes following this recall. Indeed, recalling a memory at any time is thought to open a new window of consolidation (termed reconsolidation) in which the memory becomes labile again and is reorganized, both at the scale of engrams and circuits (Clem and Schiller, 2016; Dudai, 2012), notably involving the RSP (Kwapis et al., 2015) and the PL (Stern et al., 2014). This hypothesis can and will be tested in the near future, by adding other recall tests and extinction procedures following the first recent recall during which the RSPag → PL projection is inhibited. This experiment will allow to establish if the RSPag → PL projection is important for the reconsolidation of the memory trace following recent recall.

3.2.7 The vCA1 and its projection to PL

The vCA1 is a sub-region of the HPC, which receives major inputs from the CA3 and EC, and projects back to the EC but also the mPFC, BLA and HY. In contrast, dorsal CA1 is less densely connected to regions outside of the HPC (Schultz and Engelhardt, 2014). The HPC as a whole has been largely implicated in episodic memory formation, but it is mainly the DG and dorsal CA1 (dCA1) that have been extensively studied, notably in engram cell research (see section 1.2.2.3). Nevertheless, vCA1 activity has been implicated during CFC encoding and recent recall, notably via the potentiation of its projections to the BLA (Jimenez et al., 2020; Kim and Cho, 2020), but also in remote fear generalization (Cullen et al., 2015). Interestingly, a population of vCA1 neurons that project to both mPFC and BLA is recruited during CFC encoding and context exposure, suggesting that vCA1 could convey synchronized contextual information to these target structures (Kim and Cho, 2017), and vCA1 → mPFC projection is more active when animals are anxious (Ciocchi et al., 2015).

In my observations, although vCA1 was mostly active at the encoding phase of a CFC (**Figure S2.3B**), I did not observe any specific recruitment of vCA1 → PL or vCA1 → EC projections during the different phases of fear memory consolidation (**Figure 2.3Q** and **2.7V**, respectively). Interestingly, vCA1 → EC projections were activated higher than chance only at encoding, whereas vCA1 → PL were always more active than chance. Overall, this could suggest that vCA1 sends contextual information to the PL independently of the consolidation status of the memory, but that it transmits information to the EC only upon the first presentation of a new context. It is still possible that sub-populations in vCA1, notably neurons that send collaterals to a specific set of regions (Kim and Cho, 2017), are differentially activated during learning, but this effect would have been diluted in my global tracing experiments and therefore not visible.

Thus, I could not add information on the specific roles of vCA1 projections during memory consolidation.

Altogether, I can hypothesize that at CFC encoding the PL receives information about the footshocks via the BLA, together with a potentially enhanced contextual representation via the EC, in order to integrate all the necessary components to form a memory, under the control of overall engagement by the CLA (**Figure 3.2**). This is in line with the model of memory formation proposed by Kitamura et al. (2017). However, as discussed here, the roles of the many parallel projections in this densely interconnected network are still unclear and yet remain to be functionally investigated.

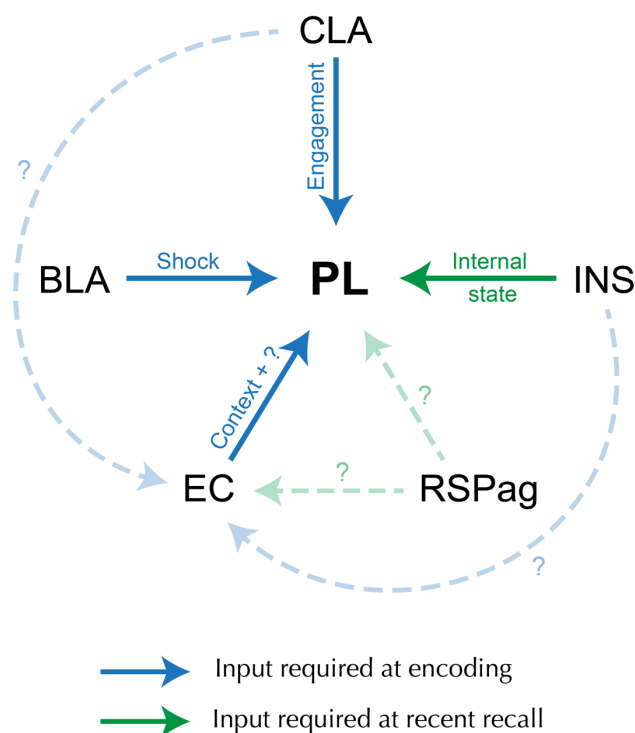


Figure 3.2. Proposed model of the information carried by PL inputs during memory encoding and recent recall. Dashed arrows indicate an increased activity that was not assessed for its functional role yet or which contribution is still unclear.

3.3 On the impossibility to find a functional connection to PL at remote recall

It has been well established that the mPFC is necessary at remote recall, both the region as a whole (Frankland et al., 2004b; Goshen et al., 2011; Restivo et al., 2009) and some of its outputs, notably to the BLA (Kitamura et al., 2017) and the PVT (Do-Monte et al., 2015a), as well as via its engram cells (DeNardo et al., 2019a; Kitamura et al., 2017; Matos et al., 2019). However, to my knowledge, there has been no report of a functional input to the mPFC at the time of remote memory recall, and I also could not identify one even with a broad screen of input regions and comparing their activity across time. Therefore, it seems that when trying to trace back the flow of information during remote recall upstream of the mPFC, researchers face a dead-end. As the functional activation of mPFC neurons still must come from somewhere, several hypotheses can explain this apparent obstacle.

First, as functional mPFC inputs have been implicated in recent recall, such as from dCA1 and BLA (Herry et al., 2008; Ye et al., 2017), the impossibility to find inputs at remote recall is likely a consequence of systems consolidation. Indeed, as the MTT stipulates, consolidated fear memories are distributed across several brain areas and thus harder to disrupt, which is apparent in the fact that they are harder to attenuate (Costanzi et al., 2011). Therefore they are probably less sensitive to specific inactivation of individual brain regions or projections, as the circuits involved in the recall process are likely to be redundant and potentially capable of taking over if one is disrupted, as was previously reported (Goshen et al., 2011). At the engram cell level as well, we know that DG engram cells are still functional at remote recall even if they are not naturally reactivated (Kitamura et al., 2017), and could then hypothetically substitute for another engram population in the PL. In that regard, inactivation studies are probably unlikely to succeed in identifying such circuits, however, activation experiments could demonstrate the capacity of a given circuit to trigger fear recall in a neutral context.

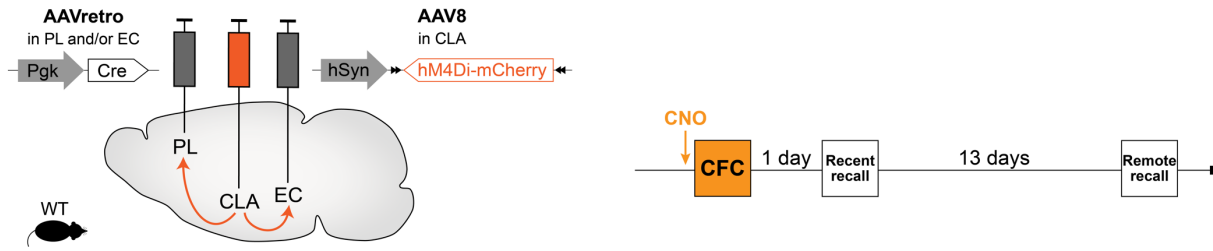
Second, it is possible that the only input that the mPFC requires to initiate a remote memory recall is purely of contextual nature. Indeed, as the memory is fully consolidated, mPFC engram cells already associated the context itself with the fear memory, and could only need an unprocessed contextual input. This is also supported by the strong interregional connectivity within the mPFC, notably highlighted in the rabies screen (**Figure 2.2H**), suggesting that further processing can be taking place in the mPFC itself. A direct consequence of this is that the use of no shock groups as controls does not permit to highlight the importance of inputs at remote recall, as the controls would be as active as the shock group. Indeed, I did not observe

any difference at remote recall in the specific recruitment of a PL input between the shock and no shock group (**Figure 2.3**). In addition, all investigated PL inputs were activated higher than chance at this time, suggesting that all of them could potentially be implicated in remote recall (**Figure S2.2 and S2.3**).

These two points are probably equally important to understand the peculiarity of remote memories. Taken together, I can therefore suggest a preliminary experimental design that could test whether a purely contextual PL input could trigger fear memory recall at a remote time point (**Figure 3.3B**). This experiment would assess if the reactivation of a projection-specific engram of a familiar context, tagged before its pairing with a fearful event, can trigger fear recall in a neutral context at a remote time point. Even if this experiment does not prevent the originally tagged context engram to be part of the later fear engram, it still allows to test in a more precise way if the activation of a contextual input only is able drive fear memory recall from the PL.

In addition, there is a large input region that I have not investigated in this project and that has received little attention in the memory circuits literature: the thalamus. Given the dense connectivity of thalamic nuclei with the mPFC but also with various regions that I have investigated here (CLA, INS, EC, BLA and RSPag) and that are implicated in memory formation, it is very likely that the thalamus plays a role beyond sensory processing. Indeed, some thalamic nuclei have been recently implicated in the encoding and recall, both recent and remote, of fear memories (Do-Monte et al., 2015a; Silva et al., 2021; Taylor et al., 2021; Vetere et al., 2021). It is therefore possible that all of us were simply looking the wrong way.

A Dual-projection inhibition



B Contextual input activation at a remote time-point

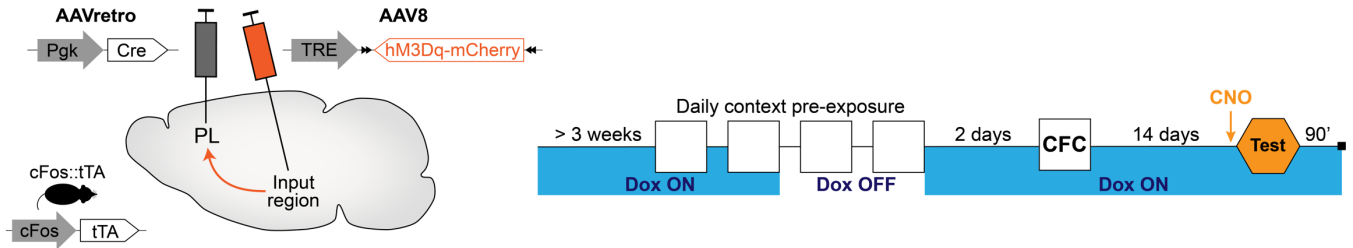


Figure 3.3. Proposed experiments to further assess the functional roles of PL inputs during fear memory consolidation.

(A) Dual projection inhibition experiment. Experimental design (left) and timeline (right) to test if CLA → PL and CLA → EC can take over from each other during memory encoding to enable remote memory consolidation. (B) Contextual input activation at a remote time-point experiment. Experimental design (left) and timeline (right) to test if a tagged PL input activated during context pre-exposure is able to later drive freezing in a neutral context at a remote time point.

3.4 On the dynamics of the PL engram

Following on the results of the chemogenetic inhibition experiments, I wondered if the memory impairment effects that I observed could be explained by an effect on PL engram reactivation. Because of the novelty of the CLA and INS findings, as well as the unexpected and specific effect on recent memory recall, I decided to pursue these engram-specific investigations on the CLA and INS manipulations. Using the cFos::tTA mouse line together with a TRE-dependent GFP reporter in the PL enabled me to measure the degree of PL engram reactivation between encoding and recall, while inhibiting the CLA → PL projection at encoding or the INS → PL projection at recent recall.

First, I confirmed the memory impairment found in WT mice in both cases, further strengthening the importance of CLA and INS inputs to PL at encoding and recent recall, respectively (**Figure 2.5E,H**). Second, I observed no overall change in engram reactivation nor in any other parameter at the time of recent recall (**Figure S2.6**). However, in both cases, I found a correlation between freezing and PL engram reactivation specifically when the memory was impaired, and not in the control groups: the less the engram was reactivated, the less the

animals froze, therefore the less they remembered (**Figure 2.5F,I**). This correlation was not found when remote memory recall was tested after CLA → PL inhibition at encoding, which did not show any behavioral impairment (**Figure S2.7**). When putting these results in perspective with the idea that PL engram cells are “silent” during early consolidation, meaning that they are always functional but only reactivated at remote and not recent recall (Kitamura et al., 2017), I was at first a little perplexed. If there is no change in overall reactivation when recent memory is impaired, which can be expected from the silent engram theory, why would there be a correlation with memory retention, and why would recent memory be impaired at all?

I first hypothesized that if recent memory expression is impaired without changes in engram reactivation, then it is not the PL engram cells themselves that are responsible for the observed behavioral effect, but potentially a neighboring set of cells. Indeed, I observed that PL engram cells receive a lot of inputs from within the mPFC as well (**Figure 2.2H**). In parallel, the emergence of a correlation between reactivation and freezing upon inhibition suggests that the role of the projections is normally to keep these two parameters decorrelated. Therefore, a possibility would be that these projections target, at least in part, some inhibitory interneurons that are not part of the engram per se, but whose role is to keep the PL engram from being functionally reactivated at the time of recent recall, and until consolidation is complete. The inhibition of the CLA and INS inputs would have therefore lifted this inhibitory control upon PL engram cells, resulting in the functional reactivation of the engram and therefore in the emergence of a correlation with behavior. Indeed, it was reported that CLA → PL projections target mainly inhibitory interneurons (Jackson et al., 2018), however the precise effect of INS projections on PL target neurons still remains to be determined.

Yet, this hypothesis still has some caveats. On one hand, a lift of inhibition on PL engram cells at recent recall does not explain why it would provoke a memory impairment. A first possibility would be that the awakening of the PL engram at a time when it should not could perturb the whole system and normal memory expression. However, a second possibility is that the behavioral effect that we observed at recent recall after the CLA and INS manipulations is due to an effect outside of the PL via the numerous axon collaterals of these two projections (**Figure 2.6** and **S2.9**). To test this hypothesis, axon terminal inhibition using local CNO delivery in the PL instead of systemic CNO administration would be preferred, thus restricting inhibition to the PL collaterals to assess where the behavioral effect comes from. On the other hand, if the silent engram theory in the PL was to be true, we would expect to see a correlation at remote recall between engram reactivation and freezing in the control group at least, as the

PL engram is functional at this point, but this is not the case (**Figure S2.7C**). However, it is possible that the measure of PL engram reactivation is not as accurate at remote recall that at recent recall, due to the highly increased GFP expression between the two time points, from around 2% at recent to around 12% at remote recall (**Figure S2.6** and **S2.7**). It is possible that once the tagging window is open, the system starts leaking and accumulates non-specific GFP expression which would then bias the engram reactivation measurement: indeed, reactivation is around 60% at recent recall, and about 30% at remote recall. Although a decrease in reactivation with time is to be expected (DeNardo et al., 2019a), the results at remote recall following CLA → PL inhibition should be taken cautiously.

Nevertheless, I found that the memory impairment at recent recall resulting from specific PL input manipulations was correlated with PL engram reactivation. It is however possible that not all engram cells are affected in the same way by each projection manipulation, and therefore reactivation might not be uniform in the PL. Indeed, I have observed that the CLA, INS and EC projections to PL do not target the same layers in the PL, suggesting different underlying structural features for each network (**Figure S2.8**). To better understand the dynamics of PL engram cells following projection-specific manipulations, it would be interesting to have a clearer idea of how the network is structured within the PL: what cell types are targeted by which projections, in which layer(s), with which electrophysiological and molecular properties, and with which output targets. This knowledge would help to test finer contribution of each sub-network in the PL, and the precise distribution of engram cells within this network is likely to shed light on the consolidation mechanisms happening within the PL, but also at the scale of the networks, to better understand the reorganization occurring during systems consolidation.

3.5 On technical limitations and how to potentially overcome them

As with any experimental design, mine had some technical limitations that need to be at least acknowledged and kept in mind when interpreting the results, but that could be potentially avoided in future experiments. Some of these limitations have been mentioned throughout the results and discussion, but I will summarize and further discuss them here.

The first point is the use of two different retrograde tracing viral vectors. All viral vectors are restricted for their entry into cells by the availability and the affinity of specific receptors at the cell surface, which result in a unique tropism for each type of viral vector, even within the same virus family, for instance the adeno-associated viruses (AAVs) and their specific serotypes

(Aschauer et al., 2013). Here, I used first a modified rabies virus from the SADB19 strain pseudotyped with EnvA (Wickersham et al., 2007), in combination with two helper AAVs to specifically express notably the TVA receptor which would allow the rabies viral particles to enter the cells (**Figure 2.2**). The helper AAVs I used were of serotype 8, thus limiting the possibility to retrogradely trace from cells that can be infected with this serotype. I used this method to broadly screen for all inputs of PL engram cells, but I might have missed some in case some PL engram cells could not be infected with the helper AAVs, or in case the expression of the reporter genes was constrained in some cells. Next, I used an AAVretro to unbiasedly trace all PL inputs, from the rAAV2-retro serotype (AAVretro, **Figure 2.3**) (Tervo et al., 2016). However, it has been reported that rabies and retrograde AAVs have different tropisms, highlighting in the fact that AAVretro is not completely unbiased (Sun et al., 2019). Indeed, even though I traced thalamic inputs in the rabies screen, these inputs were largely absent from the AAVretro screen, which was more biased towards cortical regions. Overall, I cumulated two viral tropisms, which could have resulted in the involuntary exclusion of some brain areas of interest. To overcome this limitation, the use of combined tracers (AAVretro and a synthetic tracer for instance) could help reduce the tropism constraints for general input screens. In addition, some tools have been developed to overexpress synthetic AAV receptors in the post-synaptic compartment in order to minimize the natural tropism of AAVretro (Sano et al., 2020), which could be an interesting option.

The second limitation is that I was constrained to use two different mouse lines for my engram-related experiments: the TRAP2 line for the activity-dependent rabies tracing, tamoxifen-dependent (**Figure 2.2**, DeNardo et al., 2019), and the cFos::tTA line for the engram reactivation experiment, doxycycline-dependent (**Figure 2.5**, Reijmers et al., 2007). These choices were dictated by technical constraints and availability of resources at the time of the experiments, as we had already a Cre-dependent rabies system ready to use with the TRAP2 line, as well as a projection-specific chemogenetic inhibition set of viruses that was Cre-dependent as well, which could therefore not be combined with the TRAP2 engram tagging system. However, these two mouse lines were used in independent experiments that did not require any comparison between them, and they both rely on the same cFos promoter to drive reporter expression. As such, this limitation does not alter the interpretation of my results. The divergent results that have been reported using one or the other mouse line could be explained as well by the differences in behavioral protocols than by the different mouse lines used. For instance, the study by Kitamura and colleagues and the one of DeNardo and colleagues, using the cFos::tTA and TRAP2 mouse lines, respectively, report contrasting results on the functionality of PL engram reactivation, but they used different types of conditioning and

retrieval times, as well as different inhibitory opsins and light stimulation frequencies. Perhaps the most influential discrepancy between the two systems would be the time-window of tagging, which is longer with the cFos::tTA line (at least a day) than in the TRAP2 line (a few hours). Overall, to be on the safe side, it would still be advised to only use a single engram line within a given study.

The third point of caution is the presence of axon collaterals. As this is an intrinsic aspect of the network that I am studying (**Figure 2.6** and **S2.8**), and probably even a key feature given the specific role of some neurons targeting two structures simultaneously (Kim and Cho, 2017), it is crucial to integrate it in at least my interpretations, but also ideally in future experiments. Indeed, all my chemogenetic manipulations are targeted not only to a specific projection to PL, but also encompasses the axon collaterals of these projections, as I injected CNO systemically. Therefore, even if this does not undermine the conclusions about the role of a specific projection, as the manipulated neurons are still defined by a unique projection target, I cannot exclude that the axon collaterals connected to another region are also important in the consolidation process. To overcome this limitation, the use of axon terminal inhibition would be pertinent, as it would only target the inhibition of the projections to a selected downstream target. This can be achieved via local CNO delivery via pre-implanted cannulas in the case of chemogenetic inhibition, or via axon-terminal inhibition of optogenetic modulators with an implanted optic fiber above the target structure. Such experiments could help disentangling the role of specific projections irrespective of their axon collaterals, and thus get a finer appreciation of the functional network involved in memory consolidation.

In addition, the fourth technical limitation is the slow kinetics of the inhibitory DREADD hM4Di. Indeed, the CNO-induced inhibition lasts at least an hour, and CNO is injected 30 minutes in advance to ensure that the inhibition is at its highest during behavior (**Figure 2.4**). Therefore, inhibition is not constrained to the behavioral epoch, and starts before. This could give time for global circuit reorganization to occur, as has been previously reported (Goshen et al., 2011). Consequently, an absence of behavioral effect after inhibition does not necessarily imply that a projection is not required during this behavioral phase. This could be the case for the RSPag → PL for instance, which is specifically active at recent recall but whose inhibition did not impair memory retrieval. To achieve a more precise and time-limited inhibition, the use of optogenetics instead of chemogenetics would be preferred. Indeed, optogenetics enable neuronal manipulation at the millisecond time scale, using light pulses delivered via an implanted optic fiber (Deisseroth, 2015). This technique, although more invasive than

chemogenetics, would allow for fast and therefore more accurate projection-specific manipulations by preventing circuit reorganization to occur.

In addition to the slow kinetics of DREADDs, the use of CNO raises the question of potential off-target effects of CNO itself or its metabolites. In that regard, controls receiving CNO injection but with a control virus are essential, which I did in the first functional inhibition experiments (**Figure 2.4**), ensuring that memory impairments are specifically due to the PL input inhibition. However, I used a different control in the PL engram reactivation study (**Figure 2.5**), with vehicle injections and no control virus. Even if the behavioral observations were the same, I cannot exclude that the observed effect at the level of engram reactivation is not due to the CNO itself. An additional control experiment, where animals with a control virus will receive either a CNO or vehicle injection, will allow test this hypothesis.

Lastly, the use of cFos as a proxy for neuronal activity is also accompanied with some reserves. IEGs in general are widely used to assess neuronal activity, at the RNA or at the protein level, but also employing their promoters to drive various reporters, as in the Tettag and TRAP2 systems that I previously discussed. However, several caveats can be noted here: (i) The time-window of cFos expression is much longer than the few minutes of behavioral testing, resulting in unspecific expression (hence the importance of appropriate behavioral controls) (ii) Not all activated neurons express cFos, resulting in only a subset of neurons being tagged or counted as activated. Interestingly, other IEGs can mark a different yet still functional component of the engram (Sun et al., 2020). (iii) There is no direct correlation between the strength of neuronal activation and the level of cFos expression, therefore all activated neurons are classified in the same way, preventing finer analysis of neuronal activation. Although the use of IEGs is undeniably useful and pertinent in the study of memory, given the numerous advances since the development of IEG-related tools, other methods might be preferred to gain both in temporal resolution and quantitative aspect. Typically, the use of calcium imaging recordings in freely behaving animals allow an almost real-time assessment of precise neuronal activity (Hamel et al., 2015; Nakai et al., 2001). Naturally, such a method would be impossible to use for a brain-wide screen of neuronal activity as I reported here, but it could be useful for finer analysis of engram reactivation notably. However, even if the use of IEGs prevents precise assessment of neuronal activity, it still allows for a fast evaluation of the contribution of a given neuronal population, that proved to be functionally relevant in most of the cases here.

3.6 Future directions: further unraveling the fear memory circuit

In my PhD project, I have investigated the functional aspect of specific PL inputs during fear memory consolidation. Even if I undoubtedly report new findings that help advancing our knowledge of memory circuits, these results further opened new areas of investigation, while still keeping some unresolved. As these points have been discussed in the previous sections, I will only summarize them here to give a brief overview of where this project could go now.

1) What is the precise role of the PL during memory recall as time goes by?

As we have seen, it is likely that consolidation is not over after 2 weeks, and the PL could perhaps further gain in importance with time. Typically, the use of calcium imaging, notably in specific cell sub-populations, would enable to track neuronal activity over time and further characterize the modifications arising during memory consolidation. It would also enable to target functional manipulations to the relevant populations to assess their individual role.

2) What is the exact contribution of PL inputs during fear memory consolidation?

I have reported that two new PL inputs, from the CLA and INS, are crucial for memory encoding and recall, respectively. However, their precise role still remains to be established, as well as the importance of each projection without its axon collaterals. In addition, some projections could be redundant and therefore harder to functionally isolate. For all of this, terminal inhibition of PL inputs and dual-inhibition of specific connections would help refine the role of each projection and establish what type of information it conveys to the PL.

3) Which PL inputs are necessary to drive remote memory recall?

Resolving the mystery of the absence of functional PL inputs at remote recall would help tremendously to understand the organization of the recall circuit after consolidation. The activation of specific contextual inputs would be an interesting first step towards the resolution of this open question (**Figure 3.3B**).

4) How are PL engrams distributed within the PL global circuitry?

Understanding how layer specificity within the PL relates with its global input/output organization, together with a precise electrophysiology characterization, will enable to explain how each projection affects engram formation and reactivation during recall, and what governs PL engram maturation during consolidation.

5) What are the molecular underpinnings of memory consolidation at the cellular level?

As I focused my attention on the inputs of the PL in general, and of engram cells in particular, I left aside the questions of the enduring molecular changes underlying memory formation, that are probably equally crucial to form memories. I did work on a side project attempting to address these questions by developing an *in vitro* memory-like system using primary neuronal cultures and paired chemical stimulations, but unfortunately, I could not establish a reliable enough protocol. However, I do believe that such a system would give valuable insight on how individual cells can integrate and store information related to a past event. Together with many recently developed *in vivo* techniques to manipulate molecular processes in selected cells, this field is likely to keep growing and elucidate these long-standing questions.

Eventually, when considering the general question of my research project (*Where does the engram come from?*), I think the answer I gave rather complexified the problem instead of simplifying it. However, I do believe that we are now a little bit closer to the final answer than we were before: the mechanisms involved in the process of engram formation are likely to be extremely complex, and to involve numerous inputs to finely orchestrate memory encoding in the long-term.

3.7 A side note on a research project that did not prevail

As a quickly learned in my PhD, not all projects will succeed, no matter how exciting they are or how much efforts were put in their design and execution. I would like to briefly mention my first PhD project that I really enjoyed working on, although it eventually had to stay on the side of the PhD thesis road. However, it was not all in vain, as it eventually led to an essential part of my final PhD thesis.

This first project was called the *rewiring* project, and was already aimed at investigating PL inputs during fear memory consolidation. However, there my approach was to examine whether specific PL inputs would be rewired during consolidation, which could explain the change in activity of PL engrams during the different phases of consolidation. Rewiring had been observed upon sensory deprivation (Kätzel and Miesenböck, 2014), but also associated with memory precision in hippocampal and cerebellar circuits (Ruediger et al., 2011). As such, a functional rewiring of PL inputs could have been the structural basis of long-term memory consolidation.

To address this question, I wanted to trace the inputs of PL engram cells right after the different phases of learning and compare them across time, taking into advantage a recently developed system for activity-dependent rabies-based retrograde tracing (Sakurai et al., 2016). It relied on a new mouse line, Fos^{TVA}, expressing a short-lived rabies TVA receptor under the cFos promoter, therefore allowing to directly restrict tracing from activated cells. Although very promising, this system proved to be inefficient due to various technical constraints (of note, the Fos^{TVA} line has not been used in any other published study since), and the slow kinetics of rabies tracing made it impossible to rapidly trace inputs before any rewiring could potentially happen, even in different mouse lines. In addition, it was reported that rabies tracing was influenced by the ongoing activity in the inputs, preventing a purely anatomical tracing proportional to the number of connections (Beier et al., 2017).

Overall, and despite my best efforts, I could not assess a potential network rewiring with these techniques, and I had to rethink my PhD project. Nonetheless, I still did manage to establish an activity-dependent retrograde tracing method using the TRAP mice. This experiment turned out to be an exciting entry point into a new project when applied to PL engram cells at encoding, and resulted in the rabies-based tracing screen that is presented here in Figure 2.2. These results allowed me to select relevant inputs for further investigation and consider brain regions that would have been otherwise left aside, such as the Claustrum.

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Curriculum Vitae

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Education

2016 - 2021	PhD in Neuroscience, EPFL, Switzerland
2013 - 2016	Master in Biosciences, École Normale Supérieure de Lyon, France
2014 - 2015	Exchange in the Neuroscience Master, EPFL, Switzerland
2012 - 2013	Bachelor in Biosciences, École Normale Supérieure de Lyon, France
2010 - 2012	Preparation for national entry exams in French engineering schools, biology major, Lycée St Louis, Paris, France.

Academic projects

2016 - 2022	Doctoral thesis – EPFL, Switzerland Laboratory of Johannes Gräff. Circuits in memory consolidation.
2016 (5 months)	Master internship – New-York University, USA Laboratory of Cristina Alberini. Molecular biology of memory formation.
2015 (5 months)	Master internship – Pasteur Institute, Paris, France Laboratory of Pierre-Marie Lledo. Circuits in mouse olfaction.
2015 (6 months)	Internship – EPFL, Switzerland Laboratory of Johannes Gräff. Memory and engram tagging in mouse.
2014 (6 months)	Master internship – University of California, San Francisco, USA Laboratory of Su Guo. Zebrafish behavior and molecular biology.

Technical skills

- In vivo skills: Rodent stereotaxic surgeries, viral tracing, behavioral tasks, perfusions and brain dissections. Cryostat tissue sectioning.
 - In vitro skills: P1 and P2 biosafety level techniques for cell culture, lentivirus and rabies virus production for in vitro or in vivo use.
 - Molecular biology techniques: qPCR, immunofluorescence, western-blot, bacterial cultures.
 - Image acquisition (brightfield, fluorescence, confocal microscopes), image analysis (Fiji, QuPath), data analysis (GraphPad) and visualization (Illustrator).
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Publications and conferences

- **Dixsaut L**, Gräff J. Brain-wide screen of prelimbic cortex inputs reveals a functional shift during early fear memory consolidation. Under revision.
 - **Dixsaut L**, Gräff J. The Medial Prefrontal Cortex and Fear Memory: Dynamics, Connectivity, and Engrams. *Int J Mol Sci*. 2021 Nov 9 ; 22(22):12113. doi: 10.3390/ijms222212113. Review.
 - Khalaf O, Resch S, **Dixsaut L**, Gorden V, Glauser L, Gräff J. Reactivation of recall-induced neurons contributes to remote fear memory attenuation. *Science*. 2018 Jun 15;360(6394):1239-1242. doi: 10.1126/science.aas9875.
 - Sanchez-Mut J, Heyn H, Silva B, **Dixsaut L**, ..., Gräff J. PM20D1 is a quantitative trait locus associated with Alzheimer's disease. *Nature Medicine*. 2018 May;24(5):598-603. doi: 10.1038/s41591-018-0013-y
 - Internal seminar at the Brain Mind Institute progress report series, September 2021.
 - Poster at the 2021 European Brain and Behavior Society annual meeting in Lausanne, Switzerland.
 - Poster at the 2018 Society for Neuroscience annual meeting, Chicago, USA.
 - Poster at the 2017 Lemanic Neuroscience Annual Meeting in Les Diablerets, Switzerland.
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Associative projects

- 2019 - 2020 Event manager at the Pint of Science festival in Lausanne, promoting scientific communication to the general public.
- 2019 - 2021 Elected representative of the neuroscience PhD students at EPFL.
- 2020 - 2021 Responsible of the PhD student coaching program in Life Sciences at EPFL.
- 2013 - 2014 Event manager in the student association at Ecole Normale Supérieure de Lyon.
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Language skills

English: C2

French: native language

Spanish: A2-B1