A dedicated cortico-hippocampal network changes the behavioral output of learning ensembles in the striatum

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Abstract

Fear learning relies on widely distributed networks of neuronal ensembles. These ensembles represent a fundamental mechanism through which the brain processes and organizes information, creating patterns of neural activity associated with different aspects of memory.

Projections from many brain areas involved in fear are known to converge onto the dorsomedial part of the striatum (DMS), which plays an essential role in the extinction of fear memories. However, it is still unknown whether learning ensembles are formed within this inhibitory neural circuit and, if so, what their functional role is in supporting learning and updating.

In this thesis, I provide evidence that a memory ensemble is formed in the DMS as a consequence of fear learning. Using the immediate early gene cFos as a marker of neuronal activity, I show a novel, functional role for this striatal ensemble during behavioral flexibility and characterize how its final output is orchestrated through the various phases of learning.

By combinatorial interfering with neuronal activity in the individual network nodes, I show that a dedicated cortico-hippocampal network involving the dorsal hippocampus (dHp) and the retrosplenial cortex (RSC) is specifically recruited during extinction to influence the activity of the DMS fear ensemble.

Contents

Chapter 1

Introduction

1.1 Memory and neuronal ensembles across the brain

Memory influences virtually every behavior of all conscious organisms and is thus fundamental for their survival. Accordingly, memory traces have been identified across a wide range of animal species including invertebrates, and complex structures such as the brain have evolved to enable the accurate and efficient use of memories (Koch, 2016).

During learning, memories are created, retrieved, and modified to allow organisms to remember and adapt to an experience. Memory formation is a highly dynamic process, and each learning experience is represented by the concerted activity of diverse brain areas. These representations are thought to be stored in sparse, anatomically distributed neuronal ensembles (or engram cells). The subsequent activation of these neuronal ensembles recreates the same or a similar pattern of activity that gives rise to the recall of the memory (Roy et al., 2022).

1.1.1 Definition of "ensemble"

In 1904, the term "engram" was first introduced by Richard Semon. He defined engrams as the "enduring though primarily latent modification produced by a stimulus (from experience)" (Semon, 1921). Although initially

unappreciated, Semon's insights on memory processing are currently considered the fundamental core of the modern concepts of memory.

The idea that a small population of neurons encodes a specific memory or learning event is consistent with sparse population coding. According to this theory, the number of activity patterns stored in a neural network (within biological constraints) is maximized if the population coding is sparse (Amari, 1989; McNaughton & Morris, 1987).

Over the years, the study of memory ensembles has been intensively growing thanks to the development of tools to tag, visualize and manipulate cells activated during the learning (Carrillo-Reid et al., 2017; DeNardo et al., 2019; Roy et al., 2022). Currently, engram cells are defined as a population of neurons activated by a learning experience and consequently show long-lasting plasticity changes associated with the formation of a new memory or association. These cells are reactivated by the subsequent presentation of the stimuli present during the learning session, resulting in the memory recall (Carrillo-Reid, 2022; Carrillo-Reid & Yuste, 2020; Josselyn & Tonegawa, 2020; Tonegawa et al., 2015).

1.1.2 Allocation to a neuronal ensemble

For any given learning event, many neurons across the brain are activated and eligible to become part of a memory ensemble. However, only a small subset of these cells is selected to be allocated into the final engram and committed to storing a specific memory.

Neuronal allocation has been observed in various brain regions, such as the amygdala, the hippocampus, and the cortex (Josselyn & Frankland, 2018),

and extensive work over the years has focused on understanding the mechanisms mediating this selection process.

In rodents, a large body of evidence indicates that high levels of neuronal intrinsic excitability (as the probability of a neuron firing action potentials in response to input signals) represent a key factor for allocation and ensembles' formation. Therefore, allocation is considered a "winner-take-all" process in which more excitable neurons win the competition and become part of a neuronal ensemble (Yuille & Grzywacz, 1989).

In the lateral nucleus of the amygdala (LA), it has been shown that manipulations enhancing neuronal excitability in principal neurons increase the probability for these neurons of being allocated to an ensemble supporting conditioned fear memory (Han et al., 2007; Kim et al., 2013; Rashid et al., 2016; Yiu et al., 2014). In the hippocampus, a remarkable example of allocation has emerged from studies on the development of "place fields" in the CA1 region. Here, a subset of "place cells" rapidly modulates its firing rate to a specific location as animals explore a new environment, resulting in a stable and long-lasting spatial representation (Leutgeb et al., 2005; Moser et al., 2015; O'Keefe & Dostrovsky, 1971; Wilson & McNaughton, 1993). Intracellular recordings of hippocampal neurons show that the selection of a cell to encode a specific location depends on the relative intrinsic excitability immediately before the context exploration (Cohen et al., 2017; Epsztein et al., 2011; Lee et al., 2012; Rich et al., 2014).

Allocation enhanced by neuronal excitability has also been observed to support different forms of memories in many cortical areas, such as the insular cortex, the piriform cortex, and the retrosplenial cortex (Choi et al., 2011; Czajkowski et al., 2014; Sano et al., 2014). In addition, neurons

allocated to a memory ensemble have been shown to increase the synaptic strength between each other (Choi et al., 2018).

The current view is that during a learning experience, sparse populations of active neurons that show increased intrinsic excitability during training undergo long-lasting plasticity changes and are allocated to a unique memory ensemble. Silencing of ensembles' neurons impairs memory retrieval, while artificial reactivation of these cells leads to an indiscriminate memory recall (Josselyn & Tonegawa, 2020) (Figure 1.1).

Given that memory is a complex phenomenon that relies on distributed networks, multiple sets of engram cells are formed and connected by specific circuits. Thus, neuronal ensembles are not confined to a single brain region, leading to the idea that they might support distinct aspects of a learning experience.

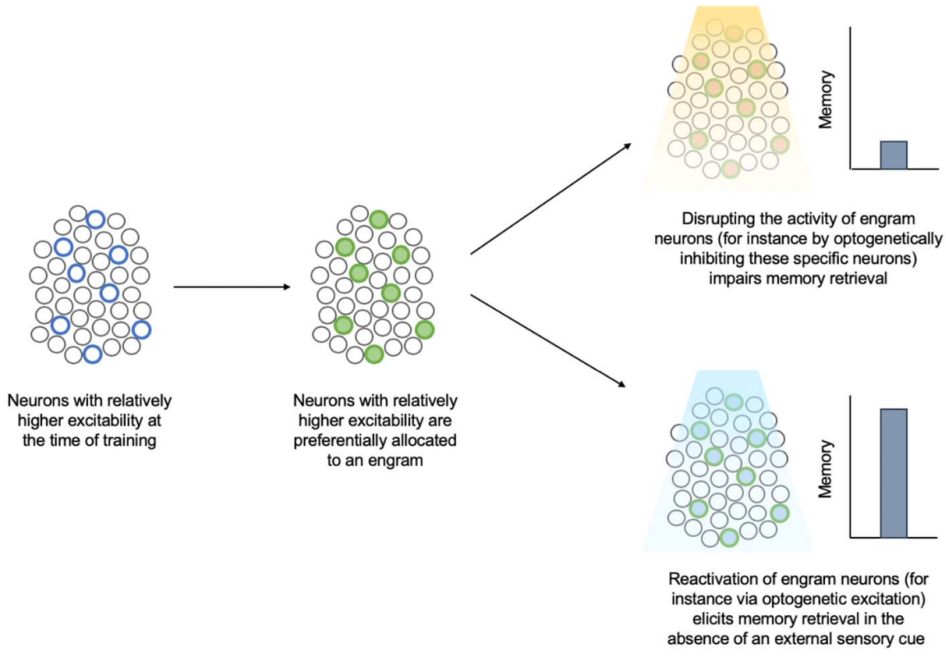

Figure 1.1. Neuronal ensembles formation. Neurons that show higher intrinsic excitability than their neighbours during training (blue circles) are

preferentially recruited into the final engram (green-filled circles). Subsequent perturbance of activity in these allocated neurons disrupts memory recall (top right), whereas artificial reactivation elicits memory recall without sensory cues (bottom right). *Adapted from Josselyn and Tonegawa (2020).*

1.1.3 Distributed memory networks

Both rodent and human studies are consistent with the concept that, during memory encoding, different kinds of information are processed and stored in distinct brain networks.

The multiple memory systems hypothesis was initially derived from studies in which lesioning one brain area would impair a specific memory feature. These early findings identified three parallel memory systems: the hippocampus, amygdala, and prefrontal cortex. This model postulates that all networks acquire information in parallel, simultaneously encoding multiple representations of a learning experience (Mizumori et al., 2004; White & McDonald, 2002; White et al., 2013).

Do these systems act as separate nodes, or can they be considered as a single integrative system? The existing literature supports the latter. One example of how memories depend on the interaction of different brain networks is the recall of recent and remote memories (system consolidation). It has been shown that shortly after learning, memory recall is strongly dependent on the hippocampal functions, while after weeks to months, memory depends on the representation stored in the prefrontal cortex (Kitamura et al., 2017; Lopez et al., 2012; Wiltgen et al., 2004).

In addition, the interplay between the infralimbic and prelimbic regions of the prefrontal cortex can compensate for the loss of hippocampal function. For instance, lesioning to the dorsal hippocampus can lead to the formation of

contextual fear memory after extensive training, but this resulting memory is temporary and differs from the one formed in the presence of an intact and functional hippocampus (Zelikowsky et al., 2012; Zelikowsky et al., 2013).

1.2 Cognitive flexibility and fear extinction

Organisms have evolved the ability to simultaneously maintain multiple memory representations and to switch between them as needed (Scott, 1962). Cognitive flexibility allows us to update memories and adjust behavioral responses to suit these. This process is incessant, and memories undergo updating throughout their existence.

At the evolutionary level, a fearful event is one of the most powerful learnings leading to strong memory and behavioral responses that are fundamental for survival. Once established, fear-induced responses can be ameliorated by re-exposing subjects to the stimuli in the absence of the aversive outcome, a procedure known as fear extinction. Behavioral fear extinction is a widely used, straightforward paradigm to study cognitive flexibility. Failure of extinction can lead to excessive and inappropriate fear and anxiety behavior as seen in certain forms of anxiety disorders, such as phobias, social anxiety disorder (SAD), and post-traumatic stress disorder (PTSD) (Lebois et al., 2019; Wen et al., 2022). Notably, brain regions implicated in fear extinction show a high degree of homology between rodents and humans (Wotjak & Pape, 2013).

1.2.1 The extinction of fear memories

Classical fear conditioning is one of the most powerful models for studying the neuronal substrates of associative learning and the mechanisms of memory formation in the mammalian brain (Davis et al., 2000; Fanselow & Poulos, 2005; Lang et al., 2000; LeDoux, 2000). Experimentally, context and cue-dependent fear conditioning paradigms represent simple forms of learning, are acquired rapidly within a single session, and elicit robust memory recall. In this Pavlovian paradigm, an initially neutral cue or context (the conditioned stimulus, CS) is paired with an aversive foot shock (the unconditioned stimulus, US). Subsequent re-exposure to the cue or the conditioning context elicits fear responses, including freezing (the conditioned response, CR), typically used to measure the fear memory.

If the CS is repeatedly presented in the absence of the US, a decline in the CRs is observed and is attributed to a process called fear extinction (Figure 1.2). Extensive work over the years demonstrated that behavioral extinction is a complex phenomenon, representing a new form of inhibitory learning that competes with, but does not erase, the original fear memory (Manahan-Vaughan et al., 2016; Mauk & Ohyama, 2004; Myers & Davis, 2007). The retrieval of a successful extinction memory (or retention of extinction) results in reduced CRs when the CS is presented.

Fear extinction has two remarkable characteristics: it is highly context-dependent, and it is transitory. If subjects are conditioned and then receive extinction training in context A, when the CS is re-presented in a different context B, the extinguished CRs reappear. This phenomenon is known as the renewal of fear (Bouton, 2004). Moreover, the reduction in CRs during and shortly after extinction training is not permanent. Over time, extinguished fear responses reappear through a process known as spontaneous recovery

(Rescorla & Heth, 1975; Westbrook et al., 2002). Interestingly, the degree of spontaneous fear recovery directly depends on the length of the retention interval. Thus, more robust CRs are elicited at extended delays (Quirk, 2002). These two behavioral phenomena constitute strong evidence that extinction does not erase the initial CS-US association but instead forms a new CS-No US association that inhibits the expression of the original memory in a contextual and time-constrained manner.

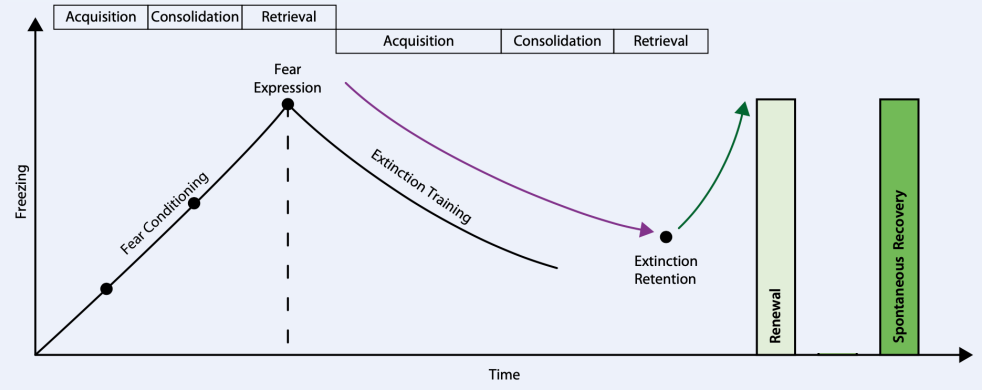

Figure 1.2. Fear conditioning and extinction paradigms. After fear conditioning, subsequent re-exposure to the CS evokes fear responses, quantified as time spent freezing. During extinction training, subjects are repeatedly presented to the CS without aversive stimulus. This procedure leads to the formation of a new inhibitory memory trace, which interferes and eventually competes with the initial fear memory. Extinction memory is tested on the next day at extinction retention. The original fear response re-emerges after extinction retention by presenting the conditioned cue in a novel context (Renewal) or spontaneously with time (Spontaneous Recovery). *Adapted from Wotjak and Pape (2013).*

1.2.2 Brain networks involved in fear extinction

Like other forms of learning, extinction is acquired and consolidated across several brain nodes, including the amygdala, the hippocampus, and the

prefrontal cortex (Herry et al., 2010; Myers & Davis, 2007; Wotjak & Pape, 2013).

The basolateral amygdala

The basolateral amygdala (BLA) is a central structure in fear conditioning, and it is composed of several anatomically and functionally distinct nuclei involved in both the acquisition and extinction of fear memories (Herry et al., 2006; Krabbe et al., 2018; Sun et al., 2020). For instance, local infusions of NMDA receptor antagonists, as well as kinase inhibitors, in the BLA prevent extinction learning, indicating that glutamatergic plasticity in this subcortical area is critical for the acquisition of this new inhibitory memory (Falls et al., 1992; Lu et al., 2001; Zhang et al., 2020).

In the basal nuclei (BA), fear and extinction are orchestrated by two distinct neuronal populations. One correlates with the initial fear state ("fear neurons"), while the other one is CS-responsive exclusively during extinction acquisition ("extinction neurons"). Notably, these two subpopulations receive different inputs, with the "fear neurons" receiving mainly from the hippocampus and the "extinction neurons" from the medial prefrontal cortex. Thus, it has been proposed that activity-switching in these BA circuits is essential for rapid adjustment and switches between behavioral states during the extinction learning (Herry et al., 2008).

The hippocampus

The hippocampus (Hp) is thought to modulate the contextual aspect of fear memories. For instance, optogenetic inhibition of the hippocampus during fear learning leads to increased generalization across contexts (Asok et al., 2019; Bernier et al., 2017).

Similarly to the amygdala, the initial fear and extinction memories are encoded in two distinct subsets of hippocampal neurons (Lacagnina et al., 2019; Mount et al., 2021; Zaki et al., 2022).

The medial prefrontal cortex

The medial prefrontal cortex (mPFC) is one of the first brain areas found to be implicated in extinction (Morgan et al., 1993). For instance, the infralimbic region (IL) seems important for the retention and expression of fear extinction, but not for its acquisition (Milad & Quirk, 2002, 2012). Notably, the IL to BLA projection is required for extinction. Enhanced activity in these cortical neurons leads to the recruitment of highly specialized GABAergic interneurons in the BLA, resulting in a decrease in the freezing response (Likhtik et al., 2008).

These findings indicate that fear extinction relies on a distributed learning network involving cortical and subcortical regions, which ultimately project to the basal ganglia system, responsible for the final integration, selection, and execution of the fear responses.

1.3 The Basal Ganglia

The term ‘basal ganglia’ refers to a group of interconnected subcortical nuclei involved in movement control, action selection and decision-making (Arber & Costa, 2022; Graybiel, 1995; Gunaydin & Kreitzer, 2016). Although the study of the mammalian basal ganglia system has traditionally been driven

by research on neurodegenerative disease and motor behavior, more recent studies indicate a role for this brain network in learning and memory processes (Goodman & Packard, 2016).

Given the wide range of functions and behaviors orchestrated by the basal ganglia system, it is not surprising that aberrant basal ganglia function is implicated in movement disorders, neuropsychiatric disorders, and drug addiction (Albin et al., 1989; Gittis & Kreitzer, 2012; Graybiel & Rauch, 2000; Gunaydin & Kreitzer, 2016; Koob & Volkow, 2016).

1.3.1 Anatomy of the basal ganglia

The basal ganglia system includes the striatum, globus pallidus, subthalamic nucleus, and *substantia nigra* (Figure 1.3).

The striatum is the largest subcortical structure in the mammalian brain and the primary input nucleus of the basal ganglia. It receives excitatory cortical and thalamic inputs, as well as dense innervation from midbrain dopaminergic neurons, representing the primary site of synaptic plasticity in the entire basal ganglia system (Kreitzer & Malenka, 2008; Lanciego et al., 2012). Striatal output diverges into two pathways: the direct pathway projects directly to the output nucleus of the basal ganglia, the *substantia nigra pars reticulate* (SNr). The indirect pathway, conversely, projects indirectly to the SNr via the external globus pallidus (GPe) and the subthalamic nucleus (STN). In addition to these canonical pathways, the STN in the indirect pathway receives direct cortical inputs from both motor and prefrontal areas (hyper-direct pathway). Notably, striatal projections to the basal ganglia output nuclei show a precisely defined spatial and functional segregation (Foster et al., 2021; Lee et al., 2020).

Basal ganglia output stations project to the thalamus, which projects back to the cortex and to locomotor regions of the brainstem.

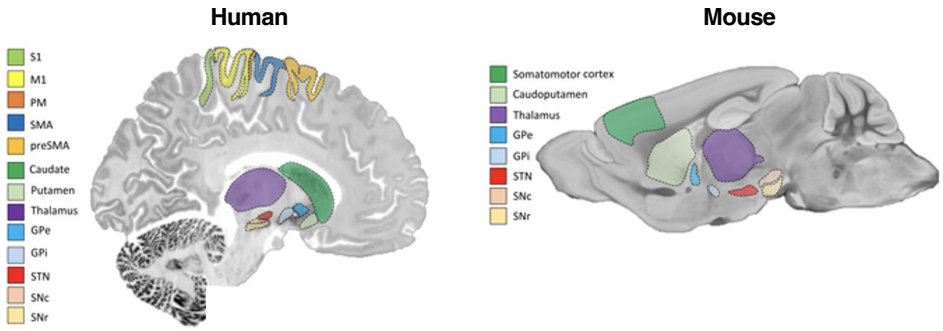

Figure 1.3. The basal ganglia system in human and mouse. S1: primary somatosensory cortex; M1: primary motor cortex; PM: premotor cortex; SMA: supplementary motor area; preSMA: pre-supplementary motor area; GPe: *globus pallidus pars externa*; GPi: *globus pallidus pars interna*; STN: subthalamic nucleus; SNc: *substantia nigra pars compacta*; SNr: *substantia nigra pars reticulate*. *Adapted from Mirzac et al. (2023).*

1.3.2 Cell types within the striatum

The striatum is unique in its lack of glutamatergic neurons: 95% of cells are GABAergic striatal projection neurons (SPNs). The remaining 5-10% are represented by small populations of interneurons: cholinergic interneurons (CINs), as well as multiple other subclasses of GABAergic neurons, such as parvalbumin and somatostatin-positive interneurons.

All striatal SPNs are inhibitory neurons that use GABA as the neurotransmitter. SPNs can be categorized into two subpopulations based on their gene expression and main downstream projections target (Gerfen et al., 1990; Smith et al., 1998). The direct pathway SPNs exhibit high expression of dopamine D1 receptors and project directly to basal ganglia output nuclei:

the internal globus pallidus (GPi) and the SNr. D1 receptors are coupled to adenylyl cyclase through G_{olf}, which positively modulates neuronal excitability. Conversely, the indirect pathway SPNs exhibit high expression of dopamine D2 and adenosine A2A receptors and project to the GPe. D2 receptors are coupled to $G_{i/o}$ proteins, which negatively modulate neuronal excitability.

This anatomical organization led to the initial hypothesis that the direct pathway promotes movements, resulting in the disinhibition of downstream motor centres, while the indirect pathway suppresses movements. However, this traditional view of the basal ganglia function has been challenged over the years by the development of molecular and genetic tools, as well as imaging and electrophysiological techniques to investigate how the direct and indirect pathways of the basal ganglia contribute to action selection and movement.

1.3.3 Beyond the classical basal ganglia model

The classical "go/no go" model has been highly influential in basal ganglia research, and it is based on the segregation of information processing into direct and indirect pathways, which act in opposing ways to control movement (Figure 1.4 A). This model has been applied to the study of neurodegenerative diseases involving the basal ganglia system. For instance, the impairment of movement observed in Parkinson's patients is thought to result from the abnormal increase in the activity of the indirect pathway. In contrast, excess movement in disorders such as Huntington's disease reflects the over-activity of the direct pathway.

Even though the go/no go model accounts for the cellular actions of dopamine on D1 and D2 receptors, data obtained with *in vivo* electrophysiology and recordings of cellular calcium dynamics have shown that the activity of both SPNs is enhanced during movements (Cui et al., 2013; Jin et al., 2014; Tecuapetla et al., 2016). Therefore, a complementary model has been proposed whereby the direct pathway SPNs select and facilitate one action, while the indirect pathway SPNs simultaneously suppress competing behavioral programs (Alexander & Crutcher, 1990; Mink, 1996) (Figure 1.4 B).

More recent work, nevertheless, has revealed that direct and indirect pathways show similar activity patterns when animals execute a particular set of movements (Barbera et al., 2016; Klaus et al., 2017; Markowitz et al., 2018; Parker et al., 2018). Thus, the indirect SPNs are unlikely to provide a blanket of inhibition over all potentially competing actions.

Recently, Bariselli et al. (2019) proposed a new competitive model, by which the balance of direct and indirect pathway output within a neuronal ensemble determines decision-making. According to this model, differential activation of direct and indirect SPNs within a specific ensemble determines whether the animal approaches, avoids, or remains in conflict with a certain stimulus. This model also accounts for the effect of dopamine-dependent plasticity that can alter the synaptic strength of inputs onto SPNs and influence future behavioral responses (Figure 1.4 C).

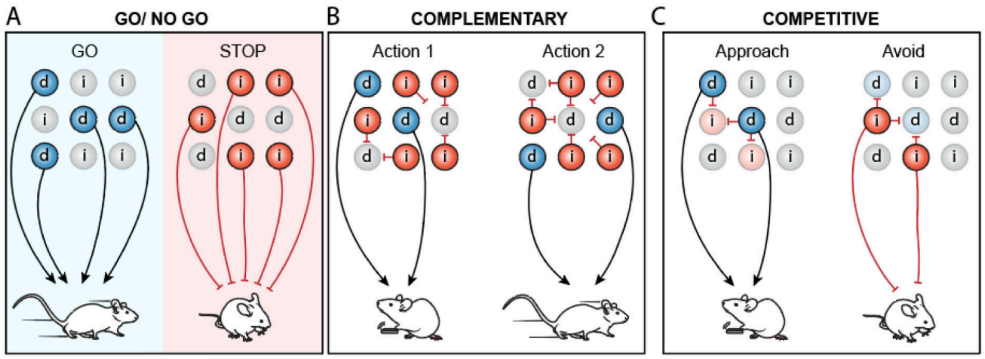

Figure 1.4. Three models of direct and indirect pathway's function. (A) Classical go/no go model: direct pathway SPNs (d, blue) activation promotes movement (GO), while indirect pathway SPNs activation (i, red) inhibits movement (STOP). **(B)** Complementary model: direct pathway SPNs select and facilitate one action, while indirect pathway SPNs simultaneously suppress competing motor programs. **(C)** Competitive model: the balance of direct and indirect pathway output within a striatal ensemble determines whether the animal approaches or avoids a stimulus. *Adapted from Bariselli et al. (2019).*

1.3.4 Cortico-basal ganglia-thalamic loops

The cortico-basal ganglia system is organized in topographically re-entrant loops. These loops involve different cortical areas projecting to a corresponding region of the striatum, with parallel projections from the striatum to other basal ganglia areas and ultimately to the thalamus and back to the same cortical area (Alexander et al., 1986; Gremel & Lovinger, 2017; Parent & Hazrati, 1995).

These loops present two remarkable characteristics. The first is the anatomical reduction observed from cortical input to the striatum to the final SNr output nucleus. For instance, in the rat brain, nearly all cortical neurons (around 20 million cells) project to the dorsal striatum (Zheng & Wilson, 2002), despite the SNr only containing about 25000 neurons (Oorschot, 1996). These anatomical limitations lead to the hypothesis that the basal

ganglia circuit might act as a low-dimensional learning system, wherein only relevant information is selected to reinforce appropriate input back to the cortex. The second remarkable feature is the presence of re-entrant loops, which could reinforce specific patterns of behaviors through the activity of midbrain dopaminergic neurons (Shen et al., 2008; Yagishita et al., 2014).
According to a recent model proposed by Athalye et al. (2020), dopaminergic inputs reinforce cortical projections at the level of the striatum. The activity of these synaptically modified striatal neurons is then propagated throughout the basal ganglia nuclei and the thalamus, and will re-enter a cortical population containing the initial and "reinforced" cortical neurons.
Currently, up to six distinct parallel loops have been delineated structurally and functionally, each organized by several precisely and highly interconnected nodes (Foster et al., 2021; Lee et al., 2020).

1.3.5 Functional segregation of the striatum

In the striatum, three main functional domains have been identified based on finely topographically organized cortical inputs, both in rodents and humans (Figure 1.5).

Dorsomedial striatum

The dorsomedial striatum (DMS), corresponding to the human caudate nucleus, receives projections mainly from limbic and higher-order cortical areas, such as the anterior cingulate cortex, parietal association cortex, prelimbic cortex, and infralimbic cortex. The DMS is involved in the encoding of action-outcome associations that underlie goal-directed learning, thus representing the "associative domain". Moreover, it has been shown that

DMS lesion or inactivation impairs flexible decision-making in a multitude of tasks that relies on the ability to shift between strategies and adapt behavioral responses to variations of the reward value and within reversal contingencies (Castañé et al., 2010; Ragozzino et al., 2002; Yin et al., 2005). The DMS plays a crucial role in action selection, and neuronal activity in the DMS reflects complex aspects of action performance, such as timing, contextual aspects, and reward specificity (Akhlaghpour et al., 2016; Emmons et al., 2017; Parker et al., 2016; Stalnaker et al., 2016). The DMS is, therefore, widely considered to be the region that facilitates flexible choice selection under changing conditions (Balleine et al., 2021).

Dorsolateral striatum

The dorsolateral striatum (DLS), homologous to the human putamen, receives projections from motor and somatosensory cortices, thus representing the "sensorimotor domain". The DLS is essential for acquiring, consolidating and expressing stimulus-response associations underlying established behavioral repertoires that facilitate automatic, habitual responding (Corbit, 2018; Yin et al., 2004; Yin et al., 2006). Neural activity in the DLS shifts with time from encoding many aspects of a task, to only the most relevant features that signal the initiation and end of a sequence of actions (Catherine A Thorn et al., 2010).

Both dorsomedial and dorsolateral subdivisions receive dense dopaminergic innervations from the *substantia nigra pars compacta* (SNc), which plays an important role in synaptic plasticity (Arbuthnott et al., 2000).

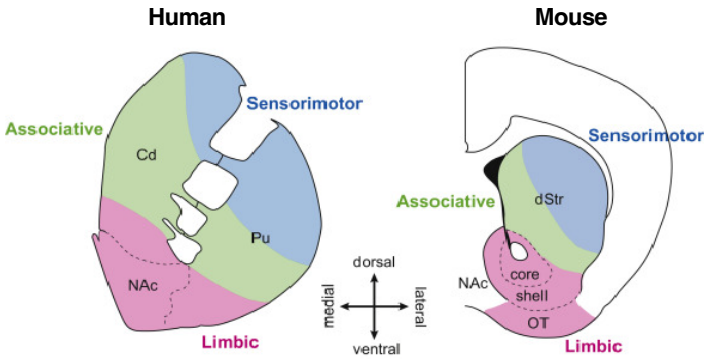

Figure 1.5. Functional domains of the striatum in human and mouse. Based on spatially and functionally segregated cortical inputs, the striatum can be divided into limbic (magenta), associative (green) and sensorimotor (blue) domains, in both human (left) and rodent (right). Cd: caudate; Pu: putamen; NAc: nucleus accumbens (core and shell); dStr: dorsal striatum; OT: olfactory tubercle. *Adapted from Chuhma et al. (2017).*

Ventral striatum

The ventral striatum (VS) includes the nucleus accumbens (core and shell subregions) and the olfactory tubercle (OT). The VS receives strong afferents from the hippocampus, prefrontal, mesocortical and limbic structures, thus representing the "limbic domain". The VS has classically been linked to outcome evaluation and motivational processes, in the formation of stimulus-outcome associations essential for Pavlovian learning, as well as in the rewarding effect of drugs of abuse, and its primary source of dopaminergic innervation is the ventral tegmental area (VTA) (Ikemoto, 2007; Root et al., 2015; Salamone et al., 2007; Yu et al., 2019).

1.4 Aims and rationale of the thesis

Learning is a complex cognitive process that allows organisms to encode, recall, and update memories about past experiences. The memory of fearful events is thought to be stored in functionally connected neuronal ensembles distributed across multiple regions. These ensembles represent a fundamental mechanism through which the brain processes and organizes information during learning.

Projections from many brain areas involved in fear learning are known to converge onto the striatum, the primary input station of the basal ganglia system. The dorsomedial part of the striatum (DMS) receives topographically organized inputs primarily from prefrontal and associative cortices and represents the striatal domain with highly associative and cognitive functions. Consequently, the DMS plays a fundamental role in many forms of cognitive flexibility, including the extinction of fear memories.

Understanding the neural basis of fear extinction and how different brain networks interact at the level of neuronal ensembles is clinically relevant, particularly in the context of human anxiety and fear disorders, such as phobias and post-traumatic stress disorder (PTSD).

Even though excitatory neuronal ensembles supporting fear memories have been identified and characterized in different brain areas, such as the cortex, the hippocampus, and the amygdala (Pettit et al., 2022; Roy et al., 2022), it remains unclear whether inhibitory ensembles exist in the striatum and what is their role in learning and memory updating.

This thesis aims to investigate whether a functionally relevant cFos+ memory ensemble is formed in the DMS upon the acquisition of contextual fear

memory and characterize how it specifically contributes to behavioral flexibility during extinction.

By comparing results obtained with a combination of specific circuit interference procedures, I aim to deepen the investigation of the brain network underlying fear extinction and I identified the retrosplenial cortex (RSC) and the dorsal hippocampus (dHp) as critical nodes in this network.

Finally, I hypothesize and test a molecular mechanism by which the activity in the dHp-RSC extinction network could be transferred and integrated into the DMS learning ensemble to select and guide flexible behaviors.

Chapter 2

Results

2.1 The DMS cFos+ ensemble formed during fear learning is required for extinction

2.1.1 cFos induction in the DMS during fear learning

To target the learning-related cell ensembles in the DMS, I carried out experiments on the Targeted Recombination in Active Populations (TRAP2) mouse line. This line uses the immediate early gene cFos locus to drive the expression of a tamoxifen-inducible Cre recombinase (iCreER^{T2}) within a defined time window, allowing for permanent labelling and genetic access to neurons activated by a specific event or experience (DeNardo et al., 2019) (Figure 2.1 A).

As the expression of cFos, as a marker for neuronal activity and plasticity, is a prerequisite for the cells' tagging, I first assessed the pattern of cFos activation in the DMS at different behavioral epochs of the contextual fear conditioning paradigm (cFC).

Mice were therefore perfused 90 minutes after cFC acquisition, recall, extinction, or 15 minutes of free context exploration, and their brains were then removed for immunohistochemical analysis of cFos expression. Tissue was also collected from naïve home cage animals, which served as a baseline control.

A significant increase of cFos+ cells was detected in response to all experimental conditions, indicating that the DMS is activated by fear encoding, recall, extinction, and contextual exploration (Figure 2.1 C). The increase was comparable between conditions.

This set of experiments showed that fear learning induces a strong neuronal activity in the DMS, leading to a robust cFos expression that can be quantified in TRAP2 mice, and provided further evidence that a specific subset of neurons undergoes plasticity changes during fear learning and its extinction.

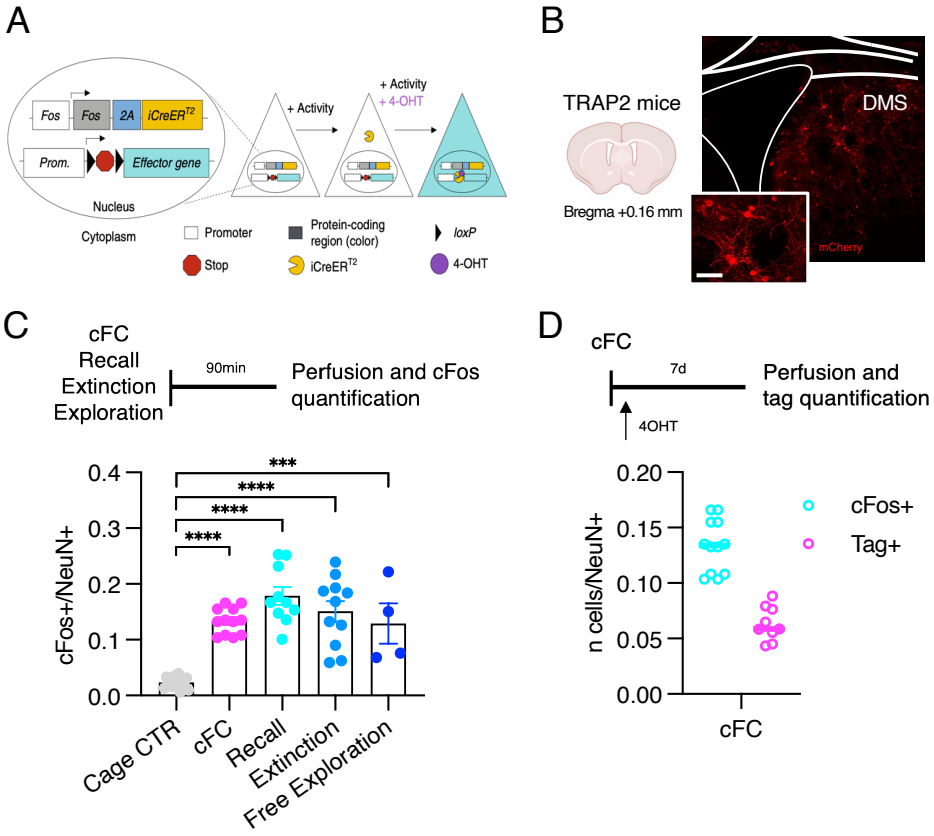

Figure 2.1. A cFos+ ensemble is formed in the DMS upon fear learning. (A) Schematic design of the TRAP2 tagging system. *Adapted from DeNardo et al. (2019).* **(B)** Representative coronal brain section showing the dorsomedial striatum (DMS) with its respective brain coordinates (left). Representative image of striatal neurons tagged upon cFC acquisition and stained with a

mCherry antibody anti-DREADD (right). Scale bars, 50 µm. **(C)** Quantification of cFos+ cells 90 minutes after cFC acquisition (magenta), recall (cyan), extinction (light blue) or 15 minutes of free context exploration (blue). A robust cFos induction was detected in the DMS in all the tested conditions compared to the home cage controls (grey). Statistical comparisons are performed using a one-way ANOVA followed by Tukey multiple comparison post-hoc tests; ***p<0.001, ****p<0.0001. Data are presented as mean ± SEM. **(D)** Quantification of DMS neurons expressing cFos (cFos+, cyan) and those tagged after 4OHT injection (Tag+, magenta) upon cFC acquisition.

2.1.2 The TRAP system allows genetic access to DMS cFos+ neurons upon fear learning

The TRAP2 method has been used successfully in many brain regions, such as the cortex, the amygdala, and the hippocampus (DeNardo et al., 2019; Roy et al., 2022), while its efficiency in inhibitory networks like the striatum is yet to be explored in sufficient depth.

To validate the tagging efficiency of this system, TRAP2 mice received intracranial injections in the DMS with excitatory, flexed Designer Receptors Exclusively Activated by Designer Drugs (DREADD, AAV9-hSyn-DIO-hM3Dq-mCherry) (Roth, 2016; Zhang et al., 2022; Zhu & Roth, 2014). To target neuronal ensembles active during fear memory encoding, mice were injected intraperitoneally with 4-hydroxytamoxifen (4OHT, 50 mg/kg) immediately after cFC acquisition. The 4OHT induces the Cre recombinase activity and drives the expression of the mCherry fluorophore associated with the DREADD channels, allowing for the visualization of the tagged neurons. Seven days after the acquisition of fear, mice were perfused, and their brains were removed for quantitative immunohistochemical analysis of labelled cells.

Immunohistochemical imaging showed a strong mCherry expression in a specific subset of neurons in the DMS (Figure 2.1 B). Quantitative analysis of mCherry+ cells revealed that, on average, half of DMS neurons expressing cFos during cFC acquisition were tagged as a neuronal ensemble for subsequent manipulations (Figure 2.1 D). Thus, DMS neurons expressing cFos upon fear learning can be efficiently tagged in the TRAP2 mouse line for later analysis and experimental manipulation.

2.1.3 Silencing of the DMS fear ensemble impairs behavioral flexibility and prevents extinction learning

The extinction of fear is considered a form of cognitive flexibility. It is believed to be mediated by new inhibitory learning that provides an alternative value (in this case, safety) to the same context, without erasing the original memory (Lacagnina et al., 2019; Martinez-Canabal et al., 2019; Sierra-Mercado et al., 2011). Experimentally, fear extinction is normally carried out after the initial learning by exposing the animals to the conditioned context for 30 minutes in the absence of footshocks. During this time, the mice progressively reduce their conditioned responses to the context and increase their exploratory behaviors. The retention of the extinction memory is then tested by exposing the animals to the conditioned context on the next day for 5 minutes and quantifying their freezing times.

Previous findings indicated that the DMS is required for behavioral flexibility and strategy updating. Silencing its activity prevents the extinction of fear memories, while it does not affect cFC acquisition or retention of the extinction (Serrano et al., 2022).

2.1 The DMS cFos+ ensemble formed during fear learning is required for extinction

Is silencing the DMS ensemble associated with the initial learning sufficient to mimic the same behavioral impairment?

To test this, wild-type mice received DMS intracranial injections with a TRAP2 vector (AAV9-Fos-ER^{T2}-Cre) and a flexed, inhibitory DREADD (AAV9-hSyn-DIO-hM4Di-mCherry). Immediately after the cFC acquisition, animals were injected intraperitoneally with 4OHT, and seven days after, they underwent an extinction session. Clozapine N-oxide (CNO, 5 mg/kg), an exogenous ligand for the DREADD channels, was administered systemically 30 minutes before extinction to inhibit the DMS fear ensemble through the entire session. Successful extinction learning was assessed by examining the ability to retain the new inhibitory memory on the next day at retention, as quantified by the percentage of time spent freezing.

Silencing of the DMS fear ensemble did not affect the freezing levels of the first five minutes of extinction, suggesting that despite the numerous inputs to this striatal region, the DMS cFC ensemble is not necessary for the recall of the fear memory. However, consistent with the hypothesis, chemogenetic inhibition of the DMS cFC ensemble selectively prevented extinction learning (Figure 2.2). Animals in which the DMS cFC ensemble was silenced during extinction kept showing freezing behavior in the conditioned context until the end of the training session and maintained those levels of freezing on the next day during the retention test, when the DMS cFC ensemble was no longer silenced. Control mice, which received the same intraperitoneal injections without the surgical procedure, gradually decreased their freezing response to the conditioned context, increasing their exploratory behavior. They reached minimal freezing levels at the end of the extinction session, and these were maintained on the following day at retention.

This set of results indicated that the cFC ensemble formed in the DMS during fear learning is not necessary to retrieve the original memory. Still, its activity is specifically required for successful extinction.

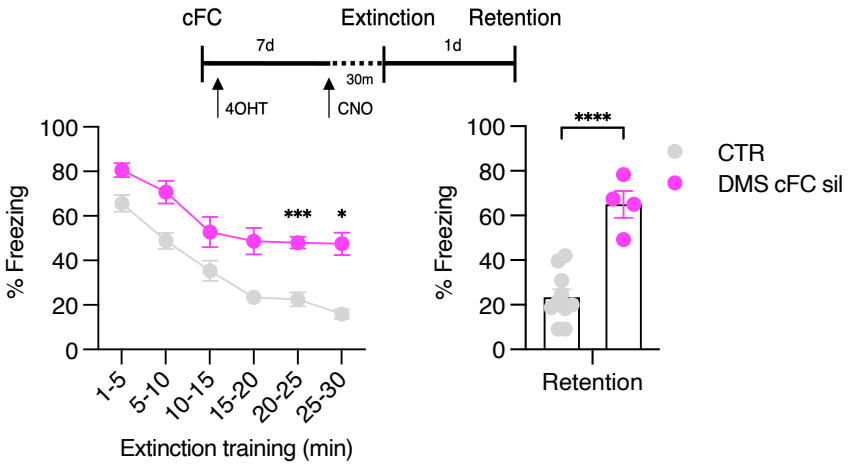

Figure 2.2. The DMS cFC ensemble is required for flexible learning during extinction. (A) Quantification of freezing levels during extinction (left) and retention (right). Silencing the DMS cFC ensemble during extinction training impairs behavioral flexibility and leads to high freezing levels at the end of training and 24 hours later at retention. [Controls (CTR, grey); DMS cFC ensemble silencing during extinction (DMS cFC sil, magenta)] Statistical comparisons are performed using a two-way ANOVA followed by Tukey multiple comparison post-hoc tests; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are presented as mean ± SEM.

2.2 Extinction learning changes the behavioral output of the DMS fear ensemble reactivation

2.2.1 Chemogenetic reactivation of the DMS cFC ensemble is sufficient to elicit a freezing response in a neutral context

Fear learning forms a memory ensemble in the DMS that is required to modify the original memory trace during extinction (Figure 2.2). Is the activity of the DMS cFC ensemble sufficient to elicit a freezing response in mice placed in a neutral context without conditioned stimuli?

To tackle this question, I performed a set of gain-of-function experiments. TRAP2 mice received DMS intracranial injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry). The neuronal ensemble active during fear memory encoding was tagged by intraperitoneal 4OHT injection immediately after cFC acquisition. Seven days later, the DMS cFC ensemble was chemogenetically reactivated via intraperitoneal CNO injection, while mice were placed for five minutes in a bucket serving as a neutral context (NC). To ensure the new context's neutrality, the bucket differed in shape, smell, and location from the conditioning context where the fear memory was initially acquired.

A significant increase in the time spent freezing was observed in animals where the DMS cFC ensemble was artificially reactivated compared to controls (Figure 2.3 A, *magenta*). Experimental animals injected with viruses and CNO showed a freezing level of around 60%, in contrast to the 20% exhibited by control mice. This indicated that activity in the DMS ensemble formed during cFC acquisition is sufficient to elicit a full freezing response in a neutral context.

Notably, the freezing levels of control animals were higher than naïve mice, which had never been conditioned before (Figure 2.3 A, *dark grey*). A possible explanation for this observation might be that the increase in freezing time is due to the novel unconditioned context still being a frightening stimulus due to the uncertainty inherently experienced by fear-conditioned animals in experimental settings. Consequently, controls slightly generalized the fear memory in the neutral context.

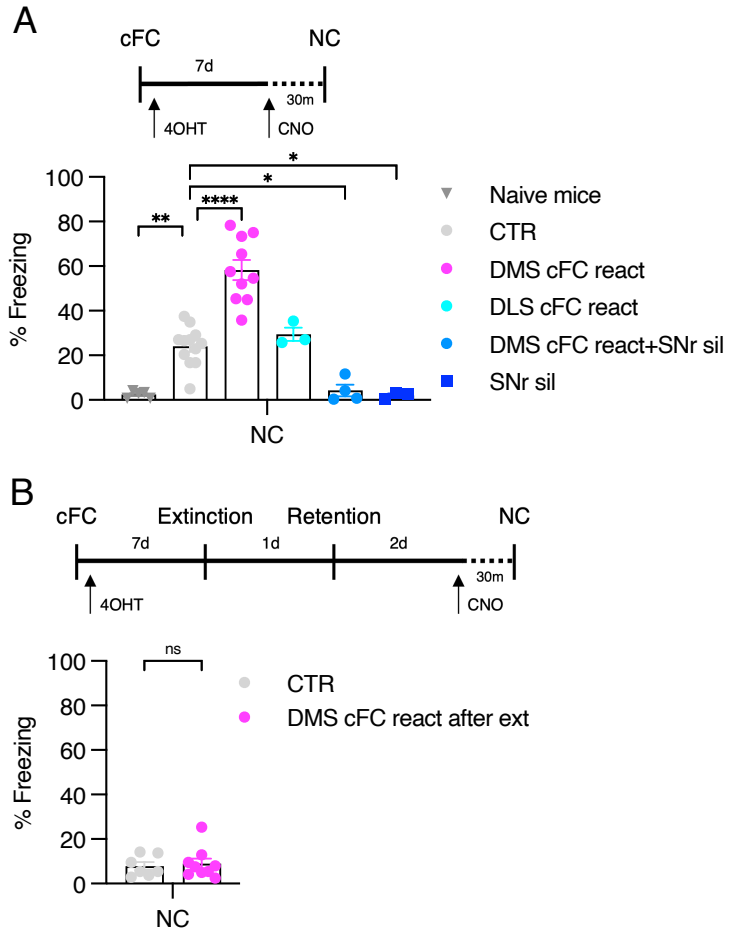

Figure 2.3. Extinction learning changes the behavioral output of the DMS cFC ensemble. (A) Quantification of freezing levels during the NC exploration after cFC. Control mice show higher freezing levels compared to naïve mice,

indicating fear memory generalization. Chemogenetic reactivation of the DMS ensemble tagged at cFC induces a significative freezing response in NC. This effect is specific to the DMS fear ensemble: chemogenetic reactivation of the DLS cFC ensemble does not affect freezing levels. Perturbing the activity of the SNr prevents DMS-induced freezing in NC. [Naïve mice (dark grey); Controls (CTR, grey); DMS cFC ensemble reactivation (DMS cFC react, magenta); DLS cFC ensemble reactivation (DLS cFC react, cyan); DMS cFC ensemble reactivation and SNr silencing (DMS cFC react+SNr sil, light blue): SNr silencing (SNr sil, blue)] **(B)** Quantification of freezing levels during the NC exploration after extinction. Chemogenetic reactivation of the DMS ensemble tagged at cFC does not induce freezing in NC after successful extinction learning. Control mice do not show memory generalization in NC after extinction learning. [Controls (CTR, grey); DMS cFC ensemble reactivation after extinction (DMS cFC react after ext, magenta)] Statistical comparisons are performed using a one-way ANOVA followed by Tukey multiple comparison post-hoc tests; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, NS: not significant. Data are presented as mean ± SEM.

2.2.2 Chemogenetic reactivation of the DLS fear ensemble

To assess whether the DMS fear ensemble is unique in its ability to elicit a freezing response in the neutral context, I reactivated neurons within the dorsolateral part of the striatum (DLS) that were expressing cFos during the cFC acquisition. Even though strict anatomical boundaries do not separate these two striatal subregions, a clear difference has been established regarding the distribution of glutamatergic inputs and their roles in learning. The DMS is important for stimulus-outcome associations underlying flexible and goal-directed behaviors, thanks to its inputs from associative regions (Balleine et al., 2007; Balleine & O'Doherty, 2010; Castane et al., 2010; C. A. Thorn et al., 2010); conversely, the DLS is specifically involved in the formation of stimulus-response associations and habitual behaviors, receiving projections from motor and somatosensory areas (C. A. Thorn et al., 2010; Yin et al., 2006).

TRAP2 mice received intracranial injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the DLS. Animals were injected with 4OHT intraperitoneally immediately after the cFC acquisition to tag the DLS fear-associated ensemble. Seven days later, CNO was injected intraperitoneally 30 minutes before the neutral context exploration to reactivate these cells.

Chemogenetic reactivation of the DLS neurons expressing cFos during fear learning did not induce any freezing response in the neutral context. Thus, the freezing levels of experimental mice did not differ from their respective controls (Figure 2.3 A, *cyan*). This experiment indicated that the ability to elicit a freezing response outside the conditioning context is an exclusive feature of the neuronal ensemble formed in the DMS and cannot be extended to the DLS.

2.2.3 The DMS-induced freezing response depends on its canonical downstream station, the SNr

In the striatum, the output of the direct and indirect pathways SPNs ultimately converges into the *substantia nigra pars reticulate* (SNr). Thus, I hypothesised that perturbing the activity of this main output nucleus would block the DMS-induced freezing response in a neutral context.

To test this, TRAP2 mice received intracranial injections with the flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the DMS, and with CamK2a-Cre and inhibitory DREADD (AAV9-hSyn-DIO-hM4Di-mCherry) in the SNr. Animals were injected with 4OHT intraperitoneally immediately after the cFC acquisition to tag the DMS ensemble and, seven days later, were exposed to the neutral context. CNO was administered 30

minutes before the start of the neutral context exploration to reactivate the DMS fear ensemble and inhibit the SNr simultaneously.

DMS-induced freezing in the neutral context was prevented by perturbing the SNr activity. This indicates that the freezing response induced by the DMS cFC reactivation depends on its canonical downstream station (Figure 2.3 A, *light blue*). Freezing levels did not differ between experimental and control animals and mice where the SNr was silenced alone as an additional control (Figure 2.3 A, *blue*).

This first set of experiments provided evidence that the DMS cFC ensemble was not only required for successful extinction learning, but it encoded "freezing", and its reactivation resulted in the specific retrieval of the original fear memory in a neutral context. This effect was specific to the ensemble formed in the dorsomedial, but not in the dorsolateral striatum, and was prevented by perturbing the activity of the SNr, the downstream output station of the basal ganglia system.

2.2.4 Chemogenetic reactivation of DMS cFC ensemble does not induce freezing in a neutral context after successful extinction learning

The activity in the DMS cFC ensemble is specifically required for extinction (Figure 2.2), raising the question of how this learning ensemble is accessed and modified to allow behavioral flexibility. Thus, I performed a set of gain-of-function experiments to test whether the freezing output of the original striatal ensemble would be modified as a consequence of extinction.

TRAP2 mice received intracranial injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the DMS. Animals were injected with 4OHT intraperitoneally immediately after the cFC acquisition

to tag the DMS ensemble, but they were subsequently allowed to undergo successful extinction and retention, two days before the start of the neutral context exploration. Before this exploration, CNO was administered intraperitoneally to reactivate the DMS cFC ensemble.

Experimental and control animals showed comparable freezing levels of around 10% (Figure 2.3 B). These results indicated that the behavioral output of the DMS cFC reactivation, which after cFC acquisition represented "freezing" and was sufficient to elicit a fear response in a neutral context, might be modified when the original memory had to be adjusted to new contingencies during extinction.

Interestingly, the effect of extinction learning could also be observed in control mice, whose freezing response was approximately half compared to control mice that did not undergo extinction (Figure 2.3 A and B, *grey*). This suggested that, while cFC acquisition creates a memory that is slightly generalized in a neutral context, this generalization is prevented when the memory is successfully extinguished.

2.3 The DMS fear and extinction ensembles can induce the same freezing response after recovery of fear, and exhibit high cFos overlap

2.3.1 Chemogenetic reactivation of the DMS cFC ensemble induces freezing in a neutral context after recovery of fear

Extinction is a temporary phenomenon. The fear memory returns approximately two weeks after the retention of extinction, inducing a full freezing response in the conditioned context. This process is called the spontaneous recovery of the fear (Bouton, 2002; Goode & Maren, 2014).

I reasoned that the spontaneous recovery might be sufficient to reinstate the original "freezing" output of the DMS cFC ensemble, resulting in a fear response when reactivated in a neutral context.

To test this, TRAP2 mice received injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the DMS. The striatal ensemble associated with cFC acquisition was tagged, and mice underwent successful extinction, retention, and fear recovery. Two days later, CNO was injected intraperitoneally 30 minutes before the neutral context exploration to reactivate the DMS fear ensemble chemogenetically.

This manipulation induced a significant increase in the freezing response to the neutral context of mice in which the DMS cFC ensemble was reactivated, compared to controls (Figure 2.4). This result indicated that, after recovery of the original fear memory, the DMS cFC ensemble changes its behavioral output, once again eliciting a freezing response.

This observation gives way to the intriguing possibility that the ensemble formed in the DMS during cFC acquisition might always represent a fear

response. In contrast, the expression or execution of this response might depend on the recruitment of specific brain networks.

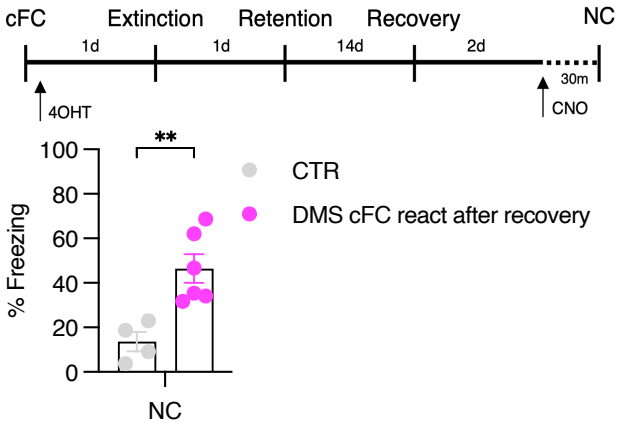

Figure 2.4. Spontaneous recovery of fear reinstates the original DMS-induced freezing in NC. Quantification of freezing levels during the NC exploration after fear recovery. Chemogenetic reactivation of the DMS cFC ensemble induces freezing in NC. [Controls (CTR, grey); DMS cFC ensemble reactivation after recovery (DMS cFC react after recovery, magenta)] Statistical comparisons are performed using a one-way or two-way ANOVA followed by Tukey multiple comparison post-hoc tests; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are presented as mean ± SEM.

2.3.2 Chemogenetic reactivation of the DMS extinction ensemble induces freezing in a neutral context after recovery of fear

The behavioral output of the DMS learning ensemble changes when the memory needs to be updated during extinction and after fear recovery. the logical implication for this finding is that tagging DMS ensembles at any relevant point after cFC acquisition would result in a freezing response when these cells are chemogenetically reactivated in a neutral context after fear recovery. To test this hypothesis, I decided to tag and reactivate the DMS extinction ensemble.

For this experiment, TRAP2 mice received injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the DMS. DMS cells expressing cFos upon extinction learning were tagged by injecting the 4OHT immediately after the 30 minutes of extinction session, when mice no longer exhibited a freezing response to the conditioning context. After the spontaneous return of fear two weeks after retention, the DMS extinction ensemble was reactivated by injecting CNO intraperitoneally 30 minutes before the neutral context exploration.

Mice in which the DMS extinction ensemble was reactivated showed a strong freezing response to the neutral context compared to controls (Figure 2.5).

These results suggested that cFos-expressing cells in the DMS, upon cFC acquisition and extinction, encode for the same freezing response, brought back by spontaneous fear recovery.

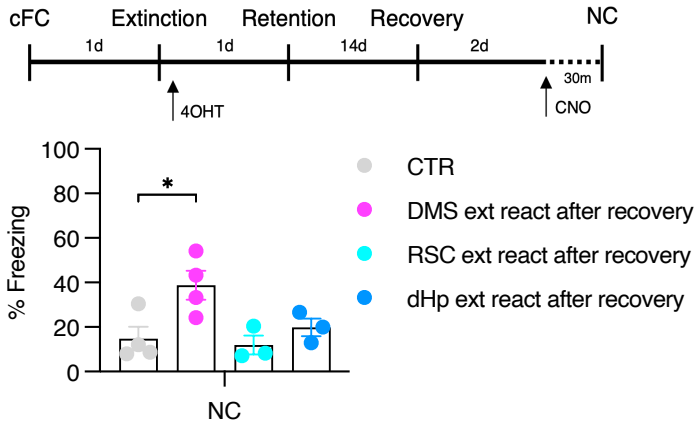

Figure 2.5. The DMS extinction ensemble elicits freezing when reactivated in the NC after spontaneous recovery of fear. Quantification of freezing levels during the NC exploration after fear recovery. Chemogenetic reactivation of the DMS ensemble tagged at the end of the extinction training induces freezing in NC after fear recovery. Reactivation of the respective ensembles tagged in the RSC or the dHp is not sufficient to elicit a freezing response. [Controls (CTR, grey); DMS extinction ensemble reactivation after fear recovery (DMS ext react after recovery, magenta)] Statistical

comparisons are performed using a one-way or two-way ANOVA followed by Tukey multiple comparison post-hoc tests; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are presented as mean ± SEM.

2.3.3 Memory recall or extinction reactivates the same proportion of cFC ensemble cells in the DMS, but not the RSC

To deepen the investigation, I once again took advantage of the TRAP2 system to test whether the cells active during cFC acquisition were similarly recruited upon recall or extinction learning.

The DMS and the retrosplenial cortex (RSC; used as a control region) of wild-type mice were injected with the AAV9-Fos-ER^{T2}-Cre TRAP2 vector and the Cre-dependent Sternson inhibitor virus (rAAV9-CAG-flox-PSAM-GFP), to tag and visualized cells, respectively.

To target neuronal ensembles in both brain areas, mice were injected intraperitoneally with 4OHT immediately after the cFC acquisition. Seven days after cFC, mice were re-exposed to the conditioning context for either 5 minutes (recall) or 30 minutes (extinction). 90 minutes later, mice were perfused, and their brains were then collected for immunohistochemical analysis of cFos expression. Tissue was also collected from naïve home cage animals, which served as a baseline control.

In all tested conditions, the general cFos quantification showed a significant increase compared to the respective home cage controls. In line with previous observations, extinction learning induced a more robust induction of cFos expression in the RSC compared to the memory recall (Serrano et al., 2022); cFos expression was, however, comparable between recall and extinction in the DMS (Figure 2.6 C).

2.3 The DMS fear and extinction ensembles can induce the same freezing response after recovery of fear, and exhibit high cFos overlap

I then quantified the proportion of cells tagged during cFC acquisition that expressed cFos upon recall or extinction (cFos overlap: double labelled cells on the total number of tagged neurons). In the RSC, the cFC ensemble was strongly reactivated by memory recall, evidenced by a cFos overlap of up to 70%. Simultaneously, extinction was associated with the formation of a new cFos+ population, with a cFos overlap of around 40%. In the DMS, 40% of cells belonging to the original cFC ensemble were reactivated during recall and extinction (Figure 2.6 D), indicating that the original ensemble is equally reactivated by both the recall and the extinction of the fear memory.

This set of results strongly suggested that unlike in the cortex, where cFC and extinction are treated as separate learning experiences encoded in dedicated neuronal ensembles, the DMS fear extinction is not associated with the formation of a new inhibitory memory. This data led to the idea that the behavioral output of the fear ensemble might be orchestrated in brain circuits upstream of the striatum. Therefore, I started reasoning about the brain areas that might coordinate its activity and be responsible for the change of DMS fear output after extinction learning.

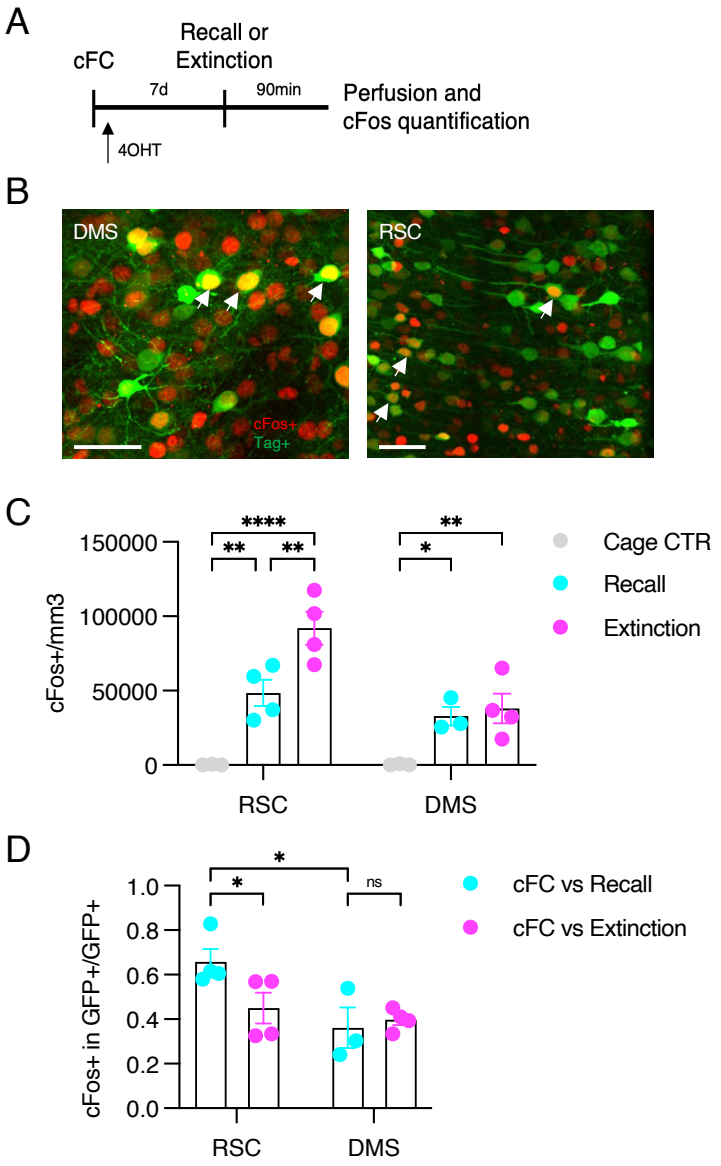

Figure 2.6. Unlike in the RSC, the recall and extinction reactivate the same portion of the original fear ensemble in the DMS. (A) Experimental timeline. **(B)** Representative images of DMS (left) and RSC (right) cFos+ neurons tagged at cFC acquisition (green cells) and cFos+ immunoreactivity upon extinction (red cells). The arrows indicate the cFos overlap between the two learning experiences. Scale bars, 30 µm. **(C)** Quantification of cFos induction in RSC and DMS upon fear recall and extinction. [Home cage controls (Cage CTR,

grey); cFos expression during recall (Recall, cyan); cFos expression during extinction (Extinction, magenta)] **(D)** cFos overlap. Quantification of double labelled cells (cFos+ and GFP+) on total tag cells (GFP+). The analysis shows in RSC a substantial overlap between cFC and recall, but not extinction, while in DMS the overlap is comparable. [cFos overlap between cFC and recall (cFC vs Recall, cyan); cFos overlap between cFC and extinction (cFC vs Extinction, magenta)] Statistical comparisons are performed using a one-way ANOVA followed by Tukey multiple comparison post-hoc tests; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns: not significant. Data are presented as mean ± SEM.

2.4 A dedicated hippocampal-cortical network changes the DMS freezing output after extinction learning

2.4.1 The DMS-RSC-dHp circuit for behavioral flexibility

Among all the cortical regions that project to the DMS and are critically involved in flexible learning, I focused my attention on the retrosplenial cortex (RSC). The RSC is an area located behind the splenium of the corpus callosum and has historically been shown to have a role in complex cognitive processes and to act as a bridge between the hippocampal formation and the neocortex (Alexander et al., 2023; Jones & Witter, 2007; Sugar et al., 2011). In a recent study by Serrano et al. (2022), the role of the RSC in flexible learning and navigation has been intensively described across different behavioral paradigms, reaching the conclusion that this cortical area is fundamental to adjusting behavior and allowing the formation of alternative associations to the original ones. Importantly for this thesis, the RSC has been shown to densely project to the DMS and silencing of the RSC to DMS

projecting neurons is sufficient to strongly impair the extinction learning (Serrano et al., 2022).

The RSC receives strong unidirectional inputs from the dorsal part of the hippocampus (dHp) (Sugar et al., 2011). The dHp represents the most anterior part of the hippocampal formation and has a well-established role in cognitive and spatial memory functions, as well as in the information processing (Fanselow & Dong, 2010; Strange et al., 2014). Notably, silencing the dHp during the cFC acquisition impairs subsequent extinction learning, despite not impacting the recall of the original fear memory (Maria Lahr, unpublished). This result suggests that the encoding of the original fear memory trace in this brain area is essential to allow subsequent modifications of the memory. Importantly for this work, dHp silencing during extinction impairs behavioral flexibility and prevents the retention of extinction (Maria Lahr, unpublished), indicating that the original fear memory, not only has to be encoded in dHp, but also has to be accessible during extinction to allow the formation of the new inhibitory association.

Given their connectivity and functional roles, both the RSC and the dHp are therefore primary candidates of this study to investigate how the freezing output of the DMS cFC ensemble is modulated after extinction learning. A schematic representation of the circuit is illustrated in Figure 2.7.

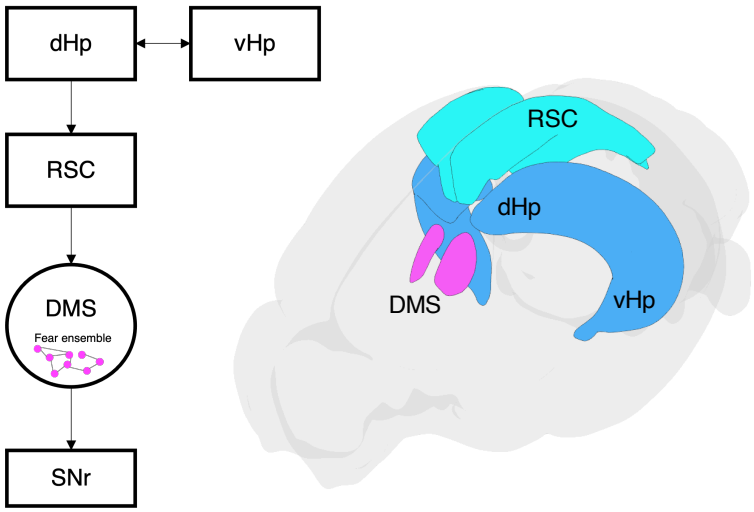

Figure 2.7. Schematic representation of the dHp-RSC-DMS connectivity. dHp: dorsal hippocampus; vHp: ventral hippocampus; RSC: retrosplenial cortex; DMS: dorsomedial striatum; SNr: *substantia nigra pars reticulate*.

2.4.2 The RSC is recruited to suppress freezing upon DMS cFC reactivation after extinction

I started my investigation of the circuit with the RSC, and, given its essential role during extinction (Serrano et al., 2022), I hypothesized that this cortical area might be responsible for conveying this signal to the striatum.

To test this, TRAP2 mice received intracranial injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the DMS, and with a mix of CamK2a-Cre and a flexed, inhibitory DREADD (AAV9-hSyn-DIO-hM4Di-mCherry) in the RSC. To target neuronal ensembles active during fear memory encoding, mice were injected intraperitoneally with 4OHT immediately after the cFC acquisition. Seven days after cFC acquisition, mice underwent successful extinction and retention. Two days after retention, CNO was administered 30 minutes before the start of neutral

context exploration to reactivate the fear ensemble in the DMS and simultaneously silence all RSC neurons.

Chemogenetic inhibition of the RSC was sufficient to reinstate the DMS-induced freezing response in the neutral context (Figure 2.8, *cyan*). Experimental mice exhibited a freezing response of up to 40% in the neutral context, compared to the 10% freezing of control mice. This indicated that activity in this cortical area suppresses the striatal-induced freezing after extinction.

2.4.3 The RSC extinction ensemble silencing does not induce fear memory generalization

In the RSC, cFC and extinction learning are computed separately, leading to the formation of two different neuronal ensembles (Figure 2.6). To study the specific contribution of these cortical ensembles, which possibly compete for the control of behavior during extinction, I asked whether silencing the RSC extinction ensemble might be sufficient to induce the generalization of the fear memory in the neutral context. Indeed, one possible explanation for the freezing induced by the RSC silencing could be that the sole inhibition of the cortical ensemble formed during extinction might result in a freezing response by itself and, therefore, not being specific to the DMS cFC reactivation.

To rule out this possibility, TRAP2 mice received injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the RSC. The cells expressing cFos upon extinction learning were tagged by injecting the 4OHT immediately after the extinction session. Two days after retention,

CNO was injected intraperitoneally 30 minutes before the start of neutral context exploration to silence the RSC extinction ensemble.

The freezing level of experimental animals in the neutral context did not differ from the control group (Figure 2.8 A, *blue*). This result indicated that silencing of the RSC extinction ensemble is not sufficient to generalize the fear memory. Therefore, the specific reactivation of the DMS cFC ensemble (in combination with RSC inhibition) is a necessary condition to observe a freezing response in the neutral context.

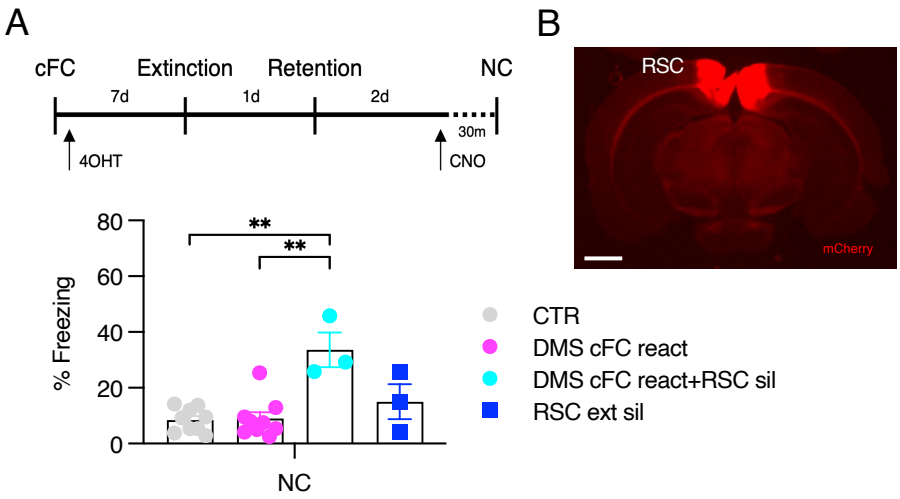

Figure 2.8. The role of RSC in suppressing DMS-induced freezing in NC after extinction. (A) Quantification of freezing levels during the NC exploration after extinction. Chemogenetic silencing of the RSC or the neurons projecting from the dHp to the RSC reinstates the DMS-induced freezing in NC. [Controls (CTR, grey); DMS cFC ensemble reactivation after extinction (DMS cFC react, magenta); DMS cFC ensemble reactivation and RSC silencing (DMS cFC react+RSC sil, cyan); RSC extinction ensemble silencing (RSC ext sil, blue)] Statistical comparisons are performed using a one-way ANOVA followed by Tukey multiple comparison post-hoc tests; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are presented as mean ± SEM. **(B)** Representative image of the RSC injection site. Scale bar, 1 mm.

2.4.4 Encoding of extinction in the dHp is required for DMS cFC ensemble behavioral output change

The RSC receives strong unidirectional projection from the dHp, which is essential for extinction. Indeed, silencing this brain area during extinction prevents flexible learning (Maria Lahr, unpublished). Therefore, to start the investigation of the role of the dHp within the circuit, I asked how an impaired extinction would be reflected in the DMS behavioral output.

To this end, TRAP2 mice received injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the DMS, and a mix of AAVs carrying CamK2a-Cre and Sternson inhibitor virus (a Cre-dependent PSAM channel selectively activated by PSEM ligand; rAAV9-CAG-flox-PSAM-GFP) in the dHp. This combination of chemogenetic tools allows for the selective inhibition of the dHp and reactivation of the DMS ensemble at two different time points. The PSEM ligand was injected intraperitoneally 20 minutes before the extinction session to inhibit the dHp, while CNO was injected intraperitoneally 30 minutes before the start of neutral context exploration to reactivate the fear ensemble in the DMS.

Perturbing the activity of the dHp during extinction impaired behavioral flexibility. Experimental mice maintained high freezing levels throughout the 30 minutes of the extinction session, and until the day of the retention (Figure 2.9 A, left panel). Two days after retention, chemogenetic reactivation of the DMS fear ensemble was sufficient to elicit a freezing response in the neutral context (Figure 2.9 A, right panel).

These data indicated that the information encoded in the dHp during extinction learning was fundamental to acquiring and retaining the new memory. If dHp activity was prevented and extinction learning did not occur,

the DMS behavioral output was not modified, and its artificial reactivation elicited the original freezing response in the neutral context.

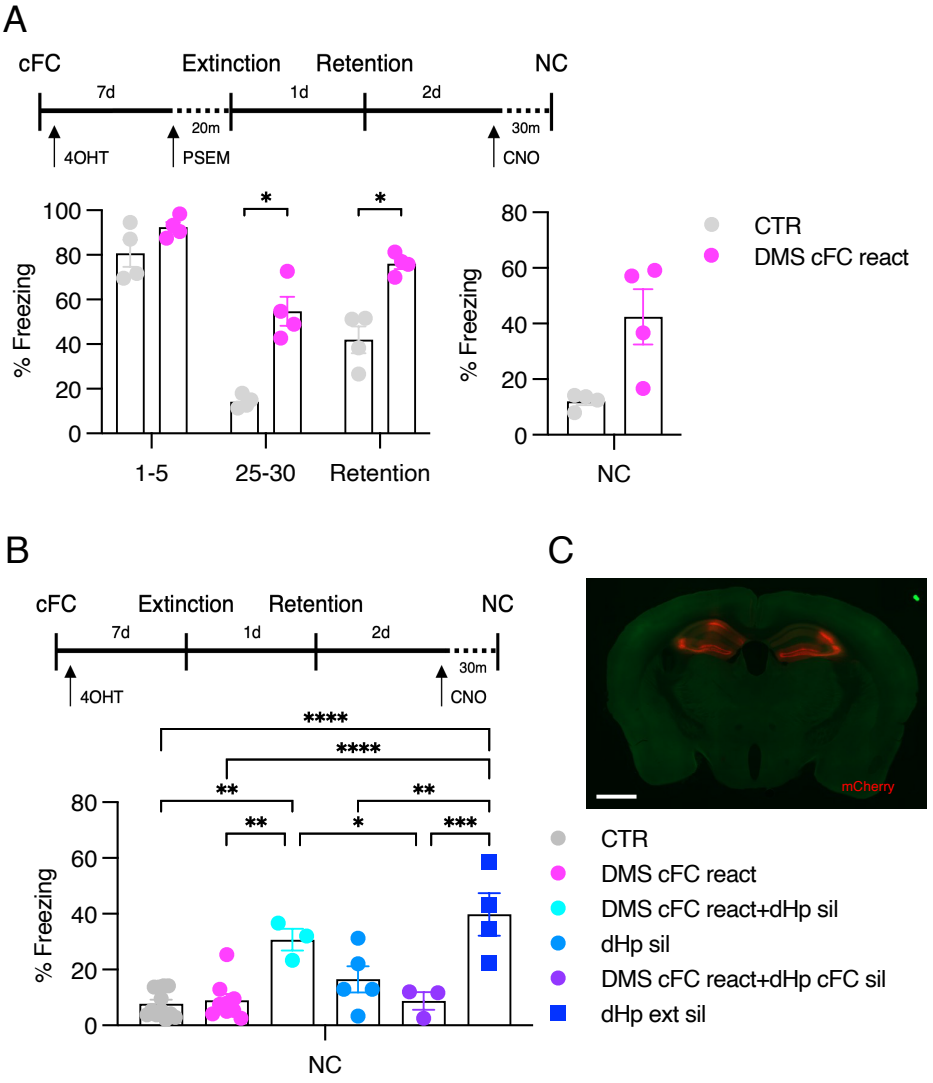

Figure 2.9. The role of dHp in suppressing DMS-induced freezing in NC after extinction. (A) Left: Quantification of freezing levels during recall (1-5), end of extinction (25-30) and retention. Chemogenetic inhibition of dHp during extinction training prevents flexible learning and leads to high freezing levels both at the end of training and 24 hours later at retention. Right: Quantification of freezing levels during the NC exploration after extinction. Chemogenetic reactivation of the original DMS cFC ensemble induces freezing

in NC when extinction is prevented by dHp silencing. [Controls (CTR, grey); dHp silencing during extinction and DMS cFC ensemble reactivation (DMS cFC react, magenta)] **(B)** Quantification of freezing levels during the NC exploration after extinction. Chemogenetic silencing of the dHp reinstates the original DMS cFC behavioral output. Silencing the dHp cFC ensemble is not sufficient to reinstate DMS freezing in NC, suggesting a specific involvement for this brain area during extinction. Chemogenetic silencing of the dHp extinction ensemble induces fear memory generalization in NC. [Controls (CTR, grey); DMS cFC ensemble reactivation after extinction (DMS cFC react, magenta); DMS cFC ensemble reactivation and dHp silencing (DMS cFC react+dHp sil, cyan); dHp silencing (dHp sil, light blue); DMS cFC reactivation and dHp cFC ensemble silencing (DMS cFC react+dHp cFC sil, purple); dHp extinction ensemble silencing (dHp ext sil, blue)] **(C)** Representative image of dHp injection site. Scale bar, 1 mm. Statistical comparisons are performed using a one-way or two-way ANOVA followed by Tukey multiple comparison post-hoc tests; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are presented as mean ± SEM.

2.4.5 The dHp is recruited after extinction to suppress freezing upon DMS cFC reactivation

Activity in the dHp is essential during online extinction to allow the change of the DMS fear ensemble output, from freezing to no-freezing. Is this region recruited also at the moment of the neutral context exploration if the DMS cFC ensemble is reactivated?

To answer this question, TRAP2 mice received intracranial injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the DMS, and with a mix of CamK2a-Cre and inhibitory DREADD (AAV9-hSyn-DIO-hM4Di-mCherry) in the dHp. Two days after retention of extinction, CNO was injected intraperitoneally 30 minutes before the start of neutral context exploration to reactivate the fear ensemble in the DMS and inhibit the dHp.

Silencing the dHp, similarly to the RSC, was sufficient to reinstate a 40% freezing response in the neutral context when the DMS fear ensemble was

reactivated (Figure 2.9 B, *cyan*). Thus, the dHp has a key role not only during online extinction but also after extinction, when it is recruited to actively suppress the DMS-induced freezing response in the neutral context.

2.4.6 Silencing of the dHp does not induce fear memory generalization

The dHp has classically been involved in the encoding of contextual information. Thus, it is important to make sure that the freezing observed in the previous experiment is due to preventing access to relevant information related to extinction, and not due to the inability of mice to recognize the novelty of the neutral context.

To exclude this possibility, the dHp was chemogenetically silenced (with a combination of CamK2a-Cre and flexed, inhibitory DREADD, AAV9-hSyn-DIO-hM4Di-mCherry) by injecting CNO intraperitoneally 30 minutes before the neutral context exploration.

Silencing of this hippocampal area was not sufficient to elicit a freezing response in the neutral context (Figure 2.9 B, *light blue*), indicating that when the original memory had been successfully extinguished, fear generalization in a neutral context was prevented. This suggested that the dHp is encoding specific information about extinction and that the DMS cFC reactivation is necessary with the dHp silencing to observe a freezing response in a neutral context after extinction.

2.4.7 Silencing of the dHp cFC ensemble

In the dHp, like in the RSC, the initial encoding of the fear memory and extinction learning form overlapping, but distinct neuronal ensembles (Maria Lahr, unpublished).

To study the specific interaction between fear ensembles encoded in the dHp and DMS, TRAP2 mice received intracranial injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the DMS and a flexed, inhibitory DREADD (AAV9-hSyn-DIO-hM4Di-mCherry) in the dHp. Two days after retention of extinction, CNO was injected intraperitoneally 30 minutes before the start of neutral context exploration to simultaneously reactivate and inhibit the cFC ensemble in the DMS and the dHp, respectively.

Experimental mice did not differ significantly from the control group (Figure 2.9 B, *purple*). This result had two main implications. First, it confirmed that preventing dHp cFC ensemble activity (like the entire brain area) does not lead to the generalization of the fear memory in the neutral context after extinction, meaning that the fear ensemble no longer encodes for contextual information. Second, this experiment showed that the dHp cFC ensemble is not involved in suppressing the DMS freezing response and strongly suggested that the dHp extinction ensemble might be specifically required for this function.

2.4.8 Silencing of the dHp extinction ensemble induces fear memory generalization

A limiting characteristic of the TRAP2 system is that it allows for the tagging of cells after exclusively one behavioral experience, such as cFC acquisition or extinction. Therefore, it is currently impossible to reactivate the DMS cFC ensemble while inhibiting the dHp extinction ensemble in the same animal.

Nevertheless, I decided to test the effect of silencing the dHp extinction ensemble in a neutral context.

TRAP2 mice received intracranial injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the dHp. I tagged the dHp cells expressing cFos upon extinction learning, injecting the 4OHT immediately after the 30 minutes of extinction session when mice no longer freeze to the conditioning context. Two days after retention of extinction, CNO was administered 30 minutes before the start of neutral context exploration to inhibit specifically the dHp extinction ensemble's cells.

Experimental mice in which the dHp extinction ensemble was silenced exhibited a strong freezing response when placed in the neutral context compared to controls (Figure 2.9 B, *blue*). Thus, preventing the activity of this specific cells' subset is sufficient to induce freezing.

However, the freezing response observed here is different and can be distinguished from the one induced by the combination of DMS cFC reactivation and dHp inhibition. Indeed, when both dHp cFC and extinction ensembles were silenced, no freezing response was induced in the neutral context. Possibly, the control of the freezing response is due to an interplay of DMS cFC reactivation and activity of the dHp extinction ensemble, mediated by the RSC.

2.4.9 Silencing of RSC to dHp projecting neurons

Having established that the RSC and the dHp play a key role in changing the DMS behavioral output after extinction, I finally wanted to investigate the directionality of the information flow within the network by silencing specifically the neurons that project from the dHp to the RSC and evaluating this effect on the striatal reactivation's output.

To this end, TRAP2 mice received intracranial injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry) in the DMS, a Flp-delivery retrograde tracer under the Camk2a promoter (rAAV-pCAG-FLPo-2A-H2B-V5) in the RSC, and a Flp-dependent inhibitory DREADDs (AAV9-hSyn1-dFRT-hM4Di-mCherry(rev)-dFRT) in the dHp. This viral combination allows the reactivation of the DMS cFC ensemble while specifically silencing the dHp to RSC projecting neurons. For this experiment only, animals were kept in home cage conditions for 2-3 weeks after the surgical procedure, ensuring the optimal expression of the retrograde tracer. CNO was injected intraperitoneally 30 minutes before the neutral context exploration.

Experimental mice in which the DMS cFC ensemble was reactivated and the dHp to RSC projection silenced showed a significant increase in their freezing levels compared to controls (Figure 2.10). Thus, specific silencing of hippocampal neurons projecting to the RSC was sufficient to reinstate the DMS-induced freezing in the neutral context after extinction. This result specified directionality to the previously described circuit for behavioral flexibility.

This set of experiments provides evidence that a dedicated hippocampal-cortical network involving the RSC and the dHp is recruited (1) during extinction to change the behavioral output of the DMS ensemble formed during the initial encoding of the fear memory, and (2) after extinction to suppress the freezing response induced by the artificial reactivation of the DMS fear ensemble.

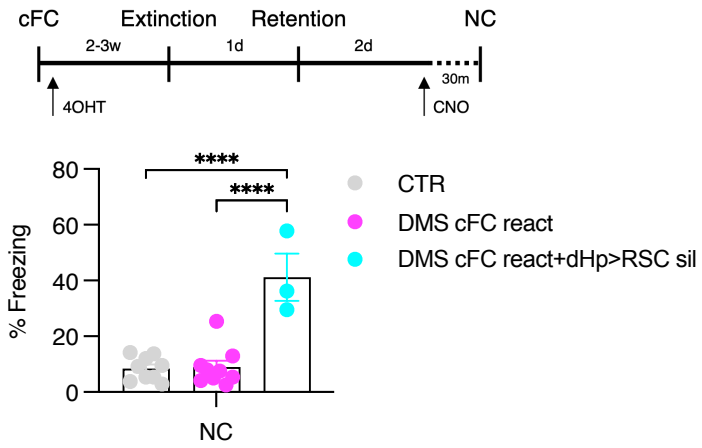

Figure 2.10. dHp to RSC projection silencing. Quantification of freezing levels during the NC exploration after extinction. Chemogenetic silencing of the dHp to RSC projecting neurons reinstates the original DMS freezing behavioral output. [Controls (CTR, grey); DMS cFC ensemble reactivation after extinction (DMS cFC react, magenta); DMS cFC ensemble reactivation and dHp to RSC projection silencing (DMS cFC react+dHp>RSC sil, cyan] Statistical comparisons are performed using a one-way ANOVA followed by Tukey multiple comparison post-hoc tests; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Data are presented as mean ± SEM.

2.4.10 The RSC cFC and extinction ensembles do not differentially recruit striatal D1 and D2-SPNs

What is the mechanism that determines whether the reactivation of a striatal ensemble would result in freezing or not?

At the computational level, it has been predicted that the key to whether an action is selected or suppressed is the relative weighting of cortical input to the D1- and D2-SPNs populations representing that action (Gurney et al., 2015). Given that the RSC strongly projects to the DMS and includes two distinct ensembles associated with the initial fear learning and with its extinction, I hypothesized that the reactivation of these two cortical

ensembles might result in a different pattern of cFos expression in the DMS D1- and D2-SPNs populations. I reasoned that the RSC cFC ensemble might recruit preferentially the D1-SPNs and promote freezing. Conversely, the RSC extinction ensemble might enhance the D2-SPNs activity and suppress freezing.

To test this, I took advantage of two mouse lines expressing the Cre-recombinase specifically in the D1- or D2-SPNs population (D1-cre and A2A-cre, respectively). These mice received intracranial injections with a mix of AAV9-Fos-ERT2-Flp TRAP2 vector and a Flp-dependent, excitatory DREADDs (AAV9-hSyn1-dFRT-hM3Dq-mCherry(rev)-dFRT) in the RSC, and the Cre-dependent Sternson inhibitor virus (rAAV9-CAG-flox-PSAM-GFP) was injected in the DMS. This viral combination allows for tagging and reactivating RSC cFC or extinction ensembles and the visualization of D1- or D2-SPNs in the striatum.

4OHT was injected intraperitoneally right after cFC acquisition or extinction to tag the RSC cFC and extinction ensembles, respectively. Ten days after tagging, CNO was injected intraperitoneally while animals were kept in cage conditions. Two hours later (30 minutes for CNO action plus 90 minutes for cFos optimal expression), mice were perfused, and their brain were collected for quantitative immunohistochemical analysis of DMS cFos expression. Tissue was also collected from naïve home cage animals, which served as a baseline control.

The reactivation of cortical ensembles within the home cage condition ensured that all detected cFos+ cells were due to the experimental manipulation without any behavioral or learning interference.

The quantitative analysis of cFos revealed that chemogenetic reactivation of both RSC cFC and extinction ensembles elicited a comparable increase of

cFos expression in the DMS compared to the home cage controls (Figure 2.11 C).

I then quantified the proportion of cFos+ D1 or D2-SPNs over the total cFos expression. This analysis showed that D1- and D2-SPNs expressed cFos equally when the RSC cFC or extinction ensembles were chemogenetically reactivated (Figure 2.11 D). The two cortical ensembles did not preferentially recruit one striatal population over the other. Thus, this mechanism is not responsible for selecting the DMS final output.

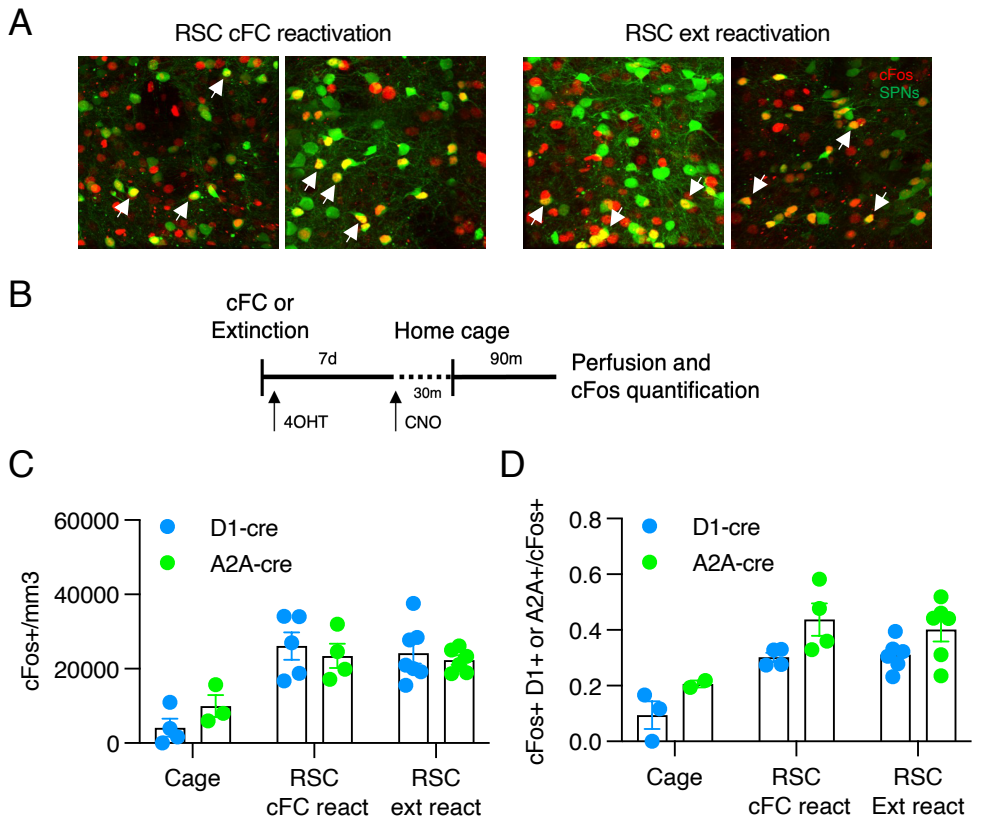

Figure 2.11. The RSC cFC and extinction ensembles do not differentially recruit D1- or D2-SPNs in the DMS. (A) Representative image of striatal SPNs (green) and cFos+ cells (red) upon RSC cFC ensemble reactivation (left) or RSC extinction ensemble reactivation (right). **(B)** Experimental timeline.

(C) Quantification of DMS cFos+ cells in D1-cre and A2A-cre mice 90 minutes after chemogenetic reactivation of the RSC cFC or extinction ensembles. **(D)** Quantification of double positive cells (cFos+ and SNP+) in D1-cre and A2A-cre mice. [Direct pathway SPNs (D1-cre, blue); Indirect pathway SPNs (A2A-cre, green)] Statistical comparisons are performed using multiple unpaired t-tests.

2.5 Memory erasure prevents the return of the DMS freezing output after fear recovery

Fear memory can be permanently erased, resulting in the absence of spontaneous recovery. If the fear memory is recalled and extinction takes place within the plasticity (reconsolidation) window initiated by the recall, the initial fear memory can be permanently updated with the new information and, consequently, mice do not show any freezing response when re-exposed to the conditioning context two weeks after retention of extinction (Kindt et al., 2009; Quirk et al., 2010). I used this behavioral paradigm to study whether this altered extinction would permanently modify the DMS fear ensemble output.

For this experiment, TRAP2 mice received DMS intracranial injections with a flexed, excitatory DREADD (AAV9-hSyn-DIO-hM3Dq-mCherry). To target the neuronal ensemble active during fear memory encoding, mice received intraperitoneal 4OHT injection immediately after cFC acquisition. The next day, mice underwent two extinction sessions (10+20 minutes) separated by a gap of three hours. This protocol resulted in the absence of spontaneous fear recovery, tested 14 days after retention. Two days after fear

recovery, CNO was administered 30 minutes before the start of neutral context exploration to reactivate the DMS fear ensemble's cells.

Experimental mice showed no significant difference from their respective controls (Figure 2.12). This indicated that behavioral erasure permanently modifies the output of the DMS fear ensemble during separate extinction, which cannot induce a freezing response when chemogenetically reactivated in the neutral context after fear recovery.

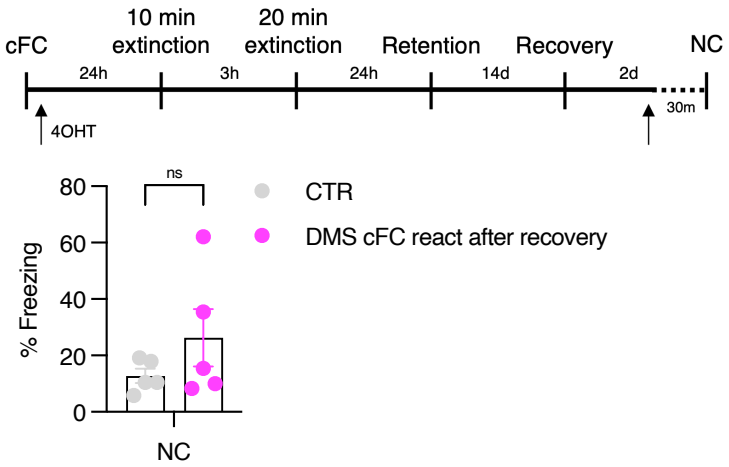

Figure 2.12. Behavioral erasure prevents the return of DMS-induced freezing after spontaneous recovery of fear. Quantification of freezing levels during the NC exploration after fear recovery. Chemogenetic reactivation of the original DMS cFC ensemble does not induce freezing in NC after behavioral erasure. [Controls (CTR, grey); DMS cFC ensemble reactivation (DMS cFC react after recovery, magenta)] Statistical comparisons are performed using a one-way ANOVA followed by Tukey multiple comparison post-hoc tests; ns: not significant.

Chapter 3

Discussion

The main goal of my PhD thesis work has been to gain new insight into the contribution of striatal ensembles to behavioral flexibility. Even though the dorsomedial striatum (DMS) has been widely implicated in flexible learning, a precise characterization of how associations are encoded and updated is still unknown.

The results presented in this thesis provide evidence that a functionally relevant neuronal ensemble is formed in the DMS upon fear learning. This ensemble presents all the classical features of a memory ensemble, and it is specifically recruited when memory needs to be updated during extinction learning. In contrast to cortical and hippocampal areas wherein primary and secondary learnings are encoded and stored in distinct ensembles, the fear ensemble in the DMS shows unique characteristics and specialized cortical-hippocampal networks might orchestrate its activity and behavioral output, allowing alternative stimulus-outcome associations to be learned during extinction.

3.1 Fear learning forms a memory ensemble in the DMS

In the first part of my thesis, I use chemogenetic tools to demonstrate that fear learning induces the formation of a cFos+ ensemble in the DMS, required specifically for behavioral flexibility during extinction (Figures 2.1 and 2.2). Even though the DMS is well-known to play a fundamental role in facilitating flexible choice selection under changing conditions (Balleine et al., 2007; Balleine & O'Doherty, 2010; Balleine et al., 2021; Serrano et al., 2022), it is remarkable that silencing of a specific subset of neurons is sufficient to recapitulate perturbations of the entire brain area.

After cFC acquisition, the reactivation of the DMS fear ensemble is sufficient to elicit the conditioned response, freezing, in a neutral context. This effect is specific to the associative domain of the striatum and depends on its canonical downstream station, the SNr (Figure 2.3 A).

Thus, the DMS fear ensemble exhibits all the characteristic features of a memory engram: it is formed as a consequence of the learning experience, it can result in the recall of the original memory when chemogenetically reactivated in the absence of conditioned stimuli, and it is required to allow subsequent modification of the initial association. Without the activity of this striatal ensemble, animals can recall the original memory but cannot update it to new environmental contingencies.

This set of experiments has two main implications. Given that the immediate early genes (IEGs) c-fos and the c-Fos protein have long been known as markers of neuronal activity (Chung, 2015; Hudson, 2018; Joo et al., 2016), this result suggests that the formation of a neuronal ensemble in an inhibitory

structure might be similar to other excitatory areas and be dependent on neuronal activity at the time of learning.
Second, it strongly suggests that even though the DMS is not necessary for the acquisition or recall of the fear memory and silencing of this brain area does not affect these behavioral epochs (Serrano et al., 2022) (Olga Matveeva, unpublished), a dedicated learning ensemble is nevertheless formed in the DMS during the initial memory encoding. A possible explanation for this phenomenon is that fear learning is acquired and stored in distributed networks (Roy et al., 2022). Therefore, the DMS fear ensemble might have a redundant function during cFC acquisition and recall, while it needs to be specifically accessed and modified during extinction.

Why is the DMS fear ensemble so important for behavioral flexibility during extinction? Given its anatomical organization, the DMS is the striatal domain wherein many cortical and thalamic inputs converge with midbrain dopamine and might represent the perfect environment for combining old and new learning. Thus, the DMS might act as a mismatch detector during extinction, recognizing the absence of unconditioned stimuli and initiating the formation of the new inhibitory memory. Another possibility is that extinction learning is computed upstream at the level of cortical areas. The activity in specific cortical ensembles might be reinforced in the DMS, resulting in the selection of the appropriate behavioral response.

3.2 The role of DMS ensembles in fear learning

To deepen the investigation of the role of the DMS fear ensemble in behavioral flexibility, I performed a gain-of-function set of experiments and reactivated this cells subset throughout the different stages of the fear paradigm. These findings revealed that the DMS fear ensemble elicits the conditioned response, freezing, in a neutral context (Figure 2.3 A). Conversely, when the original fear memory has been successfully extinguished, the reactivation of the DMS fear ensemble does not elicit any freezing response in a neutral context (Figure 2.3 B). This indicates that extinction learning changes the "freezing" output initially encoded in the DMS fear ensemble. Spontaneous fear recovery is sufficient to return the original DMS freezing output two weeks after extinction (Figure 2.4).

Does extinction result from a temporal suppression or modification of the original fear ensemble? How does the learning ensemble induce the initial freezing response after spontaneous recovery? Does the fear recovery recruit the same network associated with the initial learning? All these questions highlight the possibility that the DMS ensemble formed during the cFC acquisition might encode for the same behavioral output, freezing, and the execution or suppression of this conditioned response depends on whether the original memory is extinguished or recovered.

The data presented here support this idea. Indeed, the DMS ensembles associated with the initial learning and extinction exhibit the same freezing output when reactivated after fear recovery (Figure 2.5). In addition, in the DMS, extinction is not associated with the formation of a new neuronal ensemble: memory recall and extinction reactivate the same proportion of cells within the original DMS fear ensemble. Conversely, in the retrosplenial

cortex, memory recall highly reactivates the original fear ensemble, whereas extinction is associated with a new cFos+ population (Figure 2.6).

These findings are interesting and unexpected because they strongly imply that primary and secondary learning are processed differently in cortical and striatal neuronal ensembles.

Within the cortex, cFC acquisition and extinction are treated as separate and distinct learning processes encoded in two different ensembles. Thus, cortical areas might contain details and precise representations of the two learning experiences that can be used to compare the initial learning with the current experience and adapt to new contingencies during extinction. Conversely, the DMS could work as a point of convergence and integration of cortical inputs and serve as a low-dimensional learning system having the pivotal role of selecting and reinforcing relevant dynamics in the cortex (Athalye et al., 2020; Marton et al., 2020). Notably, this idea would be supported by the intrinsic anatomical organization of these two brain regions. In the cortex, the systematic arrangement of inputs, outputs, and interneurons facilitates the processing of complex representations. In contrast, the striatum is arranged in re-entrant loops in which the activity of striatal SPNs is driven by the combination of specific cortical, thalamic, and dopaminergic inputs and could be responsible for the final selection of the appropriate behavioral response.

3.3 The dHp-RSC-DMS network for behavioral flexibility

In the last part of my thesis, I combine gain-of-function experiments with silencing specific nodes to identify and functionally describe a dedicated hippocampal-cortical network recruited during extinction to modify the original DMS fear ensemble.

After successful extinction learning, activity within this network, involving the dorsal hippocampus (dHp) and the retrosplenial cortex (RSC) actively suppresses the freezing response induced by the chemogenetic reactivation of the DMS fear ensemble in a neutral context (Figures 2.7 and 2.8).

This set of experiments indicates that this network is specifically recruited during extinction and possibly not essential for fear learning and its recall. Therefore, the initial DMS ensemble is likely to be functionally linked to a different learning network. A possible candidate for this function could be the classical fear circuit, involving the ventral subdivision of the hippocampus (vHp), BLA and periaqueductal gray (PAG). The switches between these two learning systems in recruiting the initial fear ensemble in the striatum might account for the behavioral adjustments observed after the extinction and fear recovery.

Finally, I ask how the RSC-dHp extinction network influences the DMS freezing output and tested the hypothesis that different cortical ensembles could modulate the activity of the striatal SPNs, resulting in the enhancement or inhibition of the conditioned response if the direct or indirect pathway is promoted, respectively. I show that cFC and extinction ensembles in the RSC do not differentially recruit the two striatal subpopulations (Figure 2.9). However, this experiment does not rule out the possibility that the same

striatal neurons are recruited upon cFC and extinction. Therefore, it would be extremely interesting to investigate the calcium dynamics of the striatal ensemble formed during the initial memory encoding and follow its activity patterns during extinction and fear recovery. Inhibiting specific brain nodes would allow the identification and evaluation of the mechanisms underlying the cortical-driven selection of striatal output.

In conclusion, the findings of this thesis work characterize a previously undescribed fear memory ensemble in the DMS. This ensemble is formed during cFC acquisition and is specifically necessary during extinction, when a dedicated cortico-hippocampal network is recruited to modify its behavioral output and allow flexible learning.
Understanding the role of memory ensembles formed within the inhibitory neural circuits of the striatum, distinct from all the other memory centres of the brain, would offer key insights into how the brain achieves the formation of complex, long-lasting and yet flexible memories by using the distributed and functionally delegated network to implement behavioral flexibility.

Chapter 4

Materials and Methods

4.1 Mice

The TRAP2 mouse line ($Fos^{tm2.1(icre/ERT2)Luo/J}$) was obtained from the Jackson laboratory (JAX stock #030323); the wild-type mice (C57Bl6/J) were sourced from Janvier. Before the onset of behavioral experiments, mice were single-housed for one to three days and had access to water and food *ad libitum*. All animal procedures were approved and performed in accordance with the Veterinary Department of the Kanton Basel-Stadt.

4.2 Behavioral procedures

All behavioral experiments were carried out using mice aged between two and three months at the onset of the experiment. Male and female mice were assigned randomly to the experimental groups.

4.2.1 Contextual fear conditioning (cFC)

Contextual fear conditioning experiments were performed as described in (Donato et al., 2013). During the cFC acquisition session (total time: 5 minutes), mice were first allowed to explore the training context scented with acetic acid for 3 minutes (Habitest Unit, Coulbourn Instruments, Allentown, PA), and subsequently received 5 footshocks (1s and 0.8mA each, inter-trial interval: 30s). Contextual fear memory recall was tested by returning mice to the same conditioning chamber for 5 minutes and analysing freezing (defined as the suppression of all visible movement except that required for respiration) during a test period of 4 minutes (the first minute was excluded). For the extinction session, mice were exposed to the conditioning chamber for 30 minutes without shocks, 24 hours after cFC acquisition. The freezing response was analysed in 6 consecutive bins of 4 (for the first) or 5 minutes each. The retention of extinction was tested 24 hours after the extinction session, returning mice to the conditioning chamber for 5 minutes.

Behavioral erasure was done 24 hours after cFC acquisition by combining 10 minutes of recall in the training context with 20 minutes of exposure to the same context, which followed 3 hours after the initial recall.

Spontaneous fear recovery was tested by returning mice to the conditioning chamber for 5 minutes 14 days after retention of extinction or after the erasure protocol. The freezing response was estimated as described above.

4.2.2 Neutral context exploration

Assessment of fear generalization was tested 48 hours after the behavior of interest by exposing mice to a novel and neutral context (NC) for 5 minutes and analysing freezing during a test period of 4 minutes (the first minute was

excluded). The neutral context consisted of a white round-shaped chamber scented with Fugaten. To ensure the neutrality of the context, experiments were carried out in a behavioral room different from the one in which mice were fear conditioned.

4.3 Stereotaxic surgery

All stereotaxic surgeries were conducted under aseptic conditions using a Stereotaxic alignment system (David Kopf instruments). Half an hour before the initiation of surgery, Buprenorphine (Temgesic) (0.1 mg/kg) was applied subcutaneously as pre-emptive analgesia. Mice were anaesthetized with isoflurane (4% for induction, 1.5-2.0% during the rest of the procedure using air oxygen as a carrier gas (OXYMAT3), while body temperature was maintained with a heating pad.

After induction of anaesthesia and fixation in the stereotactic frame, mice received subcutaneous injection with a 1:1 mixture of Lidocaine (10 mg/kg) and Ropivacaine (3 mg/kg; Naropin, Astra Zeneca) around the surgery area to reduce post-operative pain. In addition, after the surgical procedure, Buprenorphine (0.1 mg/kg) was again applied subcutaneously on the day of surgery (with a break not exceeding 4-6 hours). Meloxicam (Metacam, 5 mg/kg) was given on the day of surgery when the animals were awake to ensure analgesia overnight, and again on the next two days at an interval of 24 hours.

Virus injections were carried out using glass pipettes (tip diameter 10-20 μm) connected to a picospritzer (Parker Hannifin Corporation). The glass pipette

was inserted at the desired coordinate, and viral suspensions were slowly delivered to a final volume of 100-200 nl per injection site. After the end of the injection, the pipette was left in place for a further 10-15 minutes to allow for diffusion of the virus and avoid backflow. For behavioral experiments, all viruses were injected bilaterally. Mice were left to recover for 7-10 days, which also allowed the virus to fully express in neuronal cells. Post-surgical recovery was monitored daily until the start of the behavioral protocols.

The **coordinates** used were as follows, relative to bregma (mm):

Dorsomedial striatum (DMS): AP +0.16; ML ±1.64; DV -2.4

Dorsolateral striatum (DLS): AP +0.2; ML ±2.42; DV -3.2

Substantia nigra pars reticulate (SNr): AP -3; ML ±1.4; DV -4.6

Retrosplenial cortex (RSC): AP -1.9 and -2.9; ML ±0.36; DV -0.95

Dorsal hippocampus (dHp): AP -1.7; ML ±1.65; DV -1.7 and -2.1

Ventral hippocampus (vHp): AP -3; ML ±3.2; DV -3.2 and -3.7

4.4 Genetic targeting of active neuronal populations

The Fos2A-iCreER mouse line was used for the selective manipulation of memory ensembles using the TRAP2 system (Targeted Recombination in Active Populations 2) (DeNardo et al., 2019). To label cFos-expressing neurons, 4-Hydroxytamoxifen (50 mg/kg in sunflower oil, Sigma Adrich) was injected intraperitoneally immediately after the behavioral session of interest. Mice were kept under control conditions for at least 7 days to allow for the expression of the construct.

4.5 Chemogenetic and viral vectors

For reactivation of cFos+ neuronal ensembles in TRAP2 mice, a flexed excitatory DREADD (AAV(9)-hSyn1-dlox-hM3D(Gq)_mCherry(rev)-dlox-WPRE-hGHp(A), Viral Vector Facility, Zurich) was delivered bilaterally in the brain area of interest.

For silencing of DMS cFos+ neuronal ensembles in wild-type mice, a TRAP2 vector (AAV(9)-Fos-ER^{T2}-Cre, Viral Vector Facility, Zurich) and a flexed, inhibitory DREADD (AAV(9)-hSyn1-dlox-hM3D(Gq)_mCherry(rev)-dlox-WPRE-hGHp(A), Viral Vector Facility, Zurich) were delivered bilaterally in the DMS.

For acute silencing experiments of SNr, RSC, dHp, and vHp, a flexed, inhibitory DREADD (AAV(9)-AAV-hSyn-DIO-hM4D(Gi)-mCherry, Viral Vector Facility, Zurich) was delivered bilaterally in combination with a CaMK2a promoter driven Cre-delivering (AAV(9)-CaMK2a-mCherry-Cre, Viral Vector Facility, Zurich).

For acute silencing of dHp during extinction in TRAP2 mice, a floxed, PSAM-dependent inhibitory channel (rAAV(9)-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE, Viral vector facility, Zurich) was delivered bilaterally in combination with a CaMK2a promoter driven Cre-delivering (AAV(9)-CaMK2a-mCherry-Cre, Viral Vector Facility, Zurich).

For silencing of projections from RSC to dHp in TRAP2 mice, a Flp-delivery retrograde tracer under the Camk2a promoter (rAAV-pCAG-FLPo-2A-H2B-V5-WPRE, a kind gift from the lab of Silvia Arber) was delivered bilaterally in the RSC, and a Flp-dependent inhibitory DREADDs (AAV(9)-hSyn1-dFRT-hM4Di-mCherry(rev)-dFRT-WPRE-hGHp(A), Viral Vector Facility,

Zurich) was delivered bilaterally in the dHp. For this experiment, mice were kept under control conditions for at least 14 days to allow for the expression of the construct.

For DMS and RSC cFos overlap experiments in wild-type mice, a TRAP2 vector (AAV(9)-Fos-ER^{T2}-Cre, Viral Vector Facility, Zurich) was delivered bilaterally in combination with a floxed, PSAM-dependent inhibitory channel (rAAV(9)-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE, Viral Vector Facility, Zurich) for GFP expression only.

For reactivation of RSC cFC and extinction ensembles in D1-cre and A2A-cre mouse lines, a TRAP2 vector (AAV(9)-Fos-ER^{T2}-Cre, Viral Vector Facility, Zurich) was delivered bilaterally in combination with a Flp-dependent excitatory DREADDs (AAV(9)-hSyn1-dFRT-hM3Dq-mCherry(rev)-dFRT-WPRE-hGHp(A), Viral Vector Facility, Zurich). A floxed, PSAM-dependent inhibitory channel (rAAV(9)-CBA-flox-PSAM(Leu141Phe,Tyr116Phe)GlyR-WPRE, Viral Vector Facility, Zurich) was delivered bilaterally in the DMS to visualized D1 and A2A cre-positive cells.

To allow for transgene expression, mice were kept under home cage conditions for 7-10 days before any behavioral experiment.

To chemogenetically activate the DREADD channels, Clozapine N-Oxide (Tocris) was injected intraperitoneally (5 mg/kg) 30 minutes before behavioral testing (Roth, 2016; Zhang et al., 2022; Zhu & Roth, 2014). To chemogenetically activate the PSAM channels, the PSAM agonist, PSEM308 (Apex Scientific) was injected intraperitoneally (5 mg/kg) 15-20 minutes before behavioral testing (Magnus et al., 2011).

4.6 Immunohistochemistry and image processing

Antibodies were used: Rabbit anti-cFos (Synaptic Systems) 1:1000; Goat anti-Rabbit Alexa Fluor 647 (Thermo Fisher Scientific) 1:1000.

For the immunohistochemical analysis, 90 minutes post behavior mice were transcardially perfused with 4% paraformaldehyde (PFA) in PBS (pH 7.4); brains were collected, kept overnight in 4% PFA at 4°C, and subsequently, 40 μm thick coronal sections were cut using a vibratome.

The standard procedure for immunostainings was as follows: brain sections were blocked for one hour at room temperature with 10% BSA in PBS-T (0.3% Triton X-100 in PBS). Incubation in primary antibody was done overnight in the antibody solution containing 3% BSA and 0.3% PBS-T. After three washing steps, sections were incubated in a secondary antibody solution (3% BSA and 0.3% PBS-T) at room temperature for two hours. After another three washing steps with PBS, sections were mounted in Prolong Gold antifade reagent (Molecular probes) and kept at 4°C until imaging.

Images for the analyses were acquired at 40x (objective EC Plan-Neofluar 40x/1,30 Oil DIC M27) using a spinning-disk confocal microscope (Axio Imager M2 upright microscope + Yokogawa CSU W1 Dual camera T2 spinning disk confocal scanning unit) using Visiview 4.4.0.18. All the samples belonging to one experimental set were processed in parallel and using the same imaging settings. Analysis was performed using the Imaris 9.0 software; all immunopositive cells were quantified using automatic spot detection (expected radius, 10 μm). cFos induction was quantified as a fraction of cFos-positive cells over the volume of the analysed brain area (mm^3).

4.7 Quantification and statistical analysis

All statistical analyses were based on two-tailed comparisons and were done using GraphPad Prism (GraphPad Software Inc.). Results are presented as Mean ± Standard Error of the Mean (SEM). Significant values for: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Experimental mice were always compared to CNO-treated, uninfected virus controls.

The number of animals to be used for a standard behavioral analysis was determined based on preliminary behavioral experiments.

Chapter 5

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EDUCATION

PhD – Neuroscience **Basel, Jul. 2019 – Dec. 2023**
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Thesis: "A dedicated cortico-hippocampal network changes the behavioral output of learning ensembles in the striatum."

MSc – Neurobiology **Rome, Sep. 2016 – Mar. 2019**
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Thesis: "Time course of cortico-limbic catecholamines during the exposure to a conditioned stimulus."

BSc – Biology **Rome, Sep. 2012 – Feb. 2016**
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Thesis: "The role of estrogens in the Central nervous system: neurotropism and neuroprotection."

RESEARCH EXPERIENCE

PhD Research **Basel, Jul. 2019 – Dec. 2023**
Friedrich Miescher Institute for Biomedical Research (FMI), Basel

- Independently designed, conducted, and interpreted complex *in vivo* scientific projects
- Implemented new assays or optimize existing protocols based on literature search to meet the needs of my project
- Stereotaxic surgery (AAVs-mediated chemogenetic, anterograde and retrograde tracing, Rabies virus-based retrograde tracing)
- Good knowledge of microscopy techniques and image analysis
- *Ex vivo* gene and protein analysis (DNA isolation, qRT-PCR, immunostainings)
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MSc Intern **Rome, Sep. 2017 – Mar. 2019**
European Center for Brain Research (CERC), Rome

- Performed *in vivo* micro-dialysis
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- Synthesized, characterized and stabilized gold nanoparticles for biomedical research

CERTIFICATIONS

Good Clinical Practice (GCP) – Swiss Tropical and Public Health Institute (Swiss TPH)
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