Excitatory and inhibitory ensembles of the dorsal medial prefrontal cortex during the strengthening and weakening of an appetitive association

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I declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree. The work in this thesis is entirely my own except where due acknowledgement was made.

Signature: .....

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Abstract

Animals associate relevant signals or 'cues' to food in order to efficiently gather nutrients. Sparse sets of neurons called 'neuronal ensembles' in the dorsal medial prefrontal cortex (dmPFC) play a vital role in associative memory formation. However, how these neurons are recruited into an ensemble to establish appetitive 'food-cue' associations during conditioning remains unclear.

The aims of this study were twofold: 1) examine the recruitment of both pyramidal cell and interneuron ensembles during the establishment and extinction of appetitive associations in the dmPFC and 2) investigate their function in appetitive conditioning.

Here, we took advantage of a microprism-based 2-photon imaging procedure to longitudinally image the dmPFC of *Fos-GFP x GAD-tdTomato* transgenic mice *in vivo.* These mice express GFP in strongly activated (Fos expressing) neurons and tdTomato in interneurons, which allowed us to track recently activated pyramidal cells and interneurons over conditioning and extinction. During conditioning, in which behavioural responding became cue-selective, a stable, repeatedly activated neuronal ensemble was recruited from a pyramidal cell pool activated during early learning. Furthermore, repeatedly enhancing the excitability of the initial learning activated pool with chemogenetics throughout training disrupted appetitive learning. In contrast, during extinction, a stable inhibitory ensemble emerged from interneurons activated in early extinction learning.

These novel findings reveal ensemble recruitment patterns occurring in the dmPFC during alterations in the strength of food-cue associations.

Abbreviations:

2P: 2-photon

AAV: adeno-associated virus

ACC: anterior cingulate cortex

BLA: basolateral amygdala

Cloz: clozapine

CNO: clozapine-N-oxide

CR: conditioned response

CREB: cAMP response element-binding protein

CS: conditioned stimulus

dmPFC: dorsal medial prefrontal cortex

Dox: Doxycycline

DREADD: designer receptors exclusively activated by designer drugs

DS: dorsal striatum

E1: extinction session 1

FGGT: Fos-GFP x GAD-tdTomato

GABA: gamma-aminobutyric acid

GAD: glutamic acid decarboxylase

GFP: green fluorescent protein

HC: home cage

IEG: immediate early gene

IL: infra limbic cortex

ITI: inter-trial interval

KCNQ: voltage-gated potassium channel

MAPK: mitogen-activated protein kinase

ERK: extracellular signal-regulated kinases

mPFC: medial prefrontal cortex

NAc: nucleus accumbens

NC: novel context

P: paired

PFC: prefrontal cortex

PL: prelimbic cortex

PLC: phospholipase C

PV: parvalbumin

RFI: relative fluorescence intensity

S1: conditioning session 1

Sal: saline

SD: standard deviation

SEM: standard error of the mean

SOM: somatostatin

SOP: sometimes operant process

TRE: tetracycline response element

tTa: tetracycline transactivator

UP: unpaired

UR: unconditioned response

US: unconditioned stimulus

VIP: vaso-intestinal protein

VTA: ventral tegmental area

WT: wild-type

Table of contents:

Chapter 1: General Introduction

The primary aim of the work described in this thesis was to investigate how the activation of excitatory and inhibitory neuronal populations of the dorsal medial prefrontal cortex (dmPFC) contributes to appetitive learning. More specifically, our aim was to examine how these different populations are involved in strengthening and weakening associations between food and environmental signals that predict food availability. In this introductory chapter, we will first provide a brief overview of the field of Pavlovian conditioning and extinction learning as well as the critical brain structures that mediate this type of learning with a focus on the medial prefrontal cortex. Finally, we will discuss the concept of neuronal ensembles, the use of the protein 'Fos' as a marker of activation and how these neuronal ensembles may contribute to learnt behaviours.

1.1 Appetitive conditioning and extinction

Humans and non-human animals live in dynamic, constantly changing environments, in which the availability of food or water and the presence of predators and environmental toxins can be rapidly altered. An important factor in our survival is our ability to adapt to these changes by modifying our behaviours. Identifying and learning to respond to signals, or 'cues', that predict desirable (e.g. food, mate, social bonding) and undesirable (e.g. predators, toxins) outcomes is especially crucial to maximise fitness in our environment. Such learning has been observed across the animal kingdom ranging from invertebrates to vertebrates (e.g., Aplysia (Walters et al., 1981), honey bees (Kuwabara, 1957; Takeda, 1961), dogs (Pavlov (1927), 2010) and humans (Watson and Rayner, 1920) among many others). Just as crucial, however, is the ability to rapidly and flexibly adapt when these same stimuli stop predicting these outcomes. For example, if a bird feeder is regularly filled, a bird may learn to expect to find food there and visit it often.

However, if the bird feeder is not replenished, the bird will stop searching for food in that location. Through this 'appetitive learning' process in which there is a strengthening and weakening of associations between food availability and its predictive cues, animals are able to efficiently gather nutrients while minimising expended energy (MacArthur and Pianka, 1966). The former and latter forms of learning in which the strength of learned associations is modulated are known as appetitive conditioning and extinction learning, respectively.

1.1.1 A brief history of Pavlovian conditioning

Understanding how we and non-human animals learn and recall memories has been a long-standing interest throughout history. While behavioural psychology as we know it now was only truly developed within the last century, other approaches such as Philosophy and Biology have been used to address the question of how and why we learn (Bouton, 2007). Within the field of Biology more specifically, some viewed learning as an extension of the reflexes and searched for 'psychic reflexes' (Bouton, 2007). This approach is of particular interest as it was the one employed by Pavlov and his students, whose observations of associative learning were at first a result of studying digestive reflexes in dogs. They found that repeated pairing of a neutral stimuli and food could trigger salivation in dogs in the absence of food (Pavlov (1927), 2010). Pavlov identified that this experiment and methodology could be used as a tool to study psychological processes in order to eventually identify their physiological underpinnings (Bouton, 2007; Pavlov (1927), 2010). However, to fully study the biological mechanisms responsible for learning, learning itself had to be better understood. Following Pavlov's example, methods and procedures were developed to study associative learning (e.g., Skinner's operant boxes) which have since been refined and built upon. These procedures are now at the core of the field of associative learning (Bouton, 2007).

1.1.2 Defining and modelling associative learning and extinction

The behaviour observed by Pavlov and his students was that the presentation of both a neutral stimulus (conditioned stimulus, CS) and a food reward (unconditioned stimulus, US) could lead to the CS alone triggering a conditioned, physiological preparatory response (salivation). However, following this learning if the CS was repeatedly presented alone without food delivery, it would eventually cease to evoke these conditioned responses. These are the processes of conditioning and extinction respectively (Fanselow and Wassum, 2016; Holland, 1984; Pavlov (1927), 2010; Rescorla, 1988). When Pavlov and his students first observed what later came to be known as Pavlovian (or classical) conditioning, they theorised it as a transfer of behaviours evoked by food (i.e., the unconditioned response, UR) to a previously neutral stimulus which then became a conditioned stimulus (CS) and evoked a conditioned response (CR) (Fanselow and Wassum, 2016; Pavlov (1927), 2010). However, as research into this process progressed, it was made evident that a CS could evoke behaviours that the US would not (Fanselow and Wassum, 2016; Holland, 1984). One crucial example of this is sign tracking, in which animals training to associate a cue to a reward begin to interact with the CS (e.g. sniffing the CS), despite it having no effect on the delivery of the reward (Brown and Jenkins, 1968; Costa and Boakes, 2009; Davey et al., 1981). Thus, a more recent definition of conditioning is: *"the process whereby experience with a conditional relationship between stimuli bestows these stimuli with the ability to promote adaptive behavior patterns that did not occur before the experience*." (Fanselow and Wassum, 2016). This definition elegantly conveys the core principle of associative learning.

Of note, a distinction is often made between the association of a cue with a US (Pavlovian or classical conditioning) and the association of an action with a US (operant or instrumental conditioning; Box 1). However, both learning procedures have components of associative learning and are frequently utilised to examine the neurobiological

mechanisms of processes such as motivation and reward (Fanselow and Wassum, 2016).

There are a number of models that were designed to explain and predict conditioning and extinction (for a summary, refer to Bouton, 2007). Among the most influential is the Rescorla and Wagner (1972) model, which suggests that the change in associative strength between a CS and US will depend on how surprising the US is (Rescorla and Wagner, 1972). In other words, learning will occur if events contradict expected outcomes. This model will generally correctly predict behaviours linked to associative learning and has been extremely valuable to the field of experimental psychology, although it has limitations (Fanselow and Wassum, 2016; Siegel and Allan, 1996). Other models were generated in response to some of these limitations: the Mackintosh (1975) and Pearce-Hall (1980) models, for example, include attention as a factor in the strength of learning (Mackintosh, 1975; Pearce and Hall, 1980). Later, the Wagner (1981) 'Sometimes Opponent Processes' (SOP) model also included timing between CS and US as a component, as the delay between the presentation of a CS and US has been shown to be crucial in associative learning (Wagner, 1981).

Extinction was initially suggested to act directly on the relationship between CS and US in a similar way to conditioning (although with negative rather than positive associative strength); thus, it was thought of as an 'unlearning' of the association. However, it has been shown that extinction does not rewrite the CS-US memory (Bouton, 2004; Fanselow and Wassum, 2016). Instead, extinction is now thought to suppress the CS-US association to account for phenomena such as rapid reacquisition of associations, reinstatement if exposed to the US alone, spontaneous recovery of the association following a length of time or renewal of the association in a different context to that of extinction (Bouton, 2004; Pavlov (1927), 2010). Observations of these phenomena have led to the theory that extinction is a form of new inhibitory learning where a 'CS-no US' memory is established alongside the original CS-US association (Bouton, 2004; Calton

et al., 1996; Rescorla, 1993). Furthermore, experimental evidence has demonstrated that extinction learning is highly reliant on contextual cues and less likely to generalise than the original CS-US association. This reliance on context may be a factor in the renewal of associations in new contexts following extinction (Bouton, 1993; Bouton and Bolles, 1979).

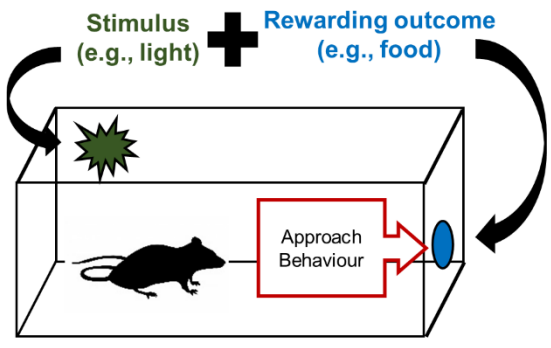
Box 1: Laboratory studies of conditioning (rewarding US)

Here, we provide concrete details of paradigms used in the study of conditioning, in particular, when the US is rewarding. The field of conditioning has divided associative learning into 2 main forms:

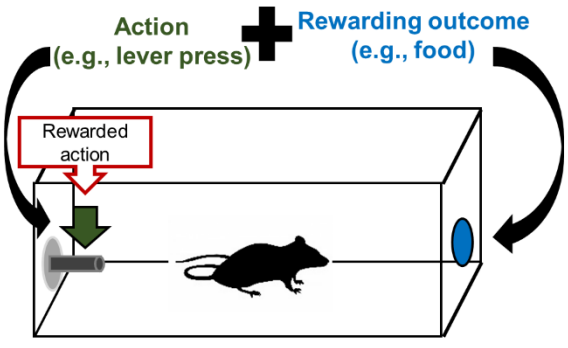
- Pavlovian (or classical) conditioning: In this form of conditioning, an association is established between a stimulus and an outcome (here, rewarding); e.g., a light cue (*Diagram: green*) and food delivery (*Diagram: blue*). This association leads to the CS gaining motivational and emotional value. As such, the presentation of the CS alone can evoke consummatory behaviours (e.g., licking, salivating) or approach behaviours (e.g., interaction with the food delivery location). Experimenters will measure the frequency of these altered behaviours to assess conditioned performance (*Diagram: in red*) (Bouton, 2007). Of note, some paradigms make use of 2 stimuli: a CS+ (rewarded) and a CS– (non-rewarded). These experiments can provide information regarding the discriminative capacities of the animal model studied (e.g., Cardinal et al., 2002).

- Operant (or instrumental) conditioning: In this form of conditioning, an association is established between an action performed by the subject and an outcome (here rewarding), e.g., a lever press (*Diagram: green*) and food delivery (*Diagram: blue*). This association will result in increased frequency of the rewarded action. Experimenters will measure the frequency of these actions in order to assess conditioning performance (*Diagram: red*) (Bouton, 2007).

Of note, these two forms of conditioning can be combined to examine the impact of Pavlovian cues on invigorating an instrumental response required to obtain a reward using the Pavlovian to instrumental transfer (PIT) procedure (Cartoni et al. 2016).



Pavlovian/classical conditioning



Operant/instrumental conditioning

1.1.3 Why study appetitive conditioning?

The drive to understand the behaviour of animals (and through this, human behaviour) has played a part in promoting the study of conditioning mechanisms. Classical conditioning paradigms are a major tool in addressing these questions and better understanding learning and memory. However, learning processes that underly conditioning have also been identified as at the root of a number of behavioural disorders in humans. For example, associations between stimuli and danger that are not properly extinguished can lead to inappropriate responses to seemingly neutral stimuli and are thought to be involved in posttraumatic stress disorder (VanElzakker et al., 2014). Similarly, associations between drugs and cues have been thought to be factors in addiction and relapse following withdrawal (Lynch et al., 1973; O'brien et al., 1992). Thus, a better understanding of conditioned behaviour may serve to inform therapies for maladaptive learning.

The way appetitive associations between food and its predictive cues affect our behaviours is of particular concern as people in the developed world live in a food-cue rich society (e.g., advertising) where high-sugar and high-fat foods are often easily accessible. Furthermore, similar to Pavlovian conditioning, these food-cues are commonly presented in a non-contingent manner. Moreover, exposure to common place food cues such as food-related advertising can lead to increased food consumption (Halford et al., 2004). This in turn leads to extra weight gain and obesity, which are risk factors in the development of numerous debilitating diseases such as cancer and cardio-vascular diseases (Guh et al., 2009). Furthermore, inability to extinguish food-cue associations has been identified as an important factor in cases of failed adjustments of unhealthy eating behaviours (van den Akker et al., 2018). The number of obese adults has been increasing in England since 1993 and is currently at 29% (briefing for the House of Commons (Baker, 2019)). Thus, there is a strong need to better understand how our

environment can influence feeding behaviours when developing therapies and preventative measures (van den Akker et al., 2018; Jansen et al., 2016).

However, looking at behaviour alone does not offer a full understanding of the processes of learning. Behavioural paradigms such as Pavlovian conditioning have been used not only to examine learning behaviours but also as tool in the search for underlying physiological mechanisms of learning. Elucidating these mechanisms may provide insights into possible neurological processes that drive these disorders.

1.2 Appetitive learning and the dorsal medial prefrontal cortex

1.2.1 Searching for the function of brain regions

Understanding what physiological processes may control the mind and how the brain may contribute are questions that have been raised and investigated for millennia (for a review of Neuroscience in Antiquity: Chapter 1 of Finger, 2001). During the late 19th and early 20th century, while Pavlov was characterising associative learning, other researchers were testing the function of the brain; for example, through lesioning sections of the brain and observing how this affected animal behaviour (e.g., Franz, 1902). One of the most famous examples of these ablation-based studies are Lashley's systematic lesions of different cortical areas in rats performing behavioural tasks (Lashley, 1920). While he did not locate an individual area that held the memory trace, he did remark upon the importance of the cortex in these behaviours and suggested a model of distributed processing in memory (Eichenbaum, 2016; Lashley, 1920). In characterising conditioning, Pavlov and his team provided a reproducible behavioural paradigm that examined learning and recall of associative memories. Researchers such as Thompson combined this behavioural paradigm to the systematic brain lesion approach. He and his team used a simple eyeblink conditioning task in rabbits to identify that the cerebellum was necessary for this form of conditioning (Thompson, 1988).

Furthermore, Pavlovian conditioning paradigms were also combined with techniques that measured real-time changes in neuronal activity (e.g., *in vivo* electrophysiology (Quirk et al., 1995)) or metabolic activity (e.g., 2-deoxyglucose intake (Gonzalez-Lima and Scheich, 1986)) within brain regions. Collectively, our understanding of the function of different brain areas in the last century has greatly increased through studies that determined the relationships between brain lesions and brain activity patterns to certain behaviours. Additionally, over the last several decades, various conditioning paradigms have been instrumental in revealing the importance of certain brain regions and the electrochemical activity within them in forming associative memories.

1.2.2 The neural circuitry underlying appetitive behaviours

In order to understand the neural basis of appetitive learning, we need to first understand the critical brain areas that underlie rewarding behaviours such as feeding. Over many decades, studies have identified brain regions that are part of a wider 'reward system'. These areas contribute to increasing the animal's likelihood of performing an action or actions that will increase survival, such as the procurement and consumption of food (Berridge, 2007). One pathway of particular importance to appetitive learning is the mesolimbic dopamine system. Of particular note in this pathway, ventral tegmental area (VTA) dopaminergic neurons project heavily to the nucleus accumbens (NAc). These dopamine connections are thought to be crucial in mediating the motivational value of stimuli such as food (Berridge and Robinson, 1998; Schultz, 1998; Wise, 2004). Accordingly, manipulations to these areas have been shown to affect feeding behaviours (Maldonado-Irizarry et al., 1995; Shimura et al., 2002; Stratford and Kelley, 1997). However, dopaminergic connections have also been shown to be crucial in learning the relationship between environmental stimuli and rewards such as foods. In appetitive learning, there is dopaminergic signalling for the US initially, but as training progresses

and the CS gains motivational value, there is dopamine signalling for the CS (Schultz, 1998).

The VTA also targets cortical areas such as the prefrontal cortex (PFC) as well as the amygdala. These areas have been shown to be involved in the acquisition of food-seeking behaviours. Crucially, lesions to these regions have been observed to disrupt appetitive learning as evidenced by disruptions to the conditioned approach elicited by CS exposure (Balleine et al., 2003; Bussey et al., 1997; Cardinal et al., 2002). Moreover, the medial PFC (mPFC) and the amygdala have been included as key regions in models of the network that mediates appetitive conditioning (Martin-Soelch et al., 2007; Petrovich and Gallagher, 2007). In particular, the amygdala is thought to be involved in assigning emotional and motivational significance to events while the mPFC is thought to be involved in promoting discriminative learning and directing behaviours (Martin-Soelch et al., 2007; Petrovich and Gallagher, 2007).

These and other interconnected cortical and limbic structures serve to drive feeding behaviours, notably those that are guided by food-associated cues. Although many brain areas are involved in cue-controlled behaviours, past and mounting recent evidence implicates the dorsal region of the mPFC as a brain structure that is involved in controlling food seeking. In the next section we will provide our arguments for examining this area in appetitive conditioning.

1.2.3 Anatomy and cytoarchitecture of the dmPFC

The mPFC is a large area composed of multiple subregions. More specifically, the mouse mPFC, as defined by Van De Werd et al., is comprised of the infralimbic (IL), prelimbic (PL), as well as both ventral and dorsal anterior cingulate cortices (ACC) and Frontal area 2 (or medial precentral cortex) (Van De Werd et al., 2010). Furthermore, the mPFC can be divided into dorsal and ventral sections according to anatomical and connectivity

similarities (Heidbreder and Groenewegen, 2003). The dorsal region (dmPFC), which is our area of study, is comprised of the dorsal PL, dorsal ACC and Frontal area 2. Here, the specific area we examined is comprised primarily of the dorsal ACC and part of the PL. Thus, due to similarities in function and connections within the dmPFC (Heidbreder and Groenewegen, 2003) and the scope of our experiment, we will often refer to our area of interest as the dmPFC more generally.

Of note, despite similarities in function of rodent and primate PFC, differences in anatomy, connectivity and organisation lead to some difficulty in comparing evidence from subregions of the PFC from one species to the next (Uylings et al., 2003). Thus, we will be primarily focusing on literature relating to the rodent mPFC.

Like most regions of the cortex, the mPFC has multiple cortical layers; however, the rodent dmPFC does not have a layer IV, which is traditionally targeted by outside inputs (Van De Werd et al., 2010). Instead, afferents arrive to and efferents leave from both superficial and deep layers of the mPFC (Riga et al., 2014). The dmPFC like other cortical areas is mainly composed of pyramidal cells that send excitatory signals both locally and to other cortical and subcortical regions (DeFelipe et al., 2002). However, 10-20% of the neurons are GABAergic interneurons which signal with the inhibitory transmitter gamma-aminobutyric acid (GABA) (Beaulieu, 1993; DeFelipe et al., 2002). Cortical interneurons primarily project locally (Kepecs and Fishell, 2014), although there is some evidence of GABAergic mPFC neurons having long-range connections (Lee et al., 2014).

Interneurons can be further subdivided to according to their firing properties and specific molecular markers that correlate with these different subtypes have been identified (Cauli et al., 1997; Kawaguchi, 1995; Kawaguchi and Kubota, 1993; Kepecs and Fishell, 2014). Thus, in the mouse cortex, the majority of interneurons either express Parvalbumin (PV), Somatostatin (SOM) or the Vaso-intestinal protein (VIP) (Rudy et al., 2011). More specifically, in layers 2/3 of the cortex which we will focus on in this study, each of these

populations represent approximately 20-30 % of the total GABAergic population (Rudy et al., 2011).

PV-expressing interneurons are generally fast-spiking (Kawaguchi, 1995; Kawaguchi and Kubota, 1993; Kepecs and Fishell, 2014) and usually synapse on the soma or axon initial segment of target neurons (Kawaguchi and Kubota, 1997; Kepecs and Fishell, 2014). Furthermore, they are thought to act synchronously due to high interconnectivity as well as gap junctions (Galarreta and Hestrin, 1999) and have a role in regulating oscillations within the brain (Cardin et al., 2009; Kim et al., 2016b). SOM-expressing interneurons will regulate the flow of information differently, often by synapsing on dendrites of target neurons (Kawaguchi and Kubota, 1997; Kepecs and Fishell, 2014). Thus, PV-expressing interneurons are thought to control the output of pyramidal cells while SOM-expressing interneurons control the input (Kvitsiani et al., 2013). Finally, VIP-expressing neurons have been shown to target other interneuron subtypes and through this, have a role in modulating inhibitory signal to excitatory neurons (Pfeffer et al., 2013).

1.2.4 The dmPFC in appetitive conditioning

The dmPFC is an interconnected area that is thought to have a number of complex functions which rely on receiving and processing information as well as directing behaviour accordingly (Dalley et al., 2004). Of particular interest to our study, the dmPFC has been shown to be involved in a number of different food-related behaviours, including feeding and food-driven foraging and exploration (Gaykema et al., 2014; Kvitsiani et al., 2013; Petykó et al., 2009; Seamans et al., 1995). However, this area also contributes to learnt food-seeking behaviours. In particular, it is thought to be crucial in promoting the expression of learnt food-related behaviours following associative learning (Calu et al., 2013; Moorman et al., 2015; Whitaker et al., 2017). Furthermore, there is evidence to suggest the dmPFC is also involved when appetitive associations (both

Pavlovian and operant) are being learnt (Baldwin et al., 2000; Otis et al., 2017). Moreover, the dmPFC has been observed to have a role in enabling cue-discrimination in associative learning as, in conditioning tasks presenting both a rewarded (CS+) and non-rewarded (CS-) cue, lesions to this area lead to decreased response specificity (Bussey et al., 1997; Cardinal et al., 2002). Finally, while a paucity of data exists regarding the role of the dmPFC in extinction, its neurons have been shown to signal during the extinction of reward seeking (Moorman and Aston-Jones, 2015) and PV activity in this area is thought to contribute to extinction of appetitive Pavlovian associations (Sparta et al., 2014).

Beyond the scope of functions that have been directly linked to appetitive or associative learning, the dmPFC has been shown to have a number of other roles. Most notably, the mPFC is well-established as being involved in mediating attention (Dalley et al., 2004). In particular, the dmPFC has been theorised to be involved in directing attention to relevant stimuli (Sharpe and Killcross, 2014, 2015), possibly through connections to sensory cortices (Zhang et al., 2014). Furthermore, the dmPFC is thought to play a role in detecting prediction error events (Bryden et al., 2011; Hyman et al., 2017; Totah et al., 2009), promoting alertness (Bryden et al., 2011; Totah et al., 2009; Wu et al., 2017) as well as allowing for flexible behaviour following changes in the environment (Karlsson et al., 2012; Seamans et al., 1995). Moreover, previous studies have suggested a role for the dmPFC in working memory (Kesner et al., 1996; Kim et al., 2016a) and action sequencing (Ostlund et al., 2009).

Thus, the dmPFC has been shown to be critical to a number of complex behaviours, including associative learning and food-seeking. Although some of the theorised functions of the dmPFC may seem only tangentially involved in appetitive learning, they are worth considering as they may still be intertwined with the role the dmPFC plays in the acquisition, recall and extinction of appetitive associations.

1.2.5 Connectivity of the dmPFC

In order to mediate these different functions, the dmPFC is connected to a large network of areas. For the sake of clarity, we will be mainly focusing on connections to a few areas that have been shown to be relevant to appetitive conditioning but it is worth keeping in mind that this list is not exhaustive (Fig. 1). The dmPFC send efferent and receives afferent projections from other mPFC regions. These projections tend to be preferentially horizontal with limited dorso-ventral connections (Condé et al., 1995; Fillinger et al., 2017, 2018). It also projects to and receives inputs from sensory areas, in particular visual areas and secondary sensory cortices (Condé et al., 1995; Fillinger et al., 2017, 2018) and has a strong reciprocal connection to the secondary motor cortex (Fillinger et al., 2017, 2018). Through this the dmPFC may exert top-down control over these areas (Zhang et al., 2014).

The dmPFC is also known to be reciprocally connected to the orbital frontal cortex (OFC) (Fillinger et al., 2017, 2018) which is thought to be involved in mediating goal-directed behaviours and processing reward-related information (Furuyashiki and Gallagher, 2007). The OFC may also be sending on information from the gustatory cortex during appetitive learning (Carleton et al., 2010).

Furthermore, although the amygdala is more robustly connected with the ventral mPFC, the dmPFC is known to have reciprocal connections with the basolateral nucleus of the amygdala (BLA) (Condé et al., 1995; Fillinger et al., 2017, 2018). These connections are thought to be involved in learning, in particular in mediating the valence of stimuli (Likhtik and Paz, 2015). Furthermore, the BLA more generally has been shown to be involved in processing changes in reward contingencies, for example in extinction of appetitive associations (Balleine et al., 2003; Burns et al., 1999).

The dmPFC also connects to different areas of the striatum; both to the dorsal striatum which is thought to be involved in learning (Cole et al., 2017), and more ventrally, to the

NAc core (Fillinger et al., 2018). This connection to the NAc core may be involved in promoting behavioural vigour in food-seeking tasks (Otis et al., 2017; Parkinson et al., 2000).

The dmPFC is also highly interconnected with the thalamus, both projecting to and receiving inputs from multiple of the nuclei (Condé et al., 1995; Fillinger et al., 2017, 2018). In appetitive conditioning, signals from the dmPFC to the paraventricular nucleus of the thalamus have been suggested to have a role in the expression of appetitive behaviour (Otis et al., 2017). Furthermore, the dorsomedial thalamus, also connected to the dmPFC (Condé et al., 1995), has been demonstrated to be involved in appetitive learning and recall (Means et al., 1975; Ostlund and Balleine, 2008; Waring and Means, 1976). These connections to the thalamus may also may serve as an indirect connection between the ventral and dorsal mPFC regions (Heidbreder and Groenewegen, 2003).

Finally, while the hypothalamus is thought to be a crucial part of the network involved in feeding behaviours (Petrovich and Gallagher, 2007), its connections with the mPFC tend to originate from the ventral areas, with only minor direct connections to the dmPFC (Fillinger et al., 2017, 2018). Therefore, connections between dmPFC and hypothalamus are likely indirect (e.g., through the BLA or ventral mPFC).

Taken together, based on its input-output relationships, the dmPFC is well situated to play a part in the network mediating appetitive learning and the execution of appetitive behaviours such as cue-evoked food seeking.

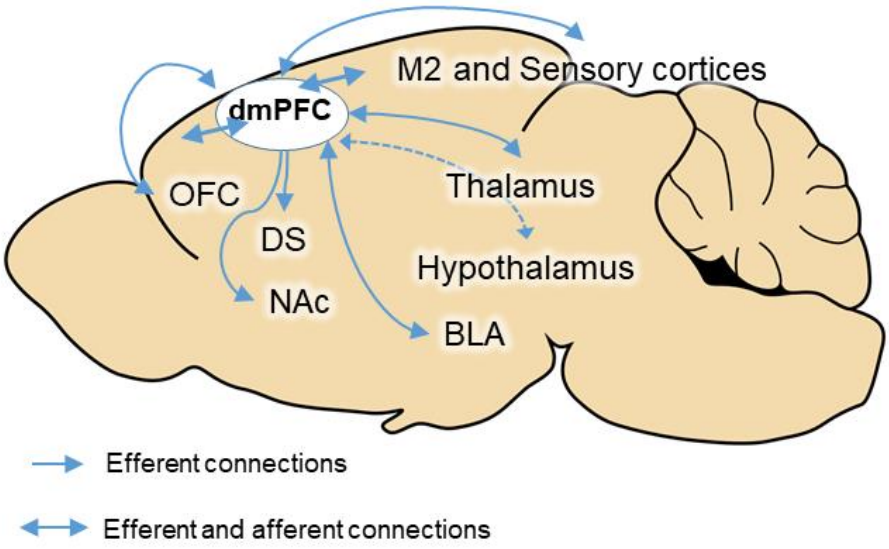


Figure 1: Connectivity of the mouse dorsal medial Prefrontal cortex (dmPFC). This diagram summarises connections highlighted in our section on 'Connectivity of the dmPFC'. The dmPFC is reciprocally connected (double-sided blue arrows) with itself, the orbital frontal cortex (OFC), the basolateral amygadala (BLA), secondary motor (M2) and sensory cortices, the thalamus and the hypothalamus (minor connections; dotted lines). The dmPFC also projects to the dorsal striatum (DS) and the nucleus accumbens (NAc).

1.3 Fos-expressing neuronal ensembles: mediating learnt behaviours through sparse minorities of neurons

1.3.1 Searching for the memory trace within groups of neurons

Investigating the general function of different brain areas and searching for regions that hold memory has yielded crucial information that we now use to understand the nervous system. However, researchers have also been concerned with how the cells, and in particular neurons, within these areas may be involved in mediating these functions. Hebb, a student of Lashley, conceptualised in the mid-20th century the idea of cell assemblies: diffuse groups of interconnected cells that had a role in perception and would form following repeated presentation of specific stimuli (Hebb, 1949; Nicolelis et al., 1997). Decades later, Pennartz, when reviewing studies on the NAc, observed that this region was involved in a number of seemingly irreconcilable functions. From these observations, he proposed that there may be functionally distinct groups of neurons, which he named 'neuronal ensembles', that were responsible for these various functions. In his own words:

"What is actually meant by the concept of "neuronal ensembles"? In the present context, the term "ensemble" refers to a group of neurons characterized by similar afferent/efferent relationships as well as closely related functions in overt behaviour, neuroendocrine regulation and sensorimotor gating." (Pennartz et al., 1994)

This idea that there are subsets of neurons that are all activated during, and responsible for, specific functions (e.g., specific learnt behaviours) has since been used as a framework when investigating learning and memory. These sparsely distributed minorities of neurons have been increasingly observed and studied in numerous brain areas using a wide variety of methods including immunohistochemistry, *in vivo* electrophysiology recordings and *in vivo* imaging (Cruz et al., 2013; Deadwyler and Hampson, 1997; Grewe and Helmchen, 2009). Crucially, certain genes, termed

‘immediate early genes’ (IEGs) have been identified as markers of high activation in neurons (Cruz et al., 2013; Minatohara et al., 2016; Morgan and Curran, 1989). These IEGs have low baseline expression but are transcribed following high external stimuli to the cell (Greenberg et al., 1985; Herdegen and Leah, 1998; Sheng and Greenberg, 1990). As such, they have been used both to measure overall activation of an area (Sharp et al., 1989; Tischmeyer and Grimm, 1999) but also as a marker of neuronal ensembles following behaviours of interest (Cruz et al., 2013; Kovács, 2008; Minatohara et al., 2016). Among them, one of the most commonly utilised to study ensembles is the immediate early gene *c-fos (or Fos)* or its protein product Fos, which will be our focus in this work.

It is worth noting here that the various techniques used to detect neuronal ensembles (e.g., *in vivo* electrophysiology, immunohistochemistry, etc.) will provide vastly different information regarding groups of neurons to the experimenter. As such, the term ‘neuronal ensemble’, which has been employed for various subsets of neurons sharing similar behaviours in response to external stimuli (e.g., similar spiking patterns during an event or similar protein expression following an event) may refer to different populations according to the method used to study them (Tanaka and McHugh, 2018; Tanaka et al., 2018). Here, we will be focusing on Fos-expressing subsets of neurons and predominantly use the term ‘ensemble’ for these neuronal populations.

1.3.2 Fos: a marker for cellular activation

c-Fos (also referred to in this thesis simply as Fos), the protein product of the *c-fos* or *Fos* gene was observed to have a dramatically increased expression following external stimuli to the cell (Greenberg et al., 1985). Later, it was demonstrated that Fos would have increased expression in neurons following robust and prolonged stimulation to the brain (e.g., seizures, chemical agonist) (Kaczmarek et al., 1988; Morgan and Curran,

1989; Morgan et al., 1987) or high input to the neuron (Sgambato et al., 1997). In neurons, the expression of Fos is in part regulated by the Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase (MAPK/ERK) pathway following glutamatergic input and calcium influx to the cell (Sgambato et al., 1998; Valjent et al., 2001). Once this expression dynamic was identified, studies comparing Fos expression to other markers of activation confirmed that the expression of Fos correlated with activation of an area (Morgan and Curran, 1991; Sharp et al., 1989). Further characterisations of the expression of the Fos protein have revealed that it has a peak expression approximately 1h following strong activation, that this expression rapidly decreases over the next few hours (approximately 6h) and that it will return to baseline 24h later (Bisler et al., 2002; Herdegen et al., 1991; Lin et al., 2018).

Thus, the expression of Fos was characterised and determined to be a robust marker of cellular activation (Cruz et al., 2013). However, there are some limitations to using Fos expression to determine activation. In particular, while it allows the identification of which neuron was highly activated within a timeframe of a few hours, it cannot provide any more precise temporal information regarding the real-time activity patterns that occurred during appetitive learning (Devan et al., 2018; Harris, 1998; Kovács, 2008; McReynolds et al., 2018). As such, there are limitations to using Fos as an activity marker, particularly with respect to revealing the activity patterns that help rapidly encode information such as the sudden presentation of food cues. Nevertheless, as we shall see in the sections below, there are advantages to this activity marker, most notably in its use in identifying activated groups of neurons that play a causal role in behaviour. Furthermore, recent brain-clearing techniques such as iDisco allow Fos expression to be examined in the entire brain, and thus potentially revealing alterations in brain-wide network changes (Renier et al., 2014, 2016).

Once expressed, Fos dimerises with the protein Jun to form a transcription factor which is part of the AP-1 family (Herdegen and Leah, 1998; Morgan and Curran, 1991).

Through this, Fos has a role in controlling responses of neurons to high activation, in part through the regulation of target genes which have a role in plasticity (Jaeger et al., 2018). In support, there is evidence for the role of Fos expression in regulating plasticity (de Hoz et al., 2018; Sanyal et al., 2003) but also in promoting learning (de Hoz et al., 2018; Swank et al., 1996).

1.3.3 Fos: a marker of neuronal ensembles

Once characterised as an activity marker, *Fos* mRNA and Fos protein expression from brain extracts and in brain slices were used to measure the overall level of activity within different regions of the brain following a variety of behaviours, including Pavlovian conditioning (Campeau et al., 1991; Morgan and Curran, 1991; Sharp et al., 1989). However, as tools such as in situ hybridization of *Fos* mRNA and immunohistochemistry of the Fos protein increased in spatial resolution, it has been possible to observe their expression from single neurons. From this, it was determined that Fos-expressing neurons represent only a minority of neurons, even following exposure to salient stimuli such as fear and drugs (Crombag et al., 2002; Han et al., 2003; Koya et al., 2009; Mattson et al., 2008; Radulovic et al., 1998). Thus, Fos has been used to identify neuronal ensembles, i.e. sparsely distributed, distinct sets of neurons that are robustly activated following exposure to different learned stimuli.

However, it was not until the last decade that Fos-expressing neurons have been determined to be functionally relevant; in other words, that these ensembles were shown to be necessary and/or sufficient to the expression of learnt behaviour. The first demonstration of this was a result of the development of the Daun02 method (Koya et al., 2009). This method utilised *Fos-LacZ* rats which co-expressed Fos and the Beta-galactosidase enzyme. When rats were injected with the pro-drug Daun02, Beta-galactosidase-expressing neurons metabolise this pro-drug to Daunorubicin which

lesions neurons (Pfarr et al., 2015). Thus, this allowed the specific inactivation of Fos-expressing neurons. This method was used to demonstrate the causal relationship between Fos-expressing neurons in the NAc and cocaine sensitization. It has since been applied to a number of different brain regions and conditioning paradigms such as appetitive and fear conditioning (Bossert et al., 2011; Grosso et al., 2015; Suto et al., 2016; Whitaker and Hope, 2018; Whitaker et al., 2017), further confirming the relevance of Fos-expressing ensembles in mediating conditioning. Following the development of this technique, other methods have emerged to demonstrate the functional relevance of Fos-expressing ensembles to learnt behaviours, most notably, reversible optogenetic and chemogenetic manipulation of Fos-expressing neurons (Liu et al., 2012; Zhang et al., 2015). Thus, the robust expression of Fos in neurons has been determined to be a marker of a functional ensemble in a number of brain areas and behaviours.

1.3.4 The Fos-GFP mouse model and TetTag DREADD method

We will now provide further information regarding two Fos-based techniques that are of particular relevance to this work: the *Fos-GFP* mouse line and the TetTag chemogenetics approach using the *Fos-tTa* mouse line.

The *Fos-GFP* mouse model was generated by including a *Fos-GFP* transgene in the genome of mice. This transgene was constructed by fusing the promoter and gene for *cfos* with the gene for the enhanced GFP (Barth et al., 2004). As such, this mouse model co-expresses Fos with a fusion ‘Fos-GFP’ non-functional protein (Barth et al., 2004; Cifani et al., 2012; Koya et al., 2012). Crucially, this fusion protein and, therefore, its fluorescence, has been shown to have a similar time course of expression following high activation of neurons as Fos (Barth et al., 2004; Cifani et al., 2012). Thus, this mouse line allows the identification of Fos-expressing neurons *in vivo* and *ex vivo*. From this model, it has been possible to measure ensemble-specific intrinsic and synaptic

properties of Fos-expressing neurons following different behavioural paradigms (Koya et al., 2012; Whitaker et al., 2017; Ziminski et al., 2017, 2018). It has also been used to examine activation patterns of Fos-expressing ensembles across multiple training sessions *in vivo* (Czajkowski et al., 2014; Milczarek et al., 2018).

The *Fos-tTa* line (Reijmers et al., 2007) has received a lot of attention in recent years as it allows targeting of Fos-expressing populations within restricted time frames with the use of Doxycycline. In this mouse model, tetracycline transactivator protein (tTa) is co-expressed with Fos. This protein then binds the tTa response element (TRE) which can be fused to a gene of interest. The TRE based transgene will allow the expression of a protein of interest to be dependent on the presence of tTa. It can be paired with the *Fos-tTa* mouse either through cross-breeding (Reijmers et al., 2007) or through delivery to the brain via a viral construct (Zhang et al., 2015). Furthermore, tTa is bound by Doxycycline when it is present in the cell. As such, it is possible to control the time frame in which the gene of interest is expressed within neurons (Reijmers et al., 2007). Thus, the *Fos-tTA* mouse allows for the expression of a gene of interest to be both dependent on Fos expression and controlled by the administration of Doxycycline (or lack thereof).

Of particular interest to us, one method that makes use of this mouse line is the TetTag DREADD method (Zhang et al., 2015). DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) are ionic receptors that are activated by non-endogenic drugs. For example, the excitatory hM3Dq DREADD is derived from a muscarinic receptor and increases the excitability of neurons when bound to by clozapine (Armbruster et al., 2007). In contrast, the hM4Di DREADD, will reduce the excitability of the neuron when bound by clozapine (Armbruster et al., 2007). When paired with *Fos-tTA* mice in the TetTag DREADD system, neurons expressing Fos within a specific timeframe can be tagged and their signalling manipulated for a period of several hours following clozapine delivery. The *Fos-tTa* mouse has also been combined with optogenetics, in which ionic channels are activated by light (Liu et al., 2012). Together,

these optogenetic and chemogenetics methods have been crucial in manipulating Fos-expressing ensembles activated during behaviours of interest. Thus, Fos based techniques such as these allow us to identify specific Fos-expressing ensembles, determine how their specific properties are altered during learning and test their relevance to learnt behaviours

1.3.5 Other IEGs

As mentioned above, Fos is only one of many identified IEGs that are strongly expressed following robust activation of the neuron. While Fos is one of the IEGs that is the most commonly used, other IEGs have also been utilised to mark robust activation, in particular through immunohistochemistry; among them Zif268 and Arc, which have comparable expression dynamics to Fos (Barry et al., 2016). Also, similar to Fos these IEGs have shown increased expression in response to reward-related cues (Fanous et al., 2013; Thomas et al., 2003).

Furthermore, the expression of proteins of interest has been linked with the promoter of these IEGs (e.g., Arc-GFP mouse model (Wang et al., 2006)). Recently, certain groups have made use of the properties of IEG promoters and modified them to create artificial promoters with altered features such as increased amplitude of expression following activation or with reduced baseline expression. E-SARE (Kawashima et al., 2013) and RAM (Sørensen et al., 2016) are examples of these artificial promoters and have been used to refine targeting of neurons that have been robustly activated.

It is worth remarking that, while the expression of other IEGs has a similar dynamic to that of Fos (Barry 2016), the overlap between populations of neurons expressing different IEGs is not perfect (Fanous et al., 2013; Guzowski et al., 2001). Moreover, different IEGs have different functions within the cell, suggesting their regulation may differ according to the needs of the cell. However, as IEGs are markers of recent

activation, populations detected with Arc or Zif268 are likely comparable (though not identical) to those detected with Fos.

1.3.6 General properties of neuronal ensembles in learning

Early manipulation studies of Fos-expressing neurons identified these neurons as necessary and sufficient to driving the expression of learnt behaviours (Cruz et al., 2013; Koya et al., 2009; Liu et al., 2012). These studies inspired other researchers to perform further investigations into learning-specific properties and recruitment patterns of these 'activated' (i.e., identified through activation markers such as IEGs) ensembles and how they compare to that of surrounding 'non-ensemble' neurons. Here, we will review some of the known properties of neuronal ensembles identified through such methods.

One property of ensembles, conceptualised by Pennartz, was that each ensemble is activated for specific functions or events. Through this, they allow single brain areas to mediate multiple different functions (Pennartz et al., 1994). Recent findings have come to support this theory. In particular, multiple studies utilising the Daun02 method to silence ensembles have revealed the co-existence of two distinct ensembles that excite and suppress reward-seeking behaviours, within the same brain region (Suto et al., 2016; Warren et al., 2016, 2019). More specifically, Warren et al. found co-existing ensembles mediating two different behaviours (conditioned responses and extinction) within the ventral mPFC (Warren et al., 2016). Moreover, within single areas, different ensembles have been shown to be activated for different stimuli (Cruz et al., 2014; Fanous et al., 2012). Thus, these findings suggest that ensembles may demonstrate specificity to the events or stimuli that activate them.

Furthermore, Fos-expressing neuronal ensembles have been observed to be 'stable'; repeatedly activated (as detected by IEG expression) at the presentation of specific stimuli or behavioural tasks (Czajkowski et al., 2014; Mattson et al., 2008; Milczarek et

al., 2018). Crucially, ensemble manipulation experiments that demonstrated the functional relevance of Fos-expressing ensembles (e.g., Daun02 method, TetTag chemogenetics and optogenetics) rely on this property in order to tag a set of neurons in one session that will most likely be reactivated in later sessions. However, in behavioural paradigms involving a longer learning period, alteration in the number of recently activated neurons has also been observed (Cao et al., 2015; Whitaker et al., 2017). Moreover, there is also evidence that the Fos-expressing ensemble may be refined as learning progresses (Milczarek et al., 2018). Thus, while there are stable subsets of neurons that are thought to be repeatedly activated across behavioural paradigms, variation in the number of Fos-expressing neurons may also play a part in shaping behaviour.

Finally, Fos-expressing neuronal ensembles have been observed to have different intrinsic and synaptic properties compared to neighbouring neurons within the same area. For example, changes in excitability in NAc ensembles has been shown to be involved in mediating the 'value' of an appetitive reward following the establishment of a food-cue association as, while it is increased compared to surrounding neurons in the recall of the association, it returns to baseline following extinction or devaluation of the reward (Ziminski et al., 2017, Sieburg et al. *in press*). Changes in excitability have also been shown to occur during appetitive learning in the dmPFC and therefore may participate in mediating learnt behaviour (Whitaker et al., 2017). However, it is worth noting here that these learning-induced alterations in excitability are not observed in all Fos-expressing neurons. For example, Ziminski et al. observed that, following cocaine memory retrieval, while the number of Fos-expressing neurons was increased in the NAc shell but not core, the excitability of Fos-expressing neurons differed from surrounding neurons in the NAc core but not shell (Ziminski et al., 2018). Similarly, synaptic properties of Fos-expressing neurons have been shown to be altered differently to surrounding neurons during learning. For example, Koya et al. observed that silent synapses formed

in Fos-expressing neurons of the NAc shell of cocaine-sensitized mice (Koya et al., 2012). In aversive conditioning, neuronal ensembles (detected with Arc) have been shown to undergo synaptic potentiation (Gouty-Colomer et al., 2016). Thus, these ensemble-specific physiological alterations in intrinsic and synaptic properties likely play a role in encoding learnt associations; although, the presence and nature of these ensemble specific alterations may depend on brain area and behavioural tasks.

While repeatedly activated neuronal ensembles seem to be present across multiple brain areas and activated by multiple forms of learning; the exact properties and recruitment dynamics seem to vary according to brain area and behaviour observed. As such, we will now focus on ensembles encoding appetitive conditioning in the dmPFC.

1.4 Investigating dmPFC ensembles in appetitive conditioning and extinction

1.4.1 dmPFC ensembles in mediating appetitive learning and extinction: what do we know?

The dmPFC has been shown to be involved in learnt food-seeking behaviours, both when learning these behaviours (Baldwin et al., 2000; Otis et al., 2017), when expressing them in recall and reinstatement (Calu et al., 2013; Whitaker et al., 2017) as well as during the extinction of these behaviours (Moorman and Aston-Jones, 2015). Moreover, dmPFC interneurons have also been implicated in food-related behaviours (Gaykema et al., 2014; Kvitsiani et al., 2013) as well as extinction of food-seeking (Sparta et al., 2014). As discussed above, Fos-expressing ensembles across multiple brain areas have been shown to be key to mediating memories. More specifically, Fos-expressing ensembles of the wider mPFC region have been causally linked to both appetitive conditioning and extinction (Cifani et al., 2012; Suto et al., 2016; Warren et al., 2016; Whitaker et al., 2017). Recently, Whitaker et al. examined ensemble recruitment during operant appetitive conditioning at multiple time points in the dmPFC. They found an increased

number of dmPFC Fos-expressing neurons during recall, suggesting that the dmPFC was involved in mediating food-seeking behaviour (Whitaker et al., 2017). Additionally, the number of neurons activated increased as learning progressed and intrinsic properties of Fos-expressing neurons modulated across learning (Whitaker et al., 2017). Thus, this suggests dmPFC ensembles are involved in mediating both appetitive conditioning and recall, although it is worth noting that these findings were observed in an operant conditioning task and therefore may differ from observations in classical conditioning paradigms (Dickinson et al., 2000; Wassum et al., 2011). Furthermore, previous studies have also established the role of dmPFC Fos-expressing ensembles in mediating reinstatement of food-seeking behaviours following extinction (Calu et al., 2013; Cifani et al., 2012). Taken together with the previously observed role of the dmPFC, these findings suggest that Fos-expressing ensembles form in the dmPFC during appetitive conditioning and are reactivated during the expression of learnt behaviours.

However, to our knowledge, there has been no longitudinal study of Fos-expressing neurons of the dmPFC during appetitive conditioning and extinction. As such, how these ensembles form during learning and what their recruitment dynamics are as appetitive conditioning and extinction progress is unclear. Furthermore, while there is evidence of the involvement of dmPFC interneurons in appetitive learning, there has been little to no investigation into dmPFC inhibitory ensembles in appetitive conditioning. Thus, while dmPFC ensembles of neurons are likely recruited during the formation of appetitive associations, how and when these neurons are consolidated into ensembles remains unclear.

1.4.2 Aims and hypotheses

We here reviewed the available evidence relating to the role of the dmPFC in appetitive conditioning and extinction and examined findings describing the part played by neuronal ensembles in encoding food-cue associations. Previous evidence has suggested that both excitatory and inhibitory signalling in the dmPFC are involved in the formation and extinction of an appetitive association. In this study, we aim to examine this signalling through the lens of Fos-expressing neuronal ensembles. These ensembles are most commonly studied at a single timepoint, once learnt behaviour has been established. As such, the dynamics and roles of Fos-expressing ensembles during the processes of appetitive conditioning and extinction in the dmPFC have yet to be fully elucidated.

To investigate these gaps in knowledge, we utilise a combination of *in vivo* 2-photon imaging and chemogenetics. Due to its location, the dmPFC is difficult to access with conventional cranial *in vivo* imaging, thus we made us of a microprism-based imaging method, which allowed us to access the dmPFC. We paired this with a transgenic *Fos-GFP x GAD-tdTomato* mouse model with which we could image recently activated neurons with GFP and interneurons with tdTomato. This allowed us to track the activity of dmPFC pyramidal cells and interneurons over multiple days during the formation of a CS-US association as well as in recall or extinction learning. Furthermore, we utilised a TetTag DREADD system to specifically tag neurons activated by early learning and to alter their excitability throughout learning. With this combination of techniques, we aimed to investigate ensemble recruitment, notably; when neurons are recruited to ensembles, what differences there are between conditioning and extinction and what contributions are made by excitatory and inhibitory neurons to conditioning and extinction learning. We also aimed to establish what causal relationship the Fos-expressing neurons may have had in learning.

Repeated activation is thought to consolidate neurons into ensembles. Thus, we hypothesise that persistently activated neurons may be recruited for conditioning and

extinction. Furthermore, we hypothesise that neuronal activation patterns are altered across learning and this modulation from early to late learning contributes to the learning process.

Chapter 2: Appetitive conditioning recruits a pyramidal cell ensemble from a neuronal pool activated in early learning

2.1 Introduction

Through Pavlovian associative learning, a conditioned stimulus (or CS) that reliably predicts food reward (unconditioned stimulus or US) is endowed with motivational significance as well as the ability to activate and retrieve food memories (van den Akker et al., 2018; Fanselow and Wassum, 2016; Holland, 1984; Pavlov (1927), 2010; Rescorla, 1988). These CS-activated food representations can elicit actions to facilitate food procurement. For animals, this maximizes caloric intake while minimizing time and energy spent searching for food (Carthey et al., 2011; MacArthur and Pianka, 1966) and in humans, can elicit food cravings and overeating (van den Akker et al., 2018; Petrovich and Gallagher, 2007). Elucidating the neurobiological mechanisms underlying the establishment of appetitive CS-US associations is important for understanding both adaptive and maladaptive feeding behaviours (van den Akker et al., 2018; Petrovich and Gallagher, 2007).

The dorsal medial prefrontal cortex (dmPFC) is an area that is implicated in food-seeking behaviours (Baldwin et al., 2000; Bussey et al., 1997; Cardinal et al., 2002; Otis et al., 2017; Petykó et al., 2009). Furthermore, neuronal ensembles of the dmPFC are thought to encode CS-evoked memory representations following appetitive learning (Calu et al., 2013; Whitaker et al., 2017). Accordingly, selective silencing of dmPFC ensembles attenuates food-seeking (Whitaker et al., 2017). These findings offer compelling evidence that CS-activated dmPFC ensembles are necessary for regulating appetitive behaviours. However, we have yet to understand how these ensembles are formed as a function of appetitive learning, i.e. how are neurons recruited into ensembles that establish a stable CS-US representation?

We addressed this key question by visualizing ensemble formation and activation patterns across conditioning through microprism-based 2-photon (2P) *in vivo* imaging (Low et al., 2014). Unlike conventional cranial window 2P imaging, this method allowed us to access the dmPFC, a region that plays a role in facilitating attentional processes and discriminating between food-predictive and non-predictive cues (Bryden et al., 2011; Cardinal et al., 2002; Parkinson et al., 2000; Totah et al., 2009). Furthermore, we crossed *Fos-GFP* and *GAD-tdTomato* mice to generate a *Fos-GFP* X *GAD-tdTomato (FGGT)* mouse line that express GFP in behaviourally-activated (GFP+) neurons and tdTomato in interneurons (Barth et al., 2004; Besser et al., 2015; Whitaker et al., 2016; Ziminski et al., 2017). This enabled us to track pyramidal cell (tdTomato–) and interneuron (tdTomato+) activation patterns across learning and recall trials in mice trained on a Pavlovian appetitive conditioning task.

We found that during conditioning a stable, repeatedly activated pyramidal cell ensemble emerged from a wider pool activated during the initial presentation of the CS-US pairing.

2.2 Methods

2.2.1 Animal breeding and housing

Heterozygous (het) male *Fos-GFP* (RRID: IMSR_JAX:014135) and *GAD-tdTomato* mice (Besser et al., 2015) (C57BL/6J-Tg(Gad2-tdTomato)DJhi; RRID:IMSR_EM:10422; were bred onto a C57BI/6 background. het Male *GAD-tdTomato* were bred with het *Fos-GFP* female mice to produce double transgenic *Fos-GFP x GAD-tdTomato* (*FGGT*) mice. *FGGT* male mice were used for 2-photon imaging experiments, *Fos-GFP* male mice were used for *ex vivo* electrophysiology experiments. Mice were housed under a 12-hours light/dark cycle (lights on at 7:00) at the maintained temperature of 21+/-1 °C and 50 +/-5% relative humidity. Animals were aged 10-13 weeks at the beginning of experimental procedures, and were food restricted (90% baseline body weight) 1 week prior to behavioural testing until the completion of behavioural experiments. Experiments were conducted in accordance with the UK 1986 Animal Scientific Procedures Act (ASPA) and received approval from the University of Sussex Animal Welfare and Ethics Review Board.

2.2.2 Microprism implantation in *FGGT* mice

At ages 10-13 weeks, *FGGT* mice were implanted with a microprism in the dmPFC. Microprism constructs were built by assembling 2 circular glass windows (5 mm and 3 mm diameter; #1 thickness, cat. no: 64-0700 and 64-0720, Warner instruments, Holliston, USA) and a 1.5 mm coated microprism (Model no: MPCH-1.5, part no: 4531-0023, Tower Optics, Boyton Beach, USA) using optical glue (Norland Optical Adhesive, Cranbury, USA), such that the microprism rested on the 3 mm window with its vertical imaging edge on the diameter. Mice were anesthetised with isoflurane 3% dilution in O_2 (0.8 L/min) and NO_2 (0.5 L/min) and maintained between 1 and 2% dilution throughout

the surgery. They first received an injection of dexamethasone (Dexadreson, 5mg/kg, s.c. or i.m.) to reduce cerebral inflammation. The skin on their scalp was sectioned off and the skin around the section was glued to the skull (Vetbond, 3M, St. Paul, USA). The bone was then scored before a set of custom headbars was fixed to the skull using dental cement (Unifast TRAD, Tokyo, Japan). A 3 mm circular opening was created in the skull centred at bregma 0.8 mm (+/-0.2 mm according to the location of blood vessels). The final area observable through the microprism spanned approximately from bregma 0.05 mm to 1.55 mm on the rostro-caudal axis and from 0 mm to 1.5 mm on the dorso-ventral axis (of note, the most dorsal section was usually obscured by the central sinus). The vast majority of this area constitutes the anterior cingulate cortex of the mPFC (Fig.2A; Paxinos and Franklin, 2001). Microprism implantation occurred similarly as described by Low et al. (Low et al., 2014). The dura was removed and the microprism construct was lowered into the brain using a custom-built holder such that the microprism was positioned between the hemispheres with the imaging surface placed against the sagittal surface of one of the hemispheres (Fig. 2B). The construct was glued with Vetbond and further fixed with dental cement. Following implantation, mice received buprenorphine (0.1 µg/kg, i.m.) and left to recover in a heated chamber for an hour. Following surgery, they received 3 days of oral Meloxicam (Metacam, Boehringer, Berks, UK). All mice recovered for a minimum of two weeks before undergoing any further procedures. The first imaging session typically occurred 3-4 weeks following surgery to allow inflammation in the imaging area to subside.

2.2.3 Behavioural experiments

Behavioural procedures were carried out similarly to Ziminski et al. (Ziminski et al., 2017). All behavioural experiments were performed in standard mouse operant chambers (15.9 × 14 × 12.7 cm; Med Associates), each housed within a sound-attenuating and light-

resistant cubicle. The chamber access panel (front), rear and ceiling were constructed from clear Plexiglas; the sidewalls were made from removable aluminium panels and the floor was a stainless steel grid. The house light was situated in the side panel and was on for the duration of the behavioural experiments. Each chamber was fitted with a protruding magazine (to accommodate mice equipped with a head-restraint device) situated in the centre of one side wall that dispensed a 10% sucrose solution serving as the unconditioned stimulus (US). An infrared beam detected head entries into the food magazine. A mechanical click generator provided a broad-frequency (0–15 kHz) sound, which served as a conditioned stimulus (CS) (Med Associates). Initiation and running of behavioural protocols, including the recording of head entries into the food magazine, was performed using Med-PC IV (MedAssociates Inc., RRID:SCR_014721).

Mice were randomly assigned to the Paired or Unpaired groups that underwent identical procedures except that Unpaired mice only received sucrose in the home cage 1–4 h at random times before or after each conditioning (acquisition) session, with the exceptions of S1, S5 and S11 (see below). Mice first received one session of magazine training, in which all mice were habituated to the chamber and Paired mice were pre-trained to the sucrose-delivery magazine by receiving a 10% sucrose solution under a random interval-30 (RI-30) schedule. One day later, mice underwent 12 acquisition sessions over a 7 d period in the morning (8:00 A.M. to 12:00 P.M.) and/or afternoon (12:00 P.M. to 5:00 P.M.) for 1–2 sessions per day. Each acquisition session lasted approximately 24 min and consisted of six 120 s CS presentations separated by 120 s RI inter-trial interval (ITI) periods. During each 120 s CS period, 13.3 µl of 10% sucrose solution was delivered into the magazine on an RI-30 s schedule (Paired mice) or was unrewarded (Unpaired mice). 12 acquisition sessions over 7 days produced selective responding to the CS. 3 days following the last acquisition session, mice were tested for Pavlovian conditioning with a cue exposure test: both Paired and Unpaired mice were placed in the conditioning chamber and tested under extinction conditions for 3 CS presentations.

To control for Fos induced by sucrose consumption, Unpaired mice received sucrose in their home cage 10 minutes before training for all acquisition sessions preceding recording (S1, S5 and S11).

2.2.4 *In vivo* 2-photon imaging

2.2.4.1 Habituation and imaging sessions

Imaging sessions took place on head-fixed, awake mice that were able to freely run on a polystyrene cylinder (Fig. 2A). For ~1 week prior to the first imaging session, mice were habituated to being restrained by being head-fixed regularly for progressively increasing durations. Following habituation, the brain surface under the microprism was assessed and 2 to 3 areas of interest were defined. In each area of interest, z-stacks in both the red and green channels were recorded simultaneously at an excitation wavelength of 970 nm (power at the objective: 70-130 mW; pixel dwell time: ~3.9 ns) from the pial surface to a depth of approximately 300 µm. Each slice of the stack was an average of two 660.14 x 660.14 µm images (corresponding to 512 x 512 pixels; pixel size: 1.2695 x 1.2695 µm). Images were captured in pre-defined areas of interest using a Scientifica multiphoton microscope (Uckfield, UK) with a 16X water immersion objective (CFI LWD Plan Fluorite Physiology objective, NA 0.8, WD 3mm; Nikon Corporation, Tokyo, Japan) and a Chameleon Vision-S Ti:Sapphire laser with dispersion precompensation (Chameleon, Coherent, Santa Clara, USA)B. The software used for recording was ScanImage r3.8 (Pologruto et al., 2003).

Imaging sessions took place 75 min following initiation of the 1st, 5th and 11th conditioning session as well as the cue exposure test (Fig. 2A). Another two imaging sessions took place directly from the home cage (2-3 days prior to conditioning and 5-8 days after the cue exposure test). Imaging sessions typically lasted 40 minutes to an

hour. Due to poor imaging quality on one or several imaging sessions, three mice (1 unpaired, 2 paired) were excluded from image analysis.

2.2.4.2 Image Analysis

Initial image processing took place in FIJI (ImageJ, (Schindelin et al., 2012)). tdTomato images within a stack were aligned to each other on x and y axes with MultiStackReg (Thevenaz et al., 1998). The resulting transformation was then applied to the GFP image stack. Stacks were aligned between sessions using the Landmark Correspondence plugin (Stephen Saalfeld). An overlapping volume within layer II/III and common to all sessions was identified and selected. All images in the selected stacks were despeckled and an FFT bandpass filter (upper threshold 40 pixels, lower threshold 5 pixels) was applied. Local maxima (noise tolerance: 30 pixels) were identified and the signal within a disk around the maxima (12 pixel diameter (15.234 µm) for GFP signal and 16 pixels diameter (20.312 µm) for tdTomato signal) was compared to the noise surrounding it (2.5390µm thick band, 1.2695µm away from the disk; Fig. 4A). If signal > noise + 2 SD (noise) for at least two consecutive slices in the stack, the cell was considered GFP+ or tdTomato+ as appropriate. Positive cells were recorded in an empty 3D matrix the size of the stack and later the x, y, z coordinates and the GFP relative fluorescent intensity (RFI = signal/noise) of each cell were extracted from the matrix using 3D object counter (Bolte and Cordelières, 2006).

A custom Matlab (2016a, MatWorks, Natick USA) script defined whether each cell was a putative ‘interneuron’ or ‘pyramidal cell’ according to whether tdTomato signal was detected in a cell for a majority of recorded sessions. GFP+ cells from each session were then sorted according to their coordinates in order to identify the activation history of neurons. In order to accomplish this, for each session, a cell’s x, y, z coordinates were compared to those obtained from previous sessions. If the x, y and z coordinates fell

within a 20 pixel interval (25.390 µm) of existing coordinates, it would be considered the same cell. If several existing coordinates fulfilled this condition, the cell was assigned to the closest set of coordinates on the x, y plane as defined by Euclidean distance. If no coordinates fulfilled this condition, the cell was considered newly activated.

To account for inter-individual difference in cell density and GFP expression, all variables relating to GFP+ cell counts were normalised to the average number of GFP+ cells detected in home cage sessions ((number of GFP+/average number GFP+ in HC) *100. GFP RFI were normalised between sessions using the average tdTomato RFI as reference. All neurons activated in HC sessions were pooled and grouped into 3 categories of brightness (High, Medium and Low) according to their GFP RFI such that a third them fell within each category. The thresholds identified through this process were used to assign a brightness category to all GFP+ neurons in S1 (Fig. 4A).

2.2.5 Data analysis

In the main text, we only report effects and interactions key to interpretation. A complete report of statistical procedures and results for all experiments can be found in the Annex (Suppl. Table 2-5). All data were analyzed using GraphPad Prism (RRID:SCR_002798; GraphPad Software) and SPSS (IBM SPSS Statistics, Version 23.0 (2015), Armonk, NY: IBM Corp). Group data are presented as mean±SEM.

Behavioural data: All behavioural data was tested either with 3-way mixed ANOVAs and 1-way repeated measures ANOVAs in SPSS or with 2-way mixed ANOVAs in Prism as appropriate. Following 2-way mixed ANOVAs, further post-hoc tests were performed (Sidak correction) if an interaction was observed ($P<0.05$).

Imaging data: GFP+ counts were tested with 2-way mixed ANOVAs and *t* tests in Prism. Following 2-way mixed ANOVAs, further post-hoc tests were performed (Sidak

correction) if an interaction was observed ($p<0.05$). Chi-squared tests were performed on pooled neurons in SPSS and further post-hoc procedures ((Beasley and Schumacker, 1995); Bonferroni correction) performed if a significant interaction was observed ($P<0.05$). Interneurons and Pyramidal cells are affected differently by Glutamatergic signalling (Riebe et al., 2016) suggesting distinct Fos induction thresholds, as such they were analysed separately.

2.3 Results

2.3.1 Appetitive conditioning shapes CS-selective approach behaviours in *FGGT* mice

We trained *Fos-GFP x GAD-tdTomato (FGGT)* mice on an appetitive conditioning task (Fig 1A). Across 12 'Acquisition' sessions, mice in the Paired group received repeated trials during which an auditory cue (CS) was paired with liquid sucrose delivery (US). Mice in the Unpaired (control) group received an equal number of CS presentations in the conditioning chamber but received sucrose only in their home cage. Three days following the last Acquisition session, mice were tested for CS-US memory recall under extinction conditions in the 'Recall' test, (Fig. 1A). Initial analysis of Acquisition and Recall test performance revealed significant interactions of Cue X Session X Group (Fig. 1B; $F_{11,220}$=5.94, P<0.001) and Group X Cue (Fig. 1C; $F_{1,11}$=15.46, P<0.01), respectively, indicating selective responding during the CS versus the Inter-Trial Interval (ITI, no cue) periods. We further assessed conditioning performance by calculating a 'Selectivity Index' (Fig. 1D). This parameter measures the robustness of selective CS responses by subtracting ITI responses and normalizing to total head entries. During Acquisition, there was a significant effect of Session ($F_{11,121}$=9.50, P<0.001) in the Paired group, indicating that Paired mice came to selectively respond to the CS as a function of conditioning.

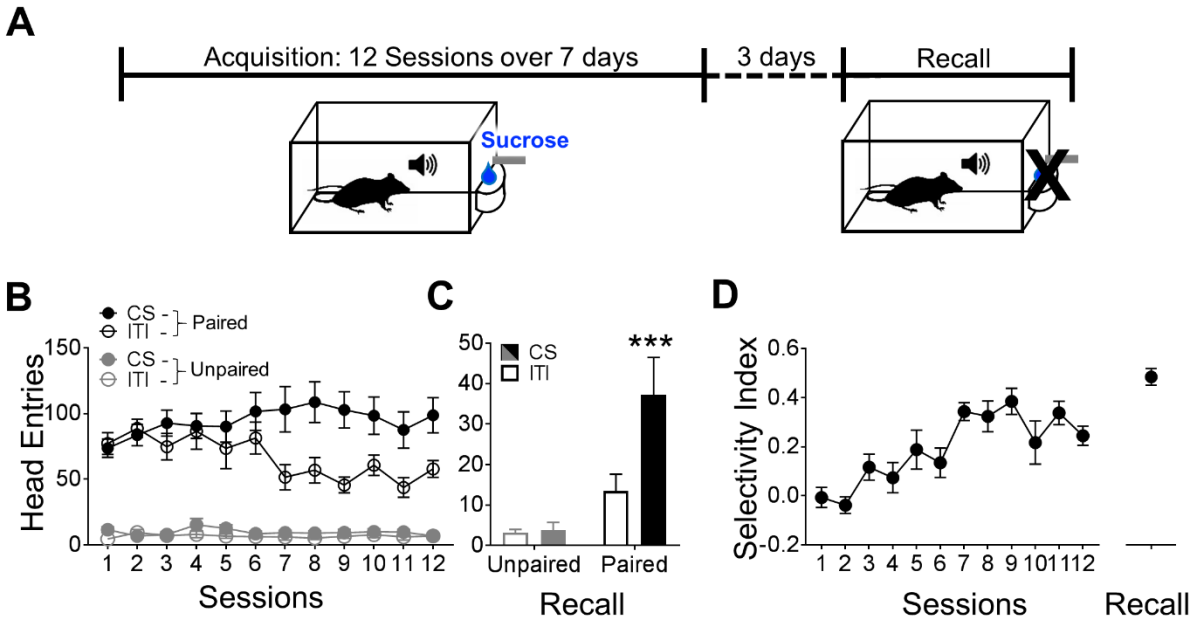


Figure 1: Experimental timeline and conditioning. (A) Timeline of conditioning and imaging. **(B)** Selective head entries into the magazine during the CS (cue) compared to ITI (no cue) periods following acquisition of conditioning and **(C)** Cue exposure test (Recall) in Paired, but not Unpaired *FGGT* mice. **(D)** 'Selectivity index' of responses (CS-ITI/total number head entries) of Paired mice during Acquisition and Recall. All data are expressed as Mean±SEM *** *P<0.001; Paired (P): n=12, Unpaired (UP): n=11*

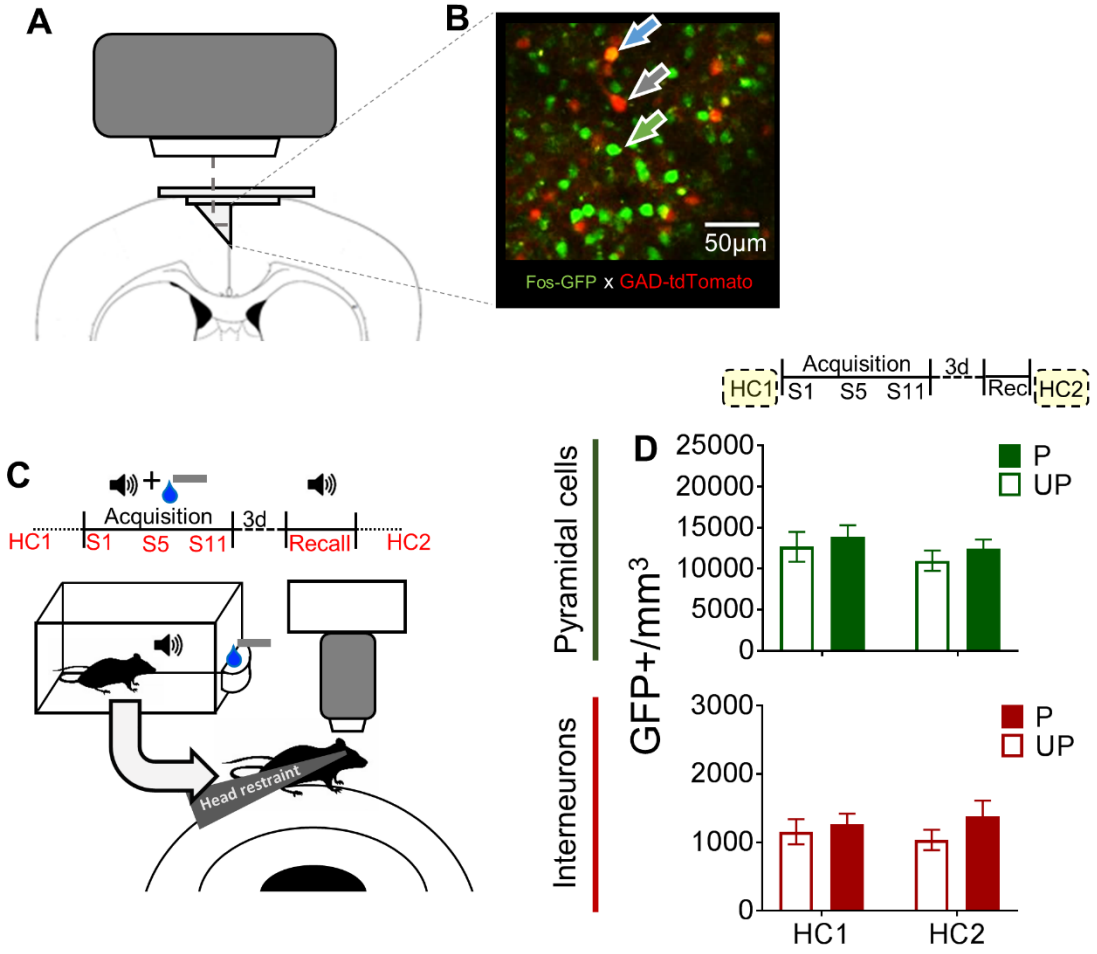


Figure 2: Experimental Timeline, Methods of 2-photon imaging, and baseline GFP expression. GFP expression was longitudinally monitored in pyramidal cells and interneurons. **(A)** Microprism placement for dmPFC imaging. **(B**) Representative *in vivo* 2-photon image of dmPFC from *Fos-GFP x GAD-tdTomato (FGGT)* mice (green arrow: GFP; grey arrow: tdTomato; blue arrow: GFP+tdTomato). GFP+ neurons were selected by comparing Signal intensity to surrounding background. **(C)** Imaging timeline and schematic representation of imaging session in head-fixed mice following behavioural training under freely moving conditions (S1, S5, S11 and Recall) or from home cage (HC1, HC2). **(D)** Number of GFP+ pyramidal cells (green) and interneurons (red) per mm^3 in imaging sessions taking place directly from home cage both before (HC1) and after (HC2) behavioural training. Data are expressed as Mean±SEM. *Paired (P) n=10, Unpaired (UP) n=9*.

2.3.2 Conditioning recruits a stable, repeatedly activated ensemble from a neuronal pool activated during the first CS-US pairing

We used 2P imaging in microprism-implanted *FGGT* mice to characterize neuronal activation patterns among pyramidal cells and interneurons in layers II/III of the dorsal medial prefrontal cortex (dmPFC) following Acquisition and Recall sessions (Fig. 2A, B, C). (Barth et al., 2004). In order to assess baseline GFP expression, we first examined the number of GFP+ pyramidal cells and interneurons per mm^3 in mice that have been in the home cage (HC) for at least 24 h. Imaging sessions were conducted both before (HC1) and after (HC2) mice underwent behavioural training. We observed no significant interaction effect of Group X Session for pyramidal cells ($F_{1,17}$=0.02, P=0.888) and interneurons ($F_{1,17}$=1.84, P=0.193; Fig. 2D). Thus, behavioural training did not modulate baseline GFP expression for both cell types. In further analyses, to account for inter-individual differences in cellular density and imaging quality, the number of HC1 and HC2 GFP+ pyramidal cells and interneurons were averaged for each mouse and used to normalize any subsequent GFP+ cell counts.

We first assessed the overall number of strongly activated, GFP+ pyramidal cells (tdTomato–) and interneurons (tdTomato+) on the 1st (S1), 5th (S5), and 11th (S11) acquisition sessions (Fig 3A). No significant interactions of Group X Session were observed in pyramidal cells $F_{2,34}$=0.20, P=0.82) or interneurons ($F_{2,34}$=0.06, P=0.95), suggesting that the total number of activated neurons across Acquisition sessions for either cell type in the dmPFC did not fluctuate as a function of conditioning in the dmPFC.

Repeated, persistent activation throughout learning is thought to consolidate neurons into an ensemble that mediates learned associations (Mattson et al., 2008). Moreover, activity in the motor cortex early in learning of a simple motor task has been shown to be a critical determinant for ensemble consolidation (Cao et al., 2015). Thus, we investigated whether appetitive Pavlovian conditioning preferentially recruits a

repeatedly activated ensemble from a pool of candidate neurons activated in S1. To this end, between Unpaired and Paired groups, we assessed and compared the number of GFP+ neurons in two distinct 'Activation History' categories: neurons that were persistently activated (+) in S5 and S11 following activation in S1 (S1+| S5+ S11+) or neurons persistently activated in S5 and S11 but that were not activated in S1 (S1-| S5+ S11+; Fig. 3B & 3C). In pyramidal cells, there was a significant interaction of Activation History X Group ($F_{1,17}$=5.97, P<0.05). There was no significant interaction of Activation History X Group in interneurons ($F_{1,17}$=0.17, P=0.68). Hence, conditioning recruited a persistently activated pyramidal cell ensemble from a pool of neurons activated in S1. An analysis performed on a less conservative criterion, which included all neurons observed to be GFP+ in more than one imaging session (e.g. S1+| S5- S11+), yielded similar results (Suppl. Fig. 1).

We also characterized neuronal activation patterns of pyramidal cells and interneurons following the Recall test (Fig. 3E). We observed a significantly higher number of pyramidal cells recruited following Recall in Paired mice compared to Unpaired mice (t_{10}=2.40, P<0.05). We did not observe a significant effect in interneurons (t_{10}= 0.67, P=0.52).

Next, we compared the number of GFP+ neurons with a S1+| S5+ S11+ or S1-| S5+ S11+ activation history that were activated following Recall (Fig. 3F). In pyramidal cells, there was a significant interaction of Activation History X Group ($F_{1,10}$=7.65, P<0.05). Post-hoc testing revealed a significant increase in the number of S1+| S5+ S11+ neurons re-recruited in Recall (S1+| S5+ S11+ R+) in Paired mice compared to Unpaired mice (P<0.01). There was no significant interaction of Activation History X Group in interneurons ($F_{1,10}$=0.24, P=0.64). Thus, similar to conditioning, memory recall recruited a persistently activated pyramidal cell ensemble with an S1 activation history.

Having established the relevance of S1 activation to the conditioning ensemble, we examined at a population level how conditioning altered neuronal reactivation likelihood following recruitment in S1 (Fig. 3D). We assessed the proportion of S1-activated neurons that were reactivated in S5 only (S1+| S5+ S11-), S11 only (S1+| S5- S11+) or S5 and S11 (S1+| S5+ S11+) as well as neurons activated in S1 but not S5 and S11 (S1+| S5- S11-). During conditioning, there was a significant interaction of Activation History X Group for both pyramidal cells (X^2_3=58.98, P<0.001) and interneurons (X^2_3=41.63, P<0.001). Notably, there was a significantly higher proportion of S1+| S5+ S11+ neurons reactivating in Paired mice compared to Unpaired mice for both cell types (P<0.05). Of note, only 29% of S1-activated pyramidal cells were recruited into the persistently activated ensemble (S1+| S5+ S11+).

We also assessed the proportion of Recall-activated neurons that had been repeatedly reactivated in Acquisition following activation in S1 ((S1+| S5+ S11-), (S1+| S5+ S11+), (S1+| S5- S11+); Fig. 3G). There was a significant interaction of Activation History X Group for both pyramidal cells (X^2_3= 77.512; P<0.001) and interneurons (X^2_3= 13.537; P<0.001). Notably, there was a significantly higher proportion of Recall-activated pyramidal cells and interneurons also activated S1, S5 and S11 of Acquisition in Paired mice compared to Unpaired mice (P<0.05). Furthermore, only 23% of Recall-activated pyramidal cells exhibited a repeated activation history during conditioning (S1+| S5+ S11+). These findings suggest that activation dynamics of neurons are altered by conditioning.

Taken together, we demonstrate that during the establishment and recall of a CS-US association, a stable, persistently activated ensemble is activated in the dmPFC from a pool of pyramidal cells that were initially recruited in S1, when the acquisition of robust CS-US representations has yet to occur. Thus, activation in early learning may be a factor in allocating neurons to a stable conditioning specific ensemble.

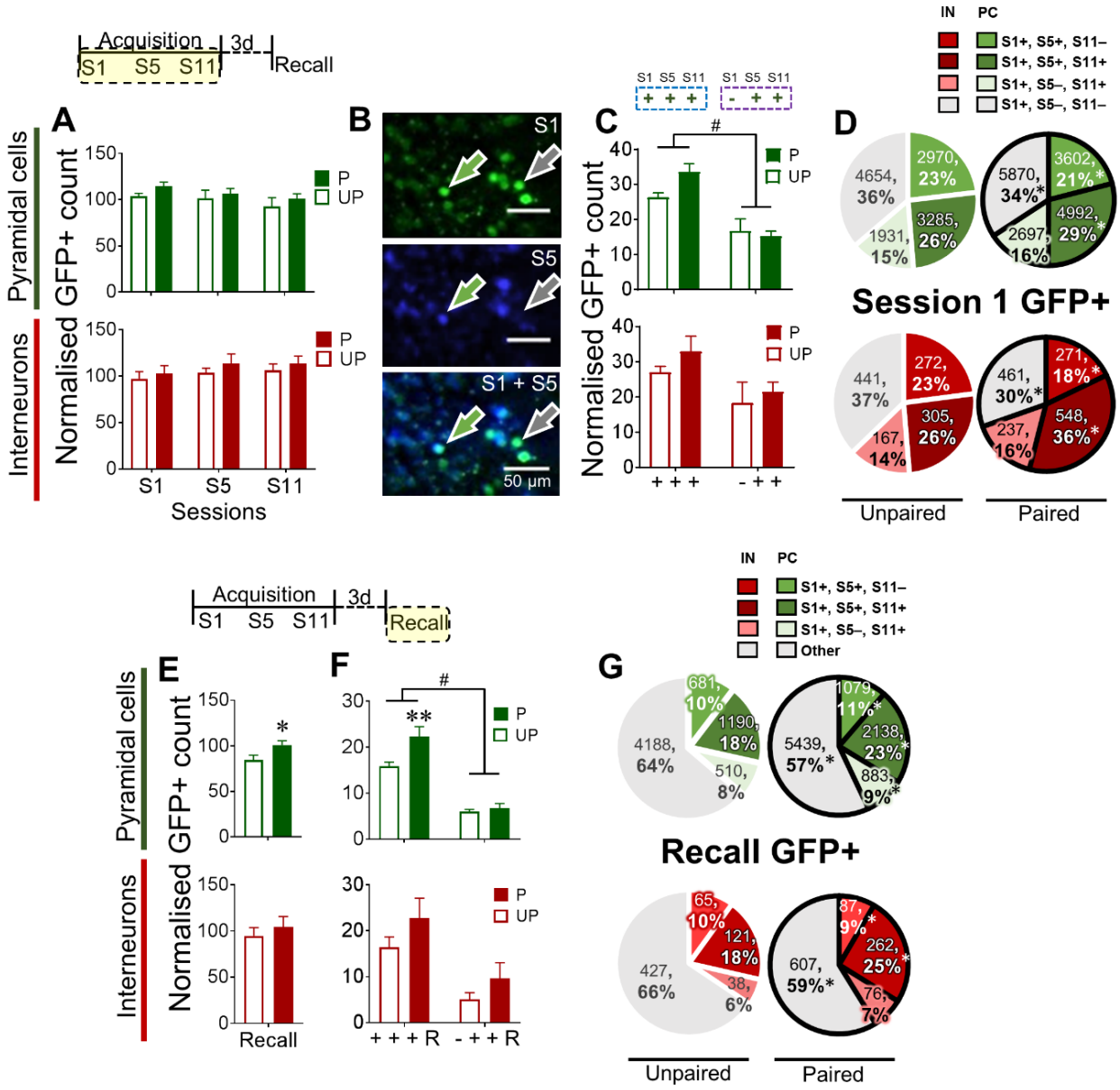


Figure 3: Conditioning and memory recall recruits a stable pyramidal cell ensemble from the initial acquisition session. (A) Normalized GFP+ counts of pyramidal cells (green) and interneurons (red) during acquisition sessions. **(B)** Representative image of longitudinal GFP imaging (S1 and S5); green arrow S1+|S5+ neurons, grey arrow S1+|S5- neurons. **(C)** Normalized GFP+ counts of pyramidal cells (PC) and interneurons (IN) with a S1 (+ + +) or no S1 (– + +) activation history. **(D)** Distribution of GFP+ pyramidal cells and interneurons activated during S1 classified according to their subsequent reactivation patterns (S1+|S5+,S11+; S1+|S5+, S11-; S1+|S5-, S11+; S1+|S5-,S11-) for Paired and Unpaired mice. **(E)** Normalized GFP+ counts of pyramidal cells and interneurons following the test for memory recall. **(F)** Normalized GFP+ counts of pyramidal cells and interneurons recruited during the test for recall that had been persistently activated during training, as a function of their S1 activation history (+ + + R or – + + R). **(G)** Distribution of GFP+ pyramidal cells (PC) and interneurons (IN) activated during the test for recall, classified according to their activation patterns from S1 onwards in Paired and Unpaired mice. 'Other' refers to neurons recruited during recall that did not demonstrate activation histories of interest (e.g. S1-|S5-, S11. Data on bar graphs are expressed as Mean±SEM. Normalization of

GFP+ counts according to HC ((number GFP+ / av. number GFP+ in HC) *100). *Interaction effect: # P<0.05*, *Post-hoc analysis: *P<0.05, **P<0.01, Paired (P) n=10, Unpaired (UP) n=9 for acquisition, Paired (P) n=6, Unpaired (UP) n=6 for recall.*

2.3.3 High GFP expression in S1 predicts reactivation, regardless of conditioning

Robust activation of the promoter of the immediate early gene *arc* has been shown to predict subsequent reactivation in motor cortex neurons during motor learning (Cao et al., 2015). This gene is expressed following activation of a similar signal transduction cascade as Fos (Barry et al., 2016).Therefore, we hypothesized that the relative intensity of GFP in early learning, an indicator of cellular Fos expression (Barth et al., 2004), would predict subsequent reactivations during conditioning. To examine this, we compared the relative GFP intensity (Signal normalized to Background; Fig. 4A) of neurons that were activated in S1 and persistently reactivated during conditioning (S1+|S5+S11+) to neurons that were persistently dismissed (S1+|S5-S11-). We classified these neurons as ‘High’, ‘Mid’ or ‘Low’ Brightness (Fig. 4A) and quantified the number of neurons in each Brightness Category according to their Activation History and the conditioning Group. In both pyramidal cells and interneurons, there was a significant interaction between Brightness Category x Activation History (Pyramidal cells: $F_{2,34}$=151.31, P<0.001; Interneurons: $F_{2,34}$=13.42, P<0.001) but no effect of Group (Suppl. Table 4; Fig. 4B). Thus, high GFP intensity is only a general predictor of neuronal reactivation during training irrespective of appetitive learning.

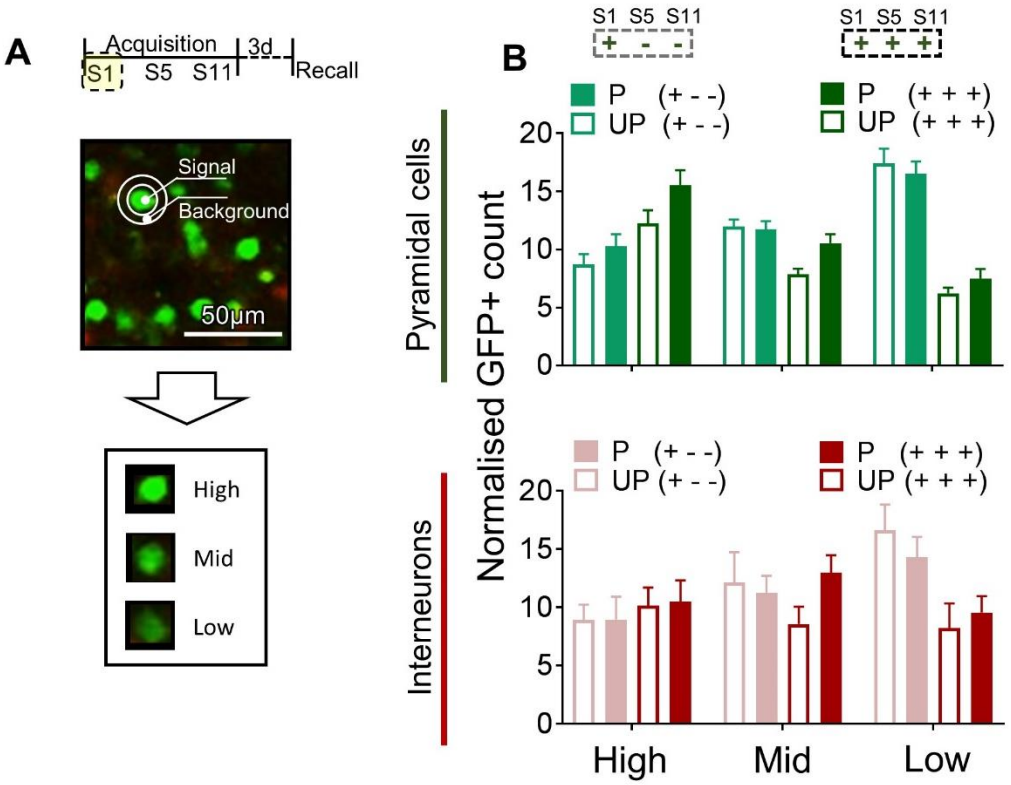


Figure 4: High GFP intensity in S1 predicts persistent reactivation, regardless of conditioning. (A) Relative GFP intensity was obtained by normalizing the signal of the cell to the surrounding background. Neurons were categorized as 'High', 'Mid' and 'Low' Brightness according to their relative intensity. **(B)** Normalized GFP+ counts in each Brightness Category for pyramidal cells (green) and interneurons (red) activated in S1 that show subsequent persistent reactivation (dark green/red) or dismissal (light green/red). Data on bar graphs is expressed as Mean±SEM. *Paired (P): n=10, Unpaired (UP) n=9.*

2.4 Discussion

Our findings show that the establishment of a CS-US association is associated with the recruitment of a repeatedly activated ensemble of pyramidal cells in the dmPFC from a wider pool of neurons activated in the initial conditioning session, when mice did not exhibit cue-selective food-seeking. Only a minority (29%) of pyramidal cells from this wider pool of candidate neurons were recruited into this stable ensemble. This recruitment was indicated by consistent reactivation across conditioning sessions and in the subsequent test for memory recall. Finally, irrespective of conditioning, pyramidal cells that were subsequently reactivated were strongly activated in the initial training session. These findings provide novel insights into how neuronal ensembles are formed to encode cue-evoked appetitive memories that elicit and guide food-seeking behaviour.

2.4.1 Appetitive memory formation recruits a stable group of pyramidal cells from the initial conditioning session

Previous studies across multiple cortico-limbic brain areas have demonstrated that learning recruits a subset of neurons that are persistently reactivated across training and memory recall (Cao et al., 2015; Czajkowski et al., 2014; Mattson et al., 2008; Tayler et al., 2013). We extend these findings to show that these persistently reactivated neurons are recruited from a neuronal pool activated in the initial conditioning stage. This suggests that activation history is important in the inclusion of neurons to stable ensembles. Furthermore, this persistently reactivated neuronal subset was recruited again during a later test for memory recall (i.e. in the absence of the US), suggesting that this ‘stable’ neuronal representation plays a role in encoding the CS-US association. These persistently activated neurons only represented 23% of neurons activated by memory recall. Selective silencing of CS-activated neurons in the mPFC has been shown to disrupt reward seeking (Bossert et al., 2011; Suto et al., 2016). Thus, our results raise

the possibility that the CS-US memory trace may be encoded in only a subset of CS-activated neurons. As such, the manipulation of CS-activated neurons may act preferentially through these subsets.

In contrast, we did not observe an increased recruitment of repeatedly activated interneurons in conditioned mice. It is worth noting, however, that we did observe increased likelihood of reactivation following S1 at a population level in interneurons as well as pyramidal cells. A contributing factor to this discrepancy may be the large variability we observed between mice, as the population level analysis does not take into account individual variability. Moreover, multiple classes of cortical interneurons exist that vary in their functional characteristics and thus in their activity patterns during food-seeking (Gaykema et al., 2014; Xu et al., 2010). Previous evidence has demonstrated that ensembles of interneurons are recruited during learning and may function to regulate the size and development of pyramidal cell ensembles (Rashid et al., 2016; Stefanelli et al., 2016) in both the amygdala and hippocampus. Thus, further work will be necessary to fully elucidate the role of specific dmPFC interneurons in the stabilization of pyramidal cell ensembles during appetitive conditioning.

Parallel work performed by Joseph Ziminski in the Koya lab identified that activated pyramidal cells were hyper-excitable following the initial, but not late conditioning session in Paired mice (Suppl. Fig. 2 & 3; Brebner et al., *in preparation*). This change in excitability was associated with a bi-directional regulation of GFP+ and GFP– firing capacity that increased and decreased across sessions, respectively (Suppl. Fig. 3). Recent evidence has determined that hyper-excitability facilitates allocation of neurons into memory-encoding ensembles (Cai et al., 2016; Yiu et al., 2014). Moreover, we observed that the neuronal pool activated by early learning was more likely to reactivate in subsequent training sessions in Paired mice compared to Unpaired mice. We cannot yet identify and measure the excitability *in vivo* from those neurons in the early learning pool that will be persistently reactivated in subsequent sessions. However, this

nevertheless suggests that increased excitability may have a role in consolidating neurons to the stable learning ensemble; for example in promoting neuronal activation in post-learning replay (de Sousa et al., 2019). Further studies will be necessary to determine the exact impact of neuronal hyper-excitability on the recruitment of ensembles in the dmPFC.

2.4.2 No observed learning-specific changes in total recruitment and GFP intensity

While more neurons are persistently reactivated in conditioning in Paired mice compared to Unpaired mice, we did not observe a significant difference in the total recruitment of neurons during conditioning. This is likely due to our use of an Unpaired group that received both novel context exposure and sucrose, and is in line with a previous study that examined Fos expression in the dmPFC with similar controls (Nordquist et al., 2003). However, we observed a general decrease of activation across training independent of conditioning group. Learning has been shown to recruit broad populations of neurons which decrease in size across learning (Cao et al., 2015; Peters et al., 2014). Furthermore, a number of studies demonstrate that novelty itself activates a large population of neuron which decreases during habituation (Struthers et al., 2005; VanElzakker et al., 2008). In our experiment, the decrease in activated neurons as learning progressed was not specific to conditioned mice, suggesting that it is dependent on habituation rather than learning. However, further work will be necessary using novelty-controls to fully characterise how ensembles are refined during associative learning and how this differs from habituation-induced disengagement of neurons.

Furthermore, persistently reactivated pyramidal cells and interneurons were more likely to display high GFP intensity in early training, regardless of conditioning. GFP is highly co-expressed with Fos (Barth et al., 2004; Cifani et al., 2012; Koya et al., 2012), therefore, this suggests that, independently of associative learning, high Fos expression

in early learning predicts subsequent reactivation. One contributing factor to this reactivation may be the pre-existing connectivity of neurons that are reactivated. Robust Fos expression has been shown to be a marker of increased excitatory inputs to the cell (Cruz et al., 2013). As such, high Fos in activated neurons may signal the presence of task-relevant connections, leading to preferential recruitment when the same stimuli are presented again. However, the level of Fos expression itself may also influence the likelihood of reactivation. Fos is a transcription factor (Morgan and Curran, 1991) that targets genes linked with neuronal plasticity and has been shown to modulate experience-dependent activity patterns (de Hoz et al., 2018; Jaeger et al., 2018). As such, increased neuronal Fos in early learning may trigger plasticity mechanisms which will promote reactivation in subsequent sessions. However, further studies will have to be performed to determine the influence of both pre-training connectivity and post-training plasticity in the recruitment of persistently activated ensembles.

Together, these findings support the existence of recruitment mechanisms and activation patterns in the dmPFC that are independent of associative learning. Both context (Hyman et al., 2012) and sucrose exposure (Gaykema et al., 2014; Petykó et al., 2009) alone have been shown to activate populations in the mPFC and may play a part in the neuronal recruitment observed here. We also add to a number of studies suggesting that it is crucial to take into account both activation history and selective adaptations of neurons that are activated by an experience (Suto et al., 2016; Ziminski et al., 2018).

2.4.3 Potential functions of the persistent activation of a dmPFC pyramidal cell ensemble throughout appetitive conditioning

We observed that a stable conditioning ensemble may arise from a pool of neurons activated during the initial conditioning session and then become persistently re-

activated throughout conditioning. This prompts the question: what function do these ensemble neurons serve during conditioning?

The dmPFC is thought to act as part of a large interconnected network in appetitive conditioning (Martin-Soelch et al., 2007). Moreover, brain-wide Fos mapping has shown that the dmPFC is co-activated with several different brain areas during fear memory recall, further supporting that it is part of larger distributed network of areas involved in encoding CS-US memories (Wheeler et al., 2013). As such, early recruitment of the conditioning ensemble may serve to activate other learning-relevant downstream targets from the first learning session. For example, dmPFC sends projections to the dorsomedial striatum (DMS) (Berendse et al., 1992; Fillinger et al., 2018), a region that has been shown to mediate early learning for both appetitive associations (Cole et al., 2017) and motor skills (Yin et al., 2009). Furthermore, dmPFC projections to the nucleus accumbens have been shown to mediate behavioural responses elicited by reward cues (Otis et al., 2017; Parkinson et al., 2000). Thus, early recruitment of the dmPFC ensemble may contribute to mediating motivational vigour necessary to learn about the various attributes of the CS and US in early conditioning sessions. Additionally, the dmPFC plays a role in selectivity of responses to food cues (Cardinal et al., 2002; Parkinson et al., 2000) and attentional processes during learning (Bryden et al., 2011; Totah et al., 2009). Recruitment of this ensemble from early learning may be associated with the engagement of conditioning-relevant attentional processes from initial exposures to CS-US pairings. As conditioning progresses, persistent activation of the dmPFC ensemble may strengthen these processes through continued activation and promote cue-selective behaviours.

Repeated activation is thought to consolidate neurons into ensembles (Mattson et al., 2008). Thus, we theorize that early recruitment of the dmPFC ensemble may maximise the number of activation these neurons undergo and, through this, contribute to strengthening of a stable ensemble during conditioning (Matsuo, 2015). However, the

stable ensemble we observed only represents 29% of the early learning pool (Fig. 3D). As such, this suggests that not all neurons in downstream areas initially targeted by the dmPFC output would have been persistently re-activated as learning progressed. Furthermore, these findings may also indicate that inputs to the dmPFChave changed as learning progressed. In support, learning-induced alterations in activation across a network of brain areas has previously been observed in appetitive conditioning, suggesting that modulations in dmPFC activation happen within the context of brain-wide adaptations (Cole et al., 2015).

In summary, repeated activation of a subset of a pool of dmPFC neurons activated in the early phases of conditioning may contribute to the establishment of a CS-US association. In turn, this may facilitate the selection of appropriate behavioural responses through output to and actions on target regions (e.g., adapted attentional and motivational vigour processes for efficient food seeking). However, further work at the network-wide level is necessary to fully determine the specific contribution of these early learning activated neurons and their reactivation patterns.

2.4.4 Conclusion

Despite the importance of appetitive conditioning for survival, few studies have established its precise mechanisms at the neuronal ensemble level in the dmPFC. We have revealed ensemble recruitment patterns that underlie the establishment of cue-evoked food-seeking. In particular, this work demonstrated that the consolidation of conditioning is associated with the emergence of a repeatedly activated conditioning ensemble from a wider early learning pool of pyramidal cells. However, one limitation of this work is that we cannot determine if neurons recruited during conditioning sessions (e.g., early learning) are specific to the learning task. Thus, our findings here warrant

further investigations into determining the behavioural relevance of the early learning pool.

Chapter 3: Repeatedly enhancing the excitability of early learning activated neurons hinders appetitive conditioning

3.1 Introduction

Appetitive conditioning is the process in which neutral stimuli (CS) gain the ability to stimulate food-related behaviours following repeated pairing with appetitive unconditioned stimuli (US) (van den Akker et al., 2018; Fanselow and Wassum, 2016; Holland, 1984; Pavlov (1927), 2010; Rescorla, 1988). The dmPFC is thought to have a role in these behaviours, in particular in establishing response selectivity and mediating attention (Bryden et al., 2011; Cardinal et al., 2002; Parkinson et al., 2000; Totah et al., 2009). Crucially, Fos-expressing ensembles of the dmPFC have been shown to be involved in mediating these behaviours following associative learning (Calu et al., 2013; Whitaker et al., 2017).

In the previous chapter, we demonstrated that consolidation of conditioning is associated with the recruitment of a stable, persistently activated ensemble from a pool of pyramidal cells activated in early learning. However, whether this early learning activated pool is relevant to the task is unclear. Furthermore, intrinsic excitability recordings revealed that activated pyramidal cells were hyper-excitable in early but not late conditioning (Suppl. Fig. 2 & 3, Brebner et al., *in preparation*). Neuronal excitability, which reflects firing properties, has been shown to be altered by learning both at a general population level (Kim et al., 2011; Mu et al., 2010; Santini et al., 2008) and within specific ensembles (Whitaker et al., 2017; Ziminski et al., 2017, 2018). Excitability contributes to modulating neuronal output and, as such, ensemble-specific intrinsic plasticity may play a crucial role in encoding properties of food-cue associations and any changes thereof (e.g., updated reward contingency (Ziminski et al., 2017; Sieburg et al, *in press*)). Furthermore, in operant appetitive conditioning, dmPFC ensembles increase their excitability as

learning progresses (Whitaker et al., 2017). Together, this suggests that the alterations in the excitability of the early learning pool may be involved in ensemble formation during appetitive learning. However, how these specific alterations might contribute to the consolidation of an appetitive association is unclear.

Thus, in this chapter, we directly tested the functional relevance of the early learning activated pool and the modulations of excitability of activated neurons during learning. To achieve this, we utilised a chemogenetic approach: the TetTag DREADD method (Zhang et al., 2015). Briefly, a viral construct containing the gene for a DREADD (Designer Receptor Exclusively Activated by Designer Drugs), whose expression is controlled by the presence of unbound tetracycline transactivator protein (tTa), is injected into the brain of *Fos-tTa* mice. tTa is bound by the antibiotic Doxycycline, hence the expression of the DREADD depends both on the absence of Doxycycline and the activation of the *Fos* promoter (Fig. 2D). In our study, the DREADD we utilized was the excitatory hM3Dq receptor. hM3Dq is a G protein coupled receptor derived from a muscarinic receptor (Armbruster et al., 2007). When activated by subclinical doses of clozapine, it will signal through the Gq pathway in order to depolarise neurons and increase firing rates (Alexander et al., 2009). With this method, we specifically tagged neurons in the dmPFC that were activated following either early learning or novel context exposure and enhanced their excitability throughout appetitive conditioning.

We found that repeatedly inducing a hyper-excitable state in early learning activated neurons interfered with conditioning, suggesting that the dissipation of this hyper-excitability is necessary for the stabilisation of associative memory.

3.2 Methods

3.2.1 Animal breeding and housing

Heterozygous (het) male *Fos-tTa* mice (RRID: MMRRC_031756-MU), were bred onto a C57Bl/6 background. Male *Fos-tTa* mice and their wild-type (WT) male littermates were used for chemogenetics experiments. As described in the previous chapter, mice were housed under a 12-hours light/dark cycle (lights on at 7:00) at the maintained temperature of 21+/-1 °C and 50 +/-5% relative humidity. Animals were aged 7-12 weeks at the beginning of experimental procedures, and were food restricted (90% baseline body weight) 1 week prior to behavioural testing until the completion of behavioural experiments. Experiments were conducted in accordance with the UK 1986 Animal Scientific Procedures Act (ASPA) and received approval from the University of Sussex Ethics Committee.

3.2.2 Surgical Procedures

3.2.2.1 Generation of AAV particles (performed by Gabriella Margetts-Smith)

All AAV transgenes were packaged into AAV capsids, serotype AAV2. HEK293 cells were co-transfected with the transgene construct plasmid pAAV-PTRE-tight-hM3Dq-mCherry which was a gift from William Wisden (Zhang et al., 2015) (Addgene plasmid # 66795), the adenovirus helper plasmid pHelper (Stratagene) and the AAV2 helper plasmid pRC (Stratagene) using the calcium phosphate method. The cells were harvested and pelleted 72 hours after transfection and re-suspended in lysis buffer (150 mM NaCl, 20 mM Tris pH 8.0). Benzonase endonuclease (Merck; E1014) was added and the cell lysate was incubated at 37°C for 30 minutes, before being centrifuged and the supernatant purified by the iodixanol gradient method. Optiseal tubes (Beckman Coulter; 361625) were prepared with iodixanol gradients overlayed in the following order; 5 ml 15% in PBS-MK, 5 ml 25% in PBS-MK with phenol red, 6 ml 40% in PBS-MK, and

9 ml 60% with phenol red. The supernatant was then overlayed and the tube sealed, then centrifuged at 461000 g for 1 hour at 18°C. The AAV particles were collected from the 20% layer by piercing the tube horizontally with an 18G needle, and concentrated using Amico Ultra-4 (Merck; UFC810008) at 2000 g for at minimum of 20 minutes. The elution was re-suspended with 250 µl dPBS and aliquoted and stored at -80°C. The final titer was $1.67x10^{10}$ copies/ml.

3.2.2.2 Virus microinjection in dmPFC of Fos-tTa and WT mice

7-12 week old *Fos-tTa* and WT mice received bilateral injections of AAV_2-TRE_{tight}-hM3Dq-mCherry (Zhang et al., 2015) in the medial prefrontal cortex (coordinates: AP: bregma +1.2, ML +/- 0.5, DV – 1.2). Mice were anesthetised with isoflurane 3% dilution in O_2 (0.8 L/min) and NO_2 (0.5 L/min) and kept between 1 and 2% dilution throughout the surgery. Using a mounted drill, openings were created at the anterior-posterior and medio-lateral coordinates. Custom-built infusers – assembled from 26G 30mm and 33G 65mm stainless steel tubes (Coopers needle works LTD., Birmingham, UK) – were then lowered to the dorsal-ventral coordinates and 0.5 µL/hemisphere of virus was injected at a rate of 0.1 µL/min. The infusers remained in the brain 7 min before being raised gradually. Mice received Meloxicam (Metacam, Boehringer, Berks, UK) orally for 1 day prior to and 3 days post-surgery for analgesia and reducing inflammation. A week following surgery and for the duration of the experiment, mice received Doxycycline in their drinking water (0.1 mg/mL) to prevent any unwanted transgene expression.

3.2.3 Behavioural experiments

3.2.3.1 General Training Procedures

Similar behavioural experimental procedures and apparatus were utilised as in the previous chapter. Briefly, behavioural experiments were performed in standard mouse operant chambers (15.9 x 14 x 12.7 cm; Med Associates, Vermont, USA). Each chamber was fitted with a recessed magazine that dispensed 10% sucrose solution serving as the unconditioned stimulus (US) and a mechanical click generator providing a sound which served as a conditioned stimulus (CS). An infrared beam detected head entries into the food magazine. Two days following Magazine training (in which mice were pre-trained to the sucrose delivery magazine), mice underwent 12 acquisition sessions over a 7 day period for 1-2 sessions per day. The first and second session were separated by 24h (Fig 1A, 2A, 3A, 4A). As previously, each 25 min acquisition session consisted of six 120s CS presentations, separated by 120s random –interval (RI) inter-trial interval periods. During each CS period 10% sucrose was delivered to the magazine for all mice. Twelve acquisition sessions produced selective responding to the CS. 3 days following the last acquisition session, mice were tested for Pavlovian conditioning with a cue exposure test: Paired mice were placed in the conditioning chamber and tested under extinction conditions for 6 CS presentations.

3.2.3.2 Experiment-specific Procedures

Repeated clozapine administration experiment: WT mice that had not undergone surgery were trained as described in the General Procedures. Mice received clozapine injections (0.1 mg/kg, i.p.) 15 minutes prior to the beginning of every two sessions (Fig. 1A). To habituate mice to injections, 4-5 saline injections were delivered to them over the week preceding training.

'S1 tag' and 'NC tag' experiments: In both experiments, *Fos-tTA* and WT mice injected with AAV_2-TRE_{tight}-hM3Dq-mCherry underwent identical behavioural procedures with the exception of the 'tagging session' that could either be a conditioning session as described above or a 25 minutes novel context exposure ('NC tag'). Previous studies have shown that novel context exposure recruits neurons that are unrelated to appetitive learning (Cruz et al., 2014), and thus, this exposure served to tag such neurons here.

Immediately following Magazine training, Doxycycline was removed from the drinking water for 48 h at which point mice underwent the 'tagging session' to label activated neurons in Fos-tTa mice with hM3Dq. An hour following this tagging session, mice received high Doxycycline drinking water (1 mg/mL) for 24h before undergoing normal a conditioning session and receiving low Doxycycline drinking water (0.1 mg/mL) for the remainder of the experiment. Conditioning sessions then proceeded as described in *General procedures* until the completion of a total of 12 conditioning sessions. Mice received clozapine injections (0.1 mg/kg, i.p.) 15 minutes prior to the beginning of every two sessions (Fig. 3A & 4A). To habituate mice to injections, 4-5 saline injections were delivered to them over the week preceding training.

3.2.4 Histology

Fos-tTa and WT mice were anesthetized with 200 mg/kg, i.p. sodium pentobarbital and transcardially perfused with 4% paraformaldehyde (PFA). To assess mCherry expression, free-floating sections were washed in Tris-buffered saline (TBS: 0.025 M Tris-HCl, 0.5 M NaCl, pH 7.5) and blocked in 10% normal goat serum (Cat # S-1000, VectorLabs, RRID:AB_2336615) in TBST (TBS, 0.2% Triton-X 100). Slices were incubated at 4'C overnight in anti-mCherry primary antibody (Cat # ab205402, Abcam, RRID: AB_2722769) diluted 1/2000 In 3% normal goat serum TBST. The following day slices were incubated 2 hours in anti-chicken 568 antibody (Cat# 20104-1, Biotium, RRID: AB_10853460) at 1/200 in TBST. Slices were mounted on Superfrost Plus slides

(Cat # UY-48512-00, Cole-Parmer), air-dried, and coverslipped with PermaFluor (Cat#TA-030-FM, Thermo Scientific, RRID: SCR_014787). Fluorescence images of mCherry staining (Fig. 5B) from both left and right hemispheres of the anterior cingulate cortex of 2-4 coronal sections per animal, corresponding approximately to Bregma 1.2 (Paxinos and Franklin, 2001), were captured using QI click camera (Qimaging) attached to an Olympus Bx53 microscope (Olympus). *Fos-tTA mice* not expressing mCherry (n=2) were excluded from the study.

3.2.5 Data analysis

In the main text, we only report effects and interactions key to interpretation. A complete report of statistical results for all experiments can be found in Suppl. Table 6. For all ANOVAs and *t* tests, data were assumed to be normally distributed although this was not formally tested. All data were analysed using GraphPad Prism (RRID:SCR_002798; GraphPad Software) and SPSS (IBM SPSS Statistics, Version 23.0 (2015), Armonk, NY: IBM Corp). Group data are presented as mean±SEM.

All behavioural data was tested either with 3-way mixed ANOVAs or with 2-way mixed ANOVAs in Prism as appropriate. Following 2-way mixed ANOVAs, further post-hoc tests were performed (Sidak correction) if a significant interaction was observed ($P<0.05$).

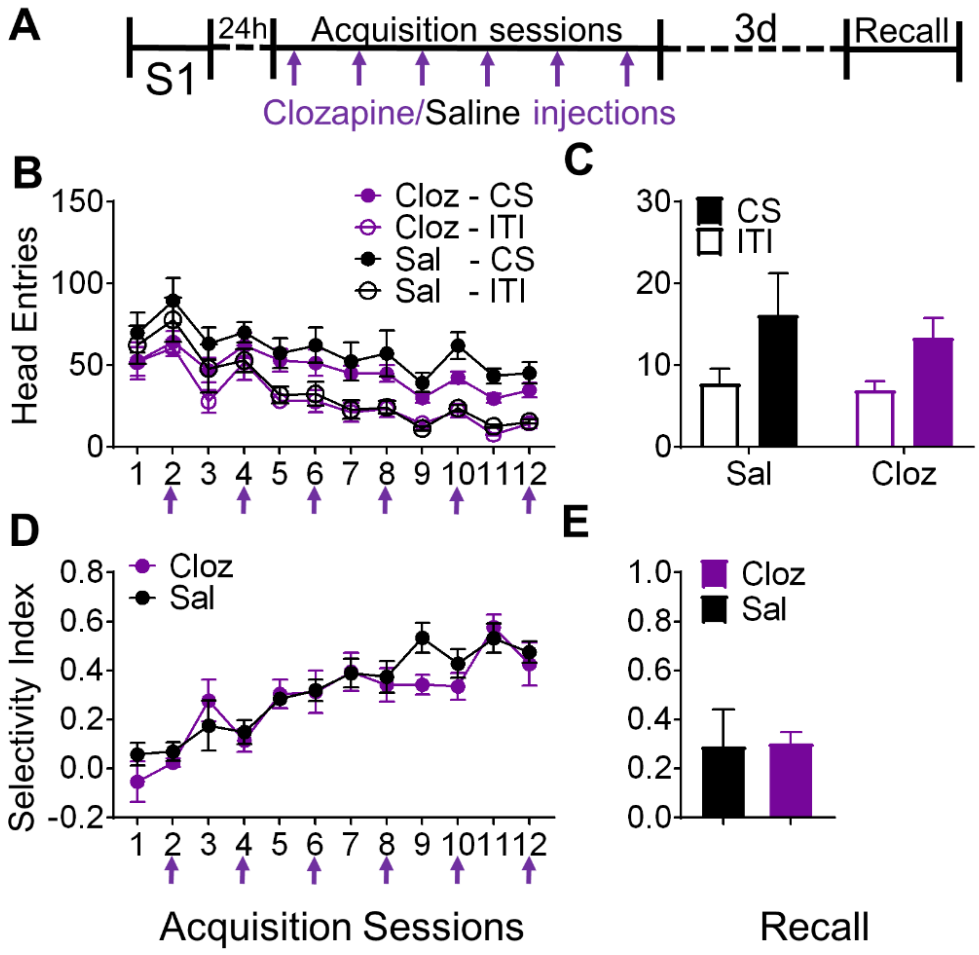


Figure 1: Repeated subclinical clozapine injections do not affect appetitive conditioning. (A) Timeline of conditioning and clozapine (Cloz) or saline (Sal) injections. **(B)** Head entries into the magazine during the CS (cue) compared to ITI (no cue) periods in mice receiving Clozapine (purple) or Saline (black) injections following acquisition of conditioning and **(C)** Cue exposure test (Recall). **(D)** 'Selectivity index' of responses (CS-ITI/total number head entries) during Acquisition and **(E)** Recall. Mice received clozapine injections on sessions 2, 4, 6, 8, 10 and 12 (purple arrows). All data are expressed as Mean±SEM. *Cloz: n=5, Sal: n=5.*

3.3 Results

We trained both *Fos-tTA* and wild-type (WT) mice in an appetitive conditioning task similar to the one described in Chapter 2. (Fig 1A, 3A, 4A). Across 12 'Acquisition' sessions, mice received repeated trials during which an auditory cue (CS) was paired with liquid sucrose delivery (US). Three days following the last Acquisition session, mice were tested in a 'Recall' test for CS-US memory recall under extinction conditions, (Fig. 1A, 3A, 4A). As previously, we also assessed all conditioning performance by calculating a 'Selectivity Index' for each mouse (Fig. 1D, 3D, 4D), a parameter measuring the robustness of selective CS responses by subtracting inter-trial interval (ITI) responses and normalizing to total head entries.

3.3.1 Repeated sub-clinical clozapine did not disrupt conditioning

We use clozapine as an agonist for the hM3Dq DREADD in TetTag DREADD experiments. Clozapine has been shown affect learning (Hou et al., 2006; Rasmussen et al., 2001; Rosengarten and Quartermain, 2002), therefore, we first assessed the effect of repeated sub-clinical clozapine (0.1mg/kg) injections on behavioural responses in our appetitive conditioning task. We trained 2 groups of WT mice (as described above); one group received repeated clozapine injections every second session while the other received repeated saline injections. During Acquisition, we observed a significant interaction of Cue X Session (Fig. 1B; $F_{11,88}$=4.705, P<0.001) and in Recall, a significant effect of Cue (Fig. 1C; $F_{1,8}$=10.23, P<0.05), indicating that mice were conditioned. We observed no significant effect of Clozapine on responses during Acquisition or Recall (Suppl. Table 6). We also assessed conditioning performance with the Selectivity Index. In Acquisition (Fig. 1D), there was no significant effect of Clozapine ($F_{1,8}$=1.12, P=0.321) and no interaction of Clozapine X Session on performance ($F_{11,88}$=0.79, P=0.645). We also observed no effect of Clozapine on performance during Recall (Fig. 1E; t_8=0.01,

P=0.942). Therefore, repeated injections of our chosen dose of clozapine did not significantly affect responding during appetitive conditioning.

However, we cannot dismiss that there may be effects of clozapine injections that were not detected here. Therefore, in subsequent experiments, we injected both *Fos-tTA* (hM3Dq+) and control WT (hM3Dq-) mice with clozapine.

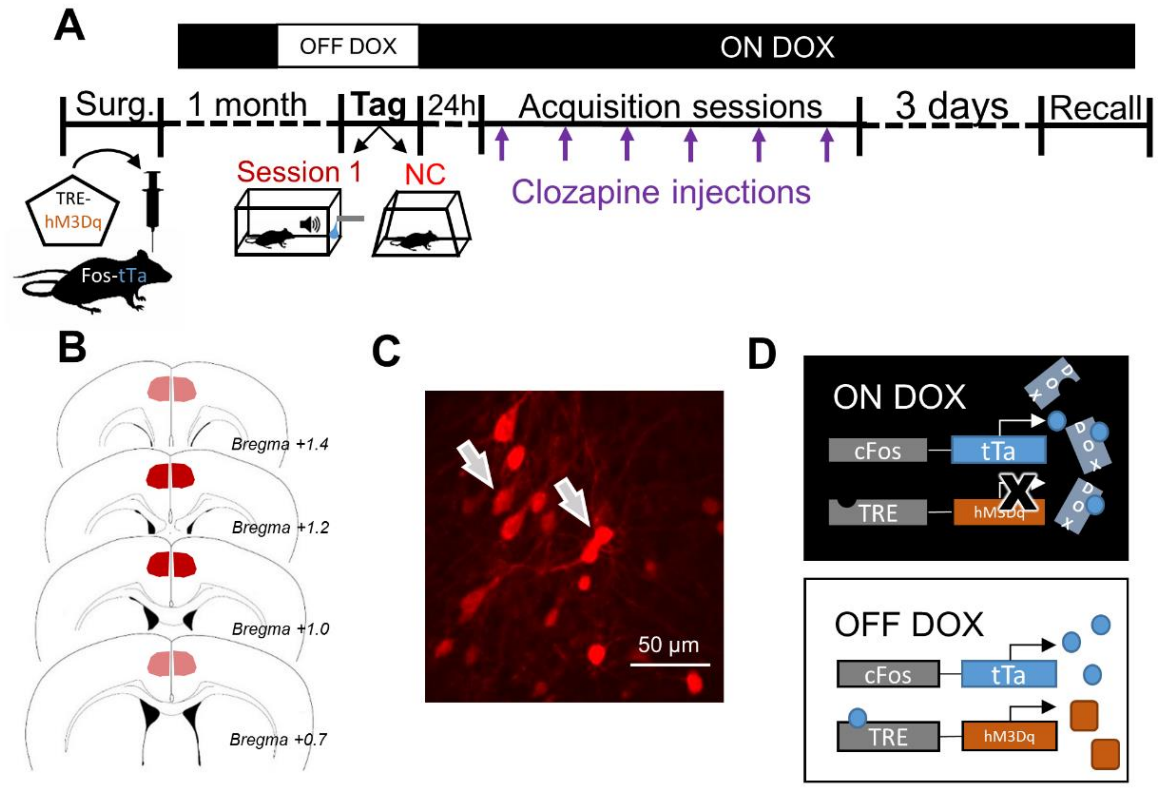


Figure 2: Timeline of experimental procedures. (A) Timeline of surgical procedures, tagging (Session 1 or Novel Context (NC), conditioning and clozapine injections. All mice received clozapine injections on sessions 2, 4, 6, 8, 10 and 12 (purple arrows). Doxycycline was presented in the drinking water of all mice for the majority of the experiment and was removed only from 48h before the tagging session to 1h after. **(B)** Schematic representation of viral microinjection site. **(C)** Representative image of mCherry staining in the dmPFC of *Fos-tTa* mice, arrows indicate hM3Dq+ neurons. **(D)** Schematic representation of TetTag DREADD system. When mice are 'ON DOX', tTa is bound to Doxycycline and hM3Dq is not expressed in *Fos-tTa mice*. When mice are 'OFF DOX', tTa is unbound, hM3Dq is expressed in *Fos-tTa mice*.

3.3.2 Enhancing the excitability of S1 neurons during conditioning impairs learning

In order to assess the relevance of the disappearance of hyper-excitability from the early learning pool, we tagged neurons activated following S1 with the excitatory DREADD hM3Dq in *Fos-tTA* mice using the TetTag DREADD approach (Figs. 2A, 2C, 3A) (Zhang et al., 2015). We repeatedly activated these tagged neurons using the hM3Dq agonist clozapine (0.1 mg/kg) (Gomez et al., 2017) to artificially enhance their excitability throughout conditioning. *Fos-tTA* mice and control wild-type (WT) mice not tagged with hM3Dq underwent Acquisition and Recall sessions as described above.

During Acquisition (Fig 3B), there was a significant interaction of Cue X Session ($F_{11,176}$=6.94, P<0.001) and a significant effect of Cue during Recall (Fig. 3C; $F_{1,16}$=9.03, P<0.01), indicating that mice were conditioned. In Acquisition, we observed a significant interaction of Cue X Session X hM3Dq ($F_{11,176}$=2.00, P<0.05) on the number of responses (Fig. 3B). Furthermore, there was also a significant interaction of hM3Dq X Session ($F_{11,176}$=3.81, P<0.001) on performance, as assessed by the Selectivity Index (Fig. 3D). Post-hoc testing revealed significantly lower performance in *Fos-tTa* mice on a number of Acquisition sessions (P<0.05). Thus, repeatedly enhancing the excitability of S1-activated neurons interfered with conditioning. During Recall, we did not observe a significant interaction of hM3Dq X Genotype on responding (Fig. 3C; $F_{1,16}$=3.82, P=0.068) and no significant effect of hM3Dq on performance (Fig. 3E; t_{16}=1.29, P=0.214).

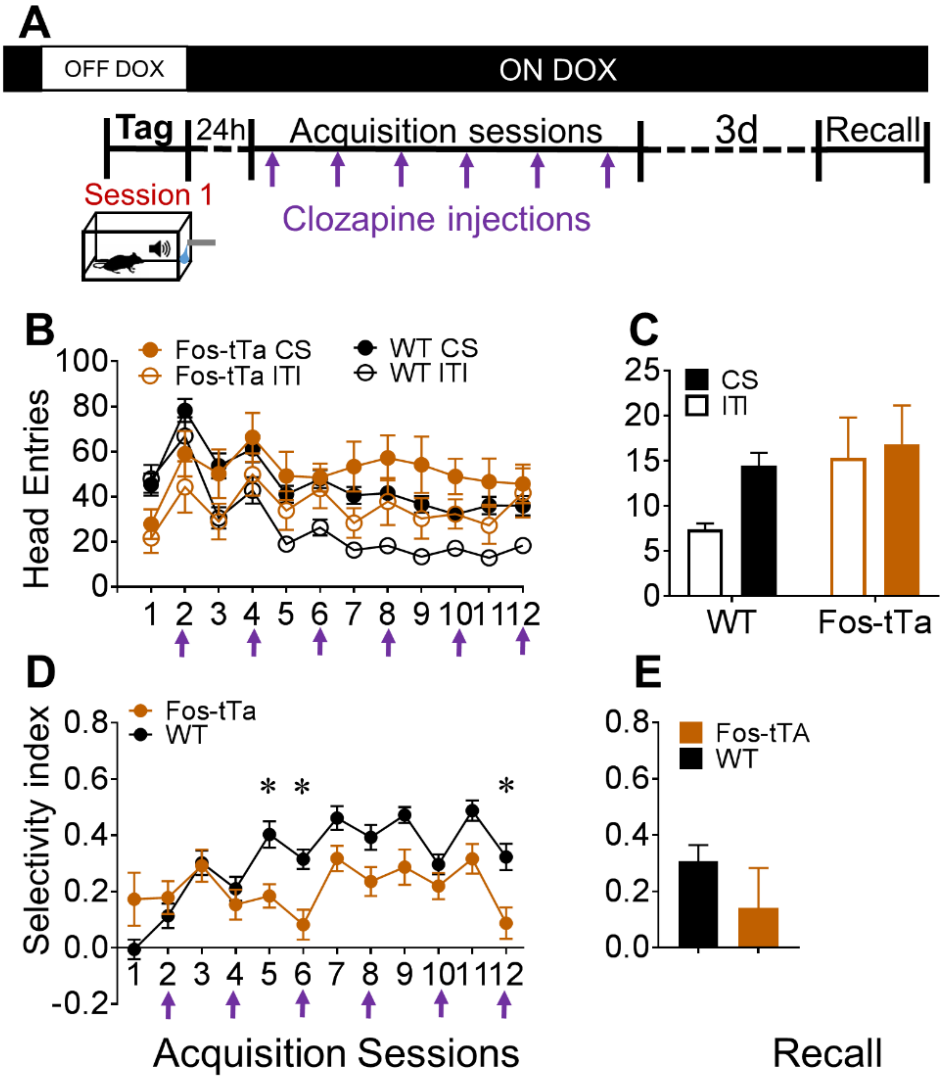


Figure 3: Repeatedly enhancing S1-activated neurons in a hyper-excitable state impairs conditioning. (A) Timeline of tagging, conditioning and clozapine injections. **(B) & (C)** Head entries into the magazine during the CS (cue) compared to ITI (no cue following Acquisition of conditioning and cue exposure test (Recall) in *Fos-tTA* (hM3Dq+; orange) and WT (hM3Dq–; black) mice. **(D) & (E)** 'Selectivity index' of responses (CS-ITI/total number head entries) during Acquisition and Recall) in *Fos-tTA* (hM3Dq+; orange) and WT (hM3Dq–; black) mice. Mice received clozapine injections on sessions 2, 4, 6, 8, 10 and 12 (purple arrows). All data are expressed as Mean±SEM * P<0.05; *Fos-tTa: n=6, WT: n=12.*

3.3.3 Enhancing the excitability of NC neurons during conditioning has no effect on learning

Next, to confirm that this effect was specific to S1-activated neurons, we enhanced the excitability of neurons tagged following neutral, novel context (NC) exposure throughout Acquisition, once more using the TetTag DREADD approach (Fig. 2A, 2B, 4A). NC exposure activates neurons that are distinct from appetitive cues (Cruz et al., 2014; Fanous et al., 2012). We observed a significant interaction of Cue X Session during Acquisition ($F_{11,176}$= 5.19, P<0.001; Fig. 4B) and a significant effect of Cue during Recall ($F_{1,16}$=45.53, P<0.001; Fig. 4C) indicating that mice are conditioned. However, we observed no significant effect of hM3Dq in either Acquisition (Fig. 4B) or Recall (Fig. 4C; Suppl. Table 6). We then assessed conditioning performance with the Selectivity Index and detected no significant interaction of hM3Dq X Session in Acquisition (Fig. 4D; $F_{11,176}$=0.33, P=0.980) and no effect of hM3Dq in Recall (Fig. 4E; t_{16}=0.45, P=0.656). Thus, in contrast to S1-activated neurons, repeatedly enhancing the excitability of NC-tagged neurons did not affect learning.

Together, these findings indicate that repeatedly enhancing the excitability of the early learning pool throughout acquisition sessions impaired conditioning.

Of note, all sessions in which clozapine was delivered were performed in the afternoon, closer to feeding time. We have previously observed in our that PM sessions often show poorer response selectivity than AM session in our task (Fig. 1D and Chapter 2 Fig. 1). Therefore, while we did observe decreased performances at sessions 2, 4, 6, 8, 10, 12 we argue that this is not due to the behavioural effects of clozapine.

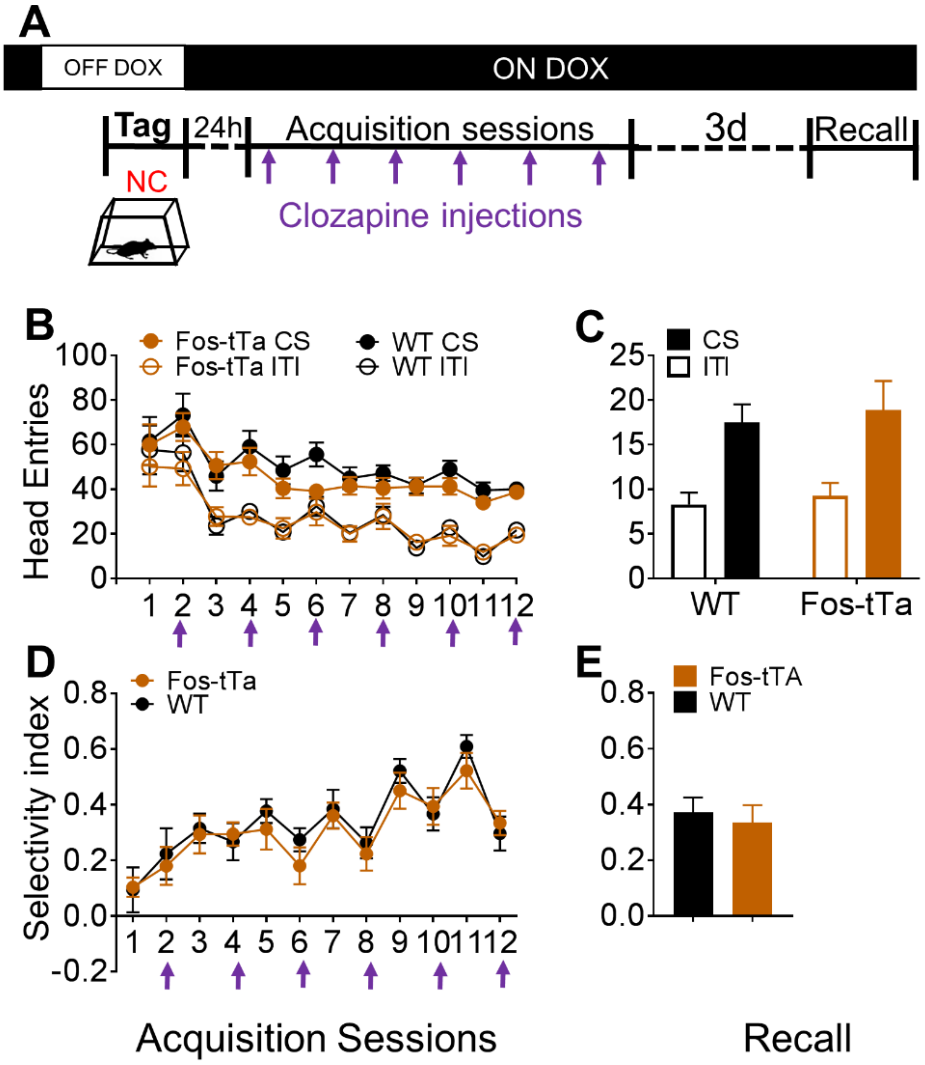


Figure 4: Repeatedly enhancing novel context-activated neurons in a hyper-excitable state does not impair conditioning. (A) Timeline of tagging, conditioning and clozapine injections. **(B) & (C)** Head entries into the magazine during the CS (cue) compared to ITI (no cue following Acquisition of conditioning and cue exposure test (Recall) in *Fos-tTA* (hM3Dq+; orange) and WT (hM3Dq–; black) mice . **(D) & (E)** 'Selectivity index' of responses (CS-ITI/total number head entries) during Acquisition and Recall) in *Fos-tTA* (hM3Dq+; orange) and WT (hM3Dq–; black) mice. Mice received clozapine injections on sessions 2, 4, 6, 8, 10 and 12 (purple arrows). All data are expressed as Mean±SEM *** P<0.001; *Fos-tTa: n=8, WT n=10.*

3.4 Discussion

In the last chapter, we determined that the establishment of an appetitive association recruited a subset of dmPFC pyramidal cells from a neuronal pool activated during initial learning. This neuronal pool was shown to be transiently hyper-excitable (Suppl. Fig. 2, Brebner et al. *in preparation*). Here we show that chemogenetically maintaining the enhanced excitability of the early learning pool across conditioning resulted in attenuated appetitive learning. Furthermore, we demonstrate that this effect was specific to the early learning pool, as chemogenetically enhancing the excitability of a novel context (NC) activated ensemble did not affect learning. Thus, disappearance of the initial hyper-excitability of the early learning neuronal pool may promote optimal appetitive learning.

3.4.1 dmPFC hyper-excitability promotes the formation of associations

Whitaker et al. demonstrated that operant appetitive conditioning was associated with the development of a hyper-excitable ensemble in the dmPFC (Whitaker et al., 2017), suggesting a role for hyper-excitability in strengthening food-cue associations. In contrast, here we show that repeatedly increasing excitability of the early learning pool, which includes the persistently activated ensemble, does not serve to strengthen the food-cue association. If so, what is the role of this neuronal hyper-excitability?

Volle et al. observed that a widespread, generalized hM3Dq-induced increase in mPFC excitability permitted conditioning in a long-delay procedure, in which conditioning is usually obstructed due to the delay between CS and US presentations (Volle et al., 2016). From this perspective, repeatedly maintaining hyper-excitability in the early learning activated pool across conditioning in our study may have led to the formation of irrelevant associations between the US and stimuli not precisely predictive of the US (e.g. components of the training chamber, arousal state), concurrently with the CS-US association. Thus, prolonged hyper-excitability may have caused the ambiguous

presence of both relevant and irrelevant associations and promoted CS-independent food-seeking.

Furthermore, increasing the excitability of neurons throughout learning affected mid and late but not early conditioning performance. We also observed that, while we only enhanced the excitability of the early learning pool every 2 sessions, performance was affected in all of mid/late conditioning sessions, including those that were clozapine-free. This suggests that reduced selectivity in late learning was not directly driven by increased firing of behaviourally-relevant neurons, as has been observed in optogenetic reactivation of ensembles (Liu et al., 2012). Instead, conditioning was likely impaired by long-lasting neurophysiological changes.

It is worth remarking that activated neurons were observed to be hyper-excitable 90 minutes following early learning in this task (Suppl. Fig. 2). Furthermore, the artificial increase in excitability induced by hM3Dq persists an hour following clozapine binding (Alexander et al., 2009). Previous studies have suggested that heightened excitability following learning has a role in encoding associations (Hsiang et al., 2014). Therefore, behavioural effects of enhanced excitability may be driven by neuronal hyper-excitability following the sessions as well as during the session. As such, in our task the hyper-excitability of the neuronal pool activated in early learning may have had a role in the initial consolidation of the CS-US association following S1. Further detailed time-course studies are required to reveal precisely how long the hyper-excitability of S1 activated neurons lasts as well as its role post-learning.

3.4.2 Early learning activates a task-specific neuronal pool from which a conditioning ensemble is recruited

In contrast with our findings when enhancing the excitability of the early learning activated pool, we found that reactivating NC activated neurons had no behavioural

effect during learning. These findings suggest that the early learning activated pool of neurons is specific for the task and may therefore encode task-related information. This is similar to previous studies which report that ablation of NC activated ensembles in the PFC does not affect cue-controlled behaviours, suggesting high specificity of learning-relevant ensembles (Cruz et al., 2014; Fanous et al., 2012). Thus, we hypothesise that CS-selective food seeking is promoted by the dismissal of hyper-excitability from a pool of neurons activated in the initial presentation of the task.

Furthermore, this ensemble specificity also provides insight into the mechanisms behind dmPFC ensemble allocation during appetitive conditioning. Had increasing the excitability of NC activated neurons resulted in their incorporation to a task-relevant ensemble in the second training session, we would expect disruptions in behaviour similar to that observed when enhancing the excitability of the early learning activated pool. Therefore, this indicates that the conditioning ensemble is likely recruited from neurons activated in the first conditioning session, as hypothesized in our previous chapter. In support, fear conditioning studies performed in the hippocampus have previously suggested that, in repeated learning, ensembles are unlikely to be reallocated once assigned (Matsuo, 2015).

Finally, we previously observed that only a minority (~29%) of pyramidal cells in the early learning pool become part of the persistently reactivated ensemble. Here, we show that maintaining all early learning neurons as ‘active’, through enhanced excitability and increased firing, interferes with learning. This suggests that the dismissal of a group of neurons from the early learning pool may also be implicated in establishing CS-selective responding. In support, multiple studies have shown that changes in neuronal recruitment are associated with learning (Cao et al., 2015; Milczarek et al., 2018; Whitaker et al., 2017). Thus, we theorise that the co-existence of reactivation and dismissal of neurons from the early learning pool underlies the establishment of appetitive conditioning, although further work will be necessary to verify that increasing

the excitability of the early learning pool results in modifications to the conditioning ensemble.

3.4.3 Potential role of alteration of intrinsic excitability of activated neurons

Our findings suggest that alterations in intrinsic excitability and, more specifically, the disappearance of hyper-excitability in neurons activated by early conditioning may have a role in discriminatory learning as enhancing the excitability of the early learning activated pool throughout conditioning decreases selectivity by increasing non-specific responses. Indeed, lesion studies suggest the dmPFC functions to promote the formation of relevant and precise associations that enable proper cue discrimination (Bussey et al., 1997; Cardinal et al., 2002; Parkinson et al., 2000). This function may be mediated by changing dmPFC output to attentional processes. In support, the dmPFC has been shown to be involved in directing attention during learning (Bryden et al., 2011; Totah et al., 2009), in part through connections to sensory regions (Zhang et al., 2014). Furthermore, the dmPFC has been previously described to have a role in directing attention to outcome-predictive cues (Sharpe and Killcross, 2014). Moreover, neurons activated by late learning were shown to have baseline excitability properties, suggesting the excitability of neurons activated by appetitive conditioning varies from early to late learning (Suppl. Fig. 2 & 3; Brebner et al., *in preparation*). Thus, we hypothesized that alterations of the excitability of neurons that were activated by learning may increase selectivity, through modulating output to attention networks.

Moreover, these alterations in excitability of dmPFC neurons may also act on other downstream targets, for example, the dmPFC-nucleus accumbens connection, which mediates behavioural vigour (Parkinson et al., 2000). With this perspective, reduced excitability of dmPFC neurons activated by learning may serve to down-regulate behavioural vigour in late learning. In our task, this translates to a decrease in total

number of food-seeking responses as conditioning progresses, a process that is impaired by artificially enhancing the excitability of the pool of neurons activated by early learning. Furthermore, connections of the dmPFC to the dorsomedial striatum (Berendse et al., 1992; Fillinger et al., 2018) which are thought to facilitate the early formation of associations (Cole et al., 2017) may also require less input in late learning. As such, decreased excitability of activated neurons in the dmPFC could function to adapt dmPFC output and behavioural responses as learning progresses.

Thus, we hypothesize that alterations of the excitability of dmPFC neurons may serve to regulate behavioural vigour and modulate attentional processes as conditioning progresses, in order to promote optimal energy-efficient food-seeking (MacArthur and Pianka, 1966).

3.4.4 Methodological consideration

Of note, we observed expression of hM3Dq in WT mice, in absence of tTa, suggesting a level of leakiness to the virus. Therefore, all mice likely expressed hM3Dq receptors in a small, random population of neurons. However, we demonstrated that hM3Dq activation affected behaviour in an ensemble-specific manner; therefore, repeatedly enhancing the excitability of the neurons that were randomly tagged with hM3Dq likely had no effect on conditioning. Furthermore, while it should be noted that our DREADD manipulation is not cell-type specific, our 2-photon data demonstrates that the vast majority (90%) of Fos+ neurons are pyramidal cells (Chapter 2, Fig. 2). Hence, our manipulation was primarily directed to these excitatory pyramidal cells.

In our previous chapter, we observed that the number of pyramidal cells activated in each session decreased slightly across conditioning. This decrease was independent of conditioning group and we hypothesized that the initial increase in activation was driven by novelty. As such, when enhancing the excitability of the early learning pool, we may

have been manipulating this novelty-activated population and, therefore, may have caused a general increase in activity in the dmPFC throughout learning. However, in our NC experiment neurons were also tagged following a novel event (NC exposure) which activates a large population in the dmPFC (Struthers et al., 2005). Thus, as increasing the excitability of NC neurons does not impair learning, generalised increased activity unrelated to conditioning in the dmPFC is unlikely to be the underlying cause of disrupted learning.

Furthermore, while we aimed to maintain hyper-excitability in the early learning pool of neurons, we cannot ensure that the level of excitability artificially generated will be similar to that presented following the early learning session. In particular, the mechanisms behind the artificial increase in excitability may be different from those occurring following early learning. Indeed, hM3Dq is thought to enhance neuronal excitability by inhibiting voltage-gated potassium channels (KCNQ channels) through the phospholipase C (PLC) pathway (Alexander et al., 2009). We cannot determine if this pathway was involved to increase excitability in early learning. Moreover, the PLC pathway induces increases in intracellular calcium concentrations, which will interact with a variety of cellular functions including gene transcription and plasticity mechanisms (Berridge, 1998). Thus, we cannot dismiss that the behavioural effects we observed are mediated through these mechanisms.

Finally, another methodological concern in this study is the use of the hM3Dq agonist, clozapine. When first developed, hM3Dq was shown to be activated by CNO (Clozapine-N-Oxide), a compound that is inert in the brain (Alexander et al., 2009); although clozapine was also known to have a high affinity for these receptors (Armbruster et al., 2007). However, recent evidence has since demonstrated that, *in vivo*, CNO is metabolized to clozapine which then mediates the effects of CNO on DREADDs within the nervous system (Gomez et al., 2017). Unlike CNO, clozapine shows high affinity for serotonergic, muscarinic, histaminergic and select dopaminergic receptors (Coward,

1992). It also has a range of behavioural effects, including on locomotor activity and learning processes for acute (Hou et al., 2006) and chronic administration of the drug (Rosengarten and Quartermain, 2002). These behavioural effects are usually described for doses above or equal to 1 mg/kg body weight (Hou et al., 2006; Rasmussen et al., 2001), although they have also been observed at lower doses (Ilg et al., 2018) . In our task, we worked to reduce these behavioural effects by using a sub-clinical dose of 0.1 mg/kg. Repeated administration of this dose did not significantly affect responses in our behavioural paradigm, suggesting this dosage of clozapine does not impact the establishment of the appetitive CS-US association. However, we did observe a small, non-significant, general decreases in behavioural responses in late conditioning, suggesting that longer-term administration of low-dosage clozapine may eventually attenuate food-seeking (Fig. 1B). Therefore, we suggest that, in future studies, care should be exercised when examining appetitive behaviours following repeated and prolonged clozapine injections.

3.4.5 Conclusion

While intrinsic properties of neurons and their alterations are thought to play a role in encoding memories, their precise functional relevance has rarely been examined. Here we demonstrate that modulations of neuronal excitability from early to late learning are crucial to forming appetitive associations. Furthermore, we provide evidence that early learning activated neurons may be task specific. Together with our findings from the previous chapter, this supports that, during appetitive conditioning, the conditioning ensemble emerges from a wider pool of neurons activated in early learning. The hyper-excitability of this early learning activated pool needs to be dismissed as learning progresses.

However, in these two chapters, we only examined learning mechanisms during the acquisition of an appetitive association. This poses the question: are these mechanisms similar to those underlying the extinction of appetitive conditioning?

Chapter 4: Extinction learning following appetitive conditioning recruits an interneuron ensemble from a neuronal pool activated in early extinction training

4.1 Introduction

To survive in a dynamic and changing environment, animals must efficiently adapt to alterations in the predictive value of environmental cues signalling the availability of food. In particular, one important aspect of this is the ability to suppress responses to cues that no longer predict food availability in order to reduce energy usage in an environment where nutrients are limited.

Extinction of appetitive behaviours is the process in which a previously established association between food (unconditioned stimulus, US) and the stimuli that predicts its availability (conditioned stimulus, CS) is weakened by presenting the CS in absence of the US. Through this process, the CS alone no longer evokes food-related behaviours (van den Akker et al., 2018; Pavlov (1927), 2010). Crucially, during extinction, the original CS-US association is inhibited rather than unlearnt; as such, CS-related behaviours can be spontaneously recovered or reinstated (Bouton, 1993; Pavlov (1927), 2010; Pearce and Hall, 1980). Furthermore, extinction learning is thought to form a new 'CS- no US' associative inhibitory memory (Bouton, 2004; Rescorla, 1993). In humans, failure to recall extinction memories is a key component to relapses following treatment of maladaptive eating. As such, elucidating the mechanisms of extinction learning is important to identifying the neurobiological substrates underlying these relapses (van den Akker et al., 2018; Jansen et al., 2016).

Extinction of CS-US associations has been shown to be encoded by sparsely distributed minorities of strongly activated neurons – neuronal ensembles – in the wider medial prefrontal cortex (mPFC) region (Warren et al., 2016). To our knowledge, extinction

specific neuronal ensembles have not been detected in the dorsal regions of the mPFC (dmPFC). However, Moorman and Aston-Jones found that dmPFC neurons would fire for adapted behavioural responses in extinction of food-seeking, suggesting the dmPFC has a role in mediating extinction behaviours (Moorman and Aston-Jones, 2015). Moreover, there is evidence that interneurons of the dmPFC are involved in extinction learning (Courtin et al., 2014; Sparta et al., 2014).

In previous chapters, we determined that a stably activated pyramidal cell ensemble is formed during conditioning in the dmPFC. This poses the question: what happens to this established conditioning ensemble during extinction learning? Moreover, while repeated neuronal activation is known to be involved in encoding learnt behaviours (Cao et al., 2015; Czajkowski et al., 2014; Mattson et al., 2008; Tayler et al., 2013), activation during extinction in the dmPFC has yet to be investigated in a longitudinal manner. As such, the existence of persistently activated dmPFC ensembles in extinction has not been examined. Furthermore, the distinct contributions and interactions between excitatory and inhibitory populations in extinction learning warrants further investigation.

Here, we address these gaps in knowledge by examining ensemble formation of pyramidal cells and interneurons in the dmPFC during the extinction of a food-cue association. Similarly to our first experimental chapter, we made use of microprism-based *in vivo* 2-photon imaging (Low et al., 2014) to record from the dmPFC of *Fos-GFP x GAD-tdTomato* (*FGGT*) mice over 3 extinction trials (1st, 3rd and 7th). Using GFP expression as a marker of high levels of activation and tdTomato as a marker of interneurons, we assessed the activation patterns of pyramidal cells (tdTomato–) and interneurons (tdTomato+) during extinction learning. Furthermore, we related neuronal activation in extinction to previously observed stable activation in appetitive conditioning (e.g., persistently activated for appetitive conditioning, as assessed in Chapter 2).

We observed that a repeatedly activated extinction ensemble emerged from a pool of interneurons activated during the first extinction session. Crucially, the composition of

this stably activated ensemble was altered to recruit reduced proportions of the neurons that had been persistently activated for conditioning.

4.2 Methods

4.2.1 Animals & Microprism-implantation

Similar breeding, microprism-implantation, imaging, and behavioural procedures were used as described in Chapter 2. Briefly, *Fos-GFP x Gad-tdTomato* (FGGT) male mice were bred in house to be used for 2-photon imaging experiments. Animals were aged 10-13 weeks at the beginning of experimental procedures, and were food restricted (90% baseline body weight) 1 week prior to behavioural testing until the completion of behavioural experiments. Experiments were conducted in accordance with the UK 1986 Animal Scientific Procedures Act (ASPA) and received approval from the University of Sussex Animal Welfare and Ethics Review Board.

FGGT mice were implanted with a microprism in the mPFC at ages approximately 10-13 weeks. The microprism was positioned such that it was between the hemispheres with the imaging surface placed against the sagittal surface of one of the hemispheres (Fig. 2B). All mice recovered for a minimum of two weeks before undergoing any further procedures. The first imaging session typically occurred 3-4 weeks following surgery to allow inflammation in the imaging area to subside.

4.2.2 Behavioural experiments

A similar behavioural apparatus was utilised in Chapter 2. Briefly, behavioural experiments were performed in standard mouse operant chambers (15.9 x 14 x 12.7 cm; Med Associates, Vermont, USA). Each chamber was fitted with a protruding magazine (to accommodate mice equipped with a head-restraint device) which dispensed 10% sucrose solution serving as the unconditioned stimulus (US) and a mechanical click generator providing a sound that served as a conditioned stimulus (CS). An infrared beam detected head entries into the food magazine.

Mice were randomly assigned to the Paired or Unpaired groups that underwent identical procedures except that Unpaired mice only received sucrose in the home cage 1–4 h at random times before or after each conditioning (acquisition) session, with the exceptions of S1, S5 and S11 (see below). One day after magazine training, in which Paired mice were pre-trained to the sucrose-delivery magazine, where they received a 10% sucrose solution under a random interval-30 (RI-30) schedule, mice underwent 12 acquisition sessions over a 7 d period in the morning (8:00 A.M. to 12:00 P.M.) and/or afternoon (12:00 P.M. to 5:00 P.M.) for 1–2 sessions per day. As previously, each acquisition session lasted approximately 24 min and consisted of six 120 s CS presentations separated by 120 s RI inter-trial interval (ITI) periods. During each 120 s CS period, 13.3 µl of 10% sucrose solution was delivered into the magazine on an RI-30 s schedule (Paired mice) or was unrewarded (Unpaired mice). Twelve acquisition sessions produced selective responding to the CS (see Chapter 2). 3 or 4 days following the last acquisition session, both Paired and Unpaired mice underwent 7 extinction sessions over 7 days. Extinction sessions resembled acquisition sessions (approximately 24 min, six 120 s CS presentations separated by 120 s RI ITI periods) with the exception that there was no delivery of sucrose solution during the session (Paired mice) or in the home cage (Unpaired).

4.2.3 *In vivo* 2-photon imaging

4.2.3.1 Habituation and imaging sessions

Similar procedures were utilised in Chapter 2. Briefly, imaging sessions took place on head-fixed, awake mice that were able to freely run on a polystyrene cylinder (Fig. 2A). For ~1 week prior to the first imaging session, mice were habituated to being restrained by being head-fixed before imaging commenced. In areas of interest, z-stacks in both the red and green channels were recorded simultaneously at an excitation wavelength

of 970 nm (power at the objective: 70-130 mW; pixel dwell time: ~3.9 ns) from the pial surface to a depth of approximately 300 µm. Each slice of the stack was an average of two 660.14 x 660.14 µm images (corresponding to 512 x 512 pixels; pixel size: 1.2695 x 1.2695 µm).

Imaging sessions took place 75 min following initiation of the 1st, 5th and 11th conditioning session as well as 1st, 3rd and 7th extinction session (Fig. 2A). Another two imaging sessions took place directly from the home cage (2-3 days prior to conditioning and 2-3 days after the final extinction session). Imaging sessions typically lasted 40 minutes to an hour. Two mice (1 Unpaired, 1 Paired) were excluded due to poor imaging quality on one or several imaging sessions and another (Unpaired) was excluded due to abnormally GFP+ counts in one session (identified with Grubbs's test, α=0.05).

4.2.3.2 Image Analysis

Similar procedures were used for Image analysis in Chapter 2. Briefly, image stacks were aligned on the x and y axis, and between sessions. An overlapping volume within layer II/III and common to all sessions was identified and selected. Images were then filtered and GFP+ and tdTomato+ cells were identified by comparing signal within to cell to the signal in the surrounding background. The x, y, z coordinates and GFP relative fluorescent intensity (RFI = signal/noise) of each cell were extracted. A custom Matlab script defined whether each cell was a putative 'interneuron' or 'pyramidal cell' and sorted cells according to their coordinates in order to identify the activation history of each neuron.

To account for inter-individual difference in cell density and GFP expression, all variables relating to GFP+ quantification were normalised to the average number of GFP+ cells detected in home cage sessions (=GFP+ number/average HC GFP+ number). GFP RFI

were normalised between sessions using the average tdTomato RFI as reference. All neurons activated in HC sessions were pooled and grouped into 3 categories of brightness (High, Medium and Low) according to their GFP RFI such that a third them fell within each category. The thresholds identified through this process were used to assign a brightness category to all GFP+ neurons in E1.

4.2.4 Data analysis

In the main text, we only report effects and interactions key to interpretation. A complete report of statistical results for all experiments can be found in the Annex (Suppl. Table 7). All data were analysed using GraphPad Prism (RRID:SCR_002798; GraphPad Software) and SPSS (IBM SPSS Statistics, Version 23.0 (2015), Armonk, NY: IBM Corp). Group data are presented as mean±SEM.

Behavioural data: Head entry responses were tested with a 3-way mixed ANOVAs in SPSS.

Imaging data: GFP+ counts were tested with 2-way mixed ANOVAs in Prism and 3-way mixed ANOVAs in SPSS. Following 2-way mixed ANOVAs, further post-hoc tests were performed (Sidak correction) if an interaction was observed ($p<0.05$). Log-linear analyses and Chi-squared tests were performed on pooled neurons in SPSS and further post-hoc procedures ((Beasley and Schumacker, 1995); Bonferroni correction) were performed for Chi-squared tests if a significant interaction was observed ($P<0.05$). Interneurons and Pyramidal cells are affected differently by Glutamatergic signalling (Riebe et al., 2016) suggesting distinct Fos induction thresholds, as such they were analysed separately.

Of note, for some animals, data recorded during conditioning was previously analysed separately in Chapter 2. For behavioural data, this corresponded to 9 mice (5 P, 4 UP) and for imaging data, 6 mice (4 P, 2 UP).

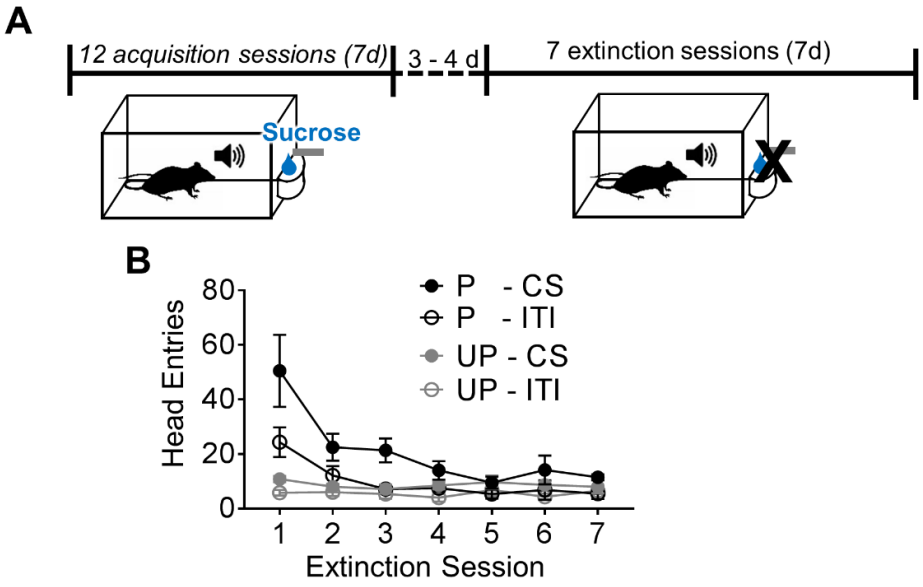


Figure 1: Experimental timeline and conditioning. (A) Timeline of conditioning, extinction and imaging. **(B)** Selective head entries into the magazine during the CS (cue) compared to ITI (no cue) periods during extinction of conditioning in Paired and Unpaired *FGGT* mice. All data are expressed as Mean±SEM. *Paired (P): n=6, Unpaired (UP): n=6*

4.3 Results

4.3.1 Extinction learning attenuates responding

Following 12 sessions of 'Acquisition', in which mice of the Paired group learnt to associate an auditory cue presentation (Conditioned stimulus, CS) to sucrose solution delivery (Unconditioned stimulus, US; see chapter 1), mice underwent 'Extinction' sessions. In each of these sessions, *FGGT* mice in both Paired and Unpaired groups received CS presentations alone without the US (Fig. 1A). During Extinction, we observed a significant interaction of Group X Cue ($F_{1,10}$=13.56, P=0.004) and Session X Group ($F_{6,60}$=5.96, P<0.001), indicating that Paired mice displayed CS-selective behaviour and that their overall responses decreased as extinction sessions progressed (Fig. 1B), suggesting extinction learning occurred, similar to our recent study (Ziminski et al., 2017).

4.3.2 Conditioning recruits a stable, repeatedly activated ensemble from an interneuron pool activated in early extinction

Similar to Chapter 2, we used 2 photon (2P) imaging in microprism-implanted *FGGT* mice, to characterise neuronal ensemble recruitment patterns of pyramidal cells and interneurons in layers 2/3 of the dorsal medial prefrontal cortex (dmPFC) during Extinction (Fig. 2A-C). We first assessed the number of strongly activated, GFP+ pyramidal cells (tdTomato–) and interneurons (tdTomato+) on the 1st (E1), 3rd (E3), and 7th (E7) extinction sessions (Fig 2D). No significant interactions of Group X Session were observed for pyramidal cells ($F_{2,14}$=0.70, P=0.513) nor interneurons ($F_{2,14}$=0.60, P=0.564). However, there was a general effect of Group in interneurons ($F_{1,7}$=7.91, P<0.05). Thus, the overall number of interneurons activated during Extinction was increased in Paired mice. In contrast, there was no significant alteration to pyramidal cell activation due to extinction learning.

Repeated activation is thought to consolidate neurons into an ensemble that mediates learned associations (Mattson et al., 2008). Furthermore, in Chapter 2, we demonstrated that a persistently activated ensemble is recruited for conditioning from neurons activated in the first conditioning session. Hence, we investigated whether extinction learning also preferentially recruited a learning-relevant ensemble from a pool of candidate neurons activated in E1. To this end, in Unpaired and Paired groups, we assessed and compared the number of GFP+ neurons in two distinct 'Activation History' categories: neurons that were persistently activated (+) in E3 and E7 following activation in E1 (E1+| E3+ E7+) or neurons persistently activated in E3 and E7 that were not activated in E1 (E1-| E3+ E7+; Fig. 2E). We observed a significant interaction of Activation History X Group in interneurons ($F_{2,14}$=6.59, P<0.05) but not pyramidal cells ($F_{2,14}$=0.43, P=0.535). Post-hoc testing in interneurons revealed a significant increase in the number of E1+| E3+ E7+ interneurons in Paired mice compared to Unpaired mice (P<0.01). Thus, extinction recruited a persistently reactivated ensemble from a pool of interneurons activated during the initial extinction session. An analysis performed on a less conservative criterion (inclusion of all neurons observed to be GFP+ in more than one imaging session, e.g. E1+| E3- E7+) yielded similar results (Suppl. Fig. 4).

Having established the relevance of E1 activation to the extinction ensemble, we next examined at a population level how extinction learning altered neuronal reactivations following E1. We assessed the proportion of E1-activated neurons that were reactivated in E3 only (E1+| E3+ E7-), E7 only (E1+| E3- E7+) or E3 and E7 (E1+| E3+ E7+) as well as neurons activated in E1 but not E3 and E7 (E1+| E3- E3-) (Fig. 2F). There was a significant interaction of Activation History X Group for pyramidal cells (X^2_3=52.837, P<0.001) but not interneurons (X^2_3=3.12, P=0.375). Notably, in pyramidal cells, there was a significantly lower proportion of E1+| E3+ E7+ neurons among E1-activate neurons in Paired mice compared to Unpaired mice and a significantly higher proportion

of E1+| E3- E7- neurons (P<0.05). Thus, in pyramidal cells, extinction learning reduced the likelihood of reactivation following E1.

Extinction learning has been shown to recruit distinct ensembles to that recruited in conditioning (Warren et al., 2016). Therefore, we examined whether a history of stable activation in conditioning would predict persistent reactivation in extinction following recruitment in E1. At a population level, we compared for Paired and Unpaired mice, the proportion of E1-activated neurons that had displayed persistent activation in conditioning ('conditioning ensemble history'). More specifically, we examined this proportion within E1-activated neurons that were subsequently reactivated in E3 only (E1+| E3+ E7-), E7 only (E1+| E3- E7+) or E3 and E7 (E1+| E3+ E7+) as well as neurons activated in E1 but not E3 and E7 (E1+| E3- E3-; Fig. 3). To this end, we performed 3-way log-linear analyses (Group X Extinction Activation History X Conditioning History). In pyramidal cells, the model retained all effects (likelihood of model: X^2_0=0, P=1), indicating a significant interaction of Group X Extinction Activation History X Conditioning History (X^2_3=9.68, P<0.05). Further chi-squared analyses of Group X Conditioning History in each Extinction Activation History category separately revealed that the proportion of neurons with a conditioning ensemble history in the E1+| E3+ E7+ category was decreased in Paired mice (X^2_1=11.43, P<0.01). In all other categories, this proportion was not significantly different between groups (Suppl. table 7). Thus, extinction learning decreased the proportion of pyramidal cells with a conditioning ensemble history specifically within the population of neurons that were persistently reactivated in extinction. In interneurons, the final log-linear model did not retain the 3rd order effect but retained 2nd order effects (likelihood of model: X^2_6=10.402, P=0.109), indicating no significant interaction of Group X Extinction Activation History X Conditioning History (X^2_3=6.53, P=0.089). Further partial associations analyses of 2nd order effects revealed a significant interaction of Group X Conditioning History (X^2_1=4.94, P<0.05). Thus, extinction learning generally decreased the proportion of interneurons

with a conditioning ensemble history recruited in E1. Taken together, these findings suggest extinction learning alters activation patterns of neurons in extinction training.

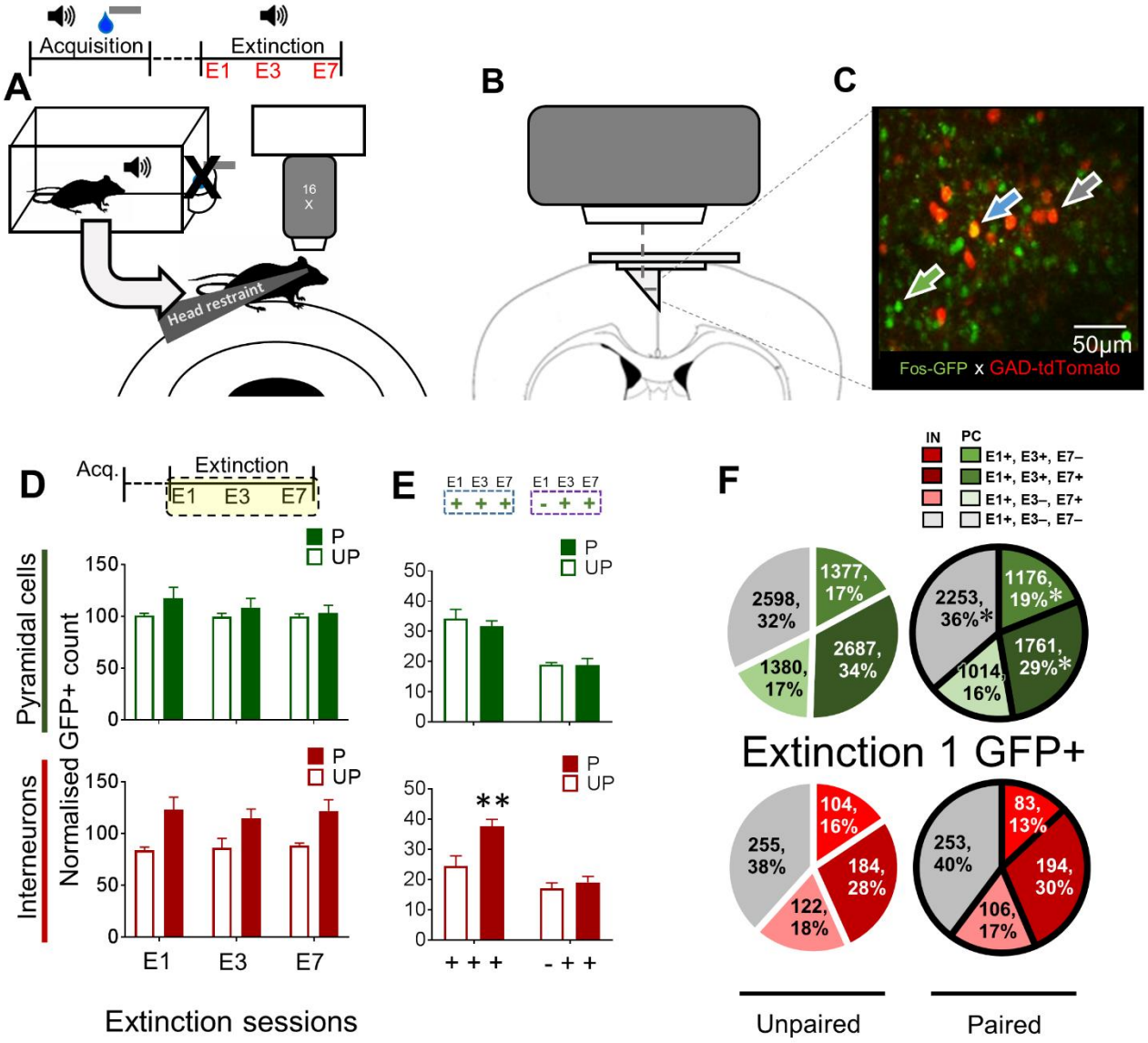


Figure 2: Extinction learning persistently recruits a stable interneuron ensemble from the initial extinction session. GFP expression was longitudinally monitored in pyramidal cells and interneurons. **(A)** Schematic representation of imaging session in head-fixed mice following behavioural training under freely moving conditions. **(B)** Microprism placement for mPFC imaging. **(C)** Representative *in vivo* 2-photon image of mPFC from *Fos-GFP x GAD-tdTomato (FGGT)* mice (green arrow: GFP; grey arrow: tdTomato; blue arrow: GFP+tdTomato). **(D)** Normalized GFP+ counts during Acquisition for pyramidal cells (green) and interneurons (red) **(E)** Normalized GFP+ counts of persistently activated pyramidal cells and interneurons with a E1 (+ + +) or no E1 (– + +) activation history. **(F)** Distribution of GFP+ pyramidal cells and interneurons activated in E1 classified according to their subsequent reactivation patterns (E1+|E3+,E7+; E1+|E3+,E7-; E1+|E3-,E7+; E1+|E3-,E7-) in Paired and Unpaired mice during acquisition. Data on bar graphs is expressed as Mean±SEM. *Interaction effect: # P<0.05*, *Post-hoc analysis: *P<0.05, ** P<0.01, Paired (P): n=5, Unpaired (UP) n=4.*

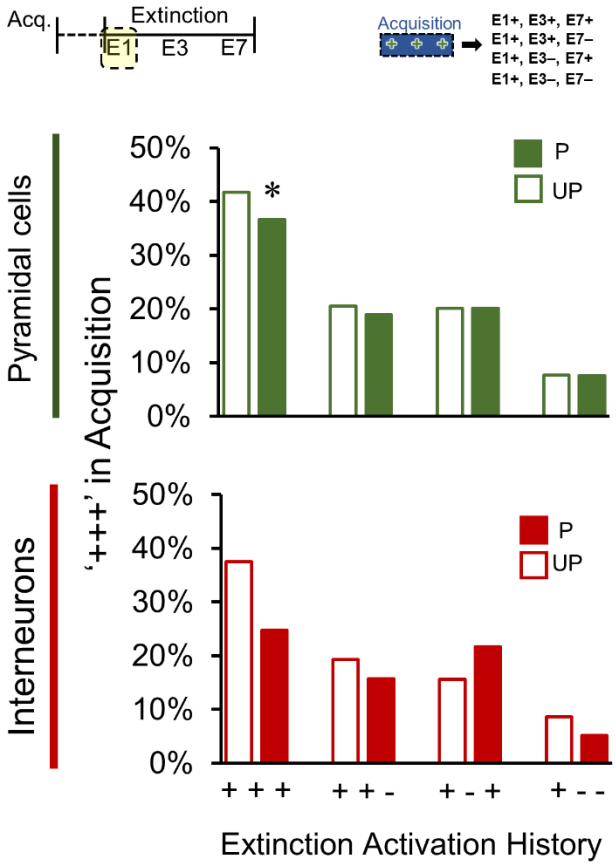


Figure 3: Extinction learning is less likely to recruit neurons persistently activated in conditioning. Activation history of (persistently activated or not) was assessed for all E1-activated pyramidal cells and interneurons according to their subsequent reactivation patterns (E1+|E3+,E7+; E1+|E3+,E7-; E1+|E3-,E7+ or E1+|E3-,E7-). Data on bar graphs is expressed as proportion of total number of neurons in each category. *Post-hoc analysis: * P<0.05, Paired (P): n=5, Unpaired (UP): n=4.*

4.3.3 High GFP expression in E1 predicts reactivation regardless of conditioning

Robust activation of the promoter of the immediate early gene *arc* has been shown to predict subsequent reactivation in motor cortex neurons during motor learning (Cao et al., 2015). This gene is expressed following activation of a similar signal transduction cascade as *Fos* (Barry et al., 2016). In our first experimental chapter, we demonstrated that GFP intensity in early conditioning was a predictor of neuronal reactivation. Therefore, we hypothesized that the relative intensity of GFP in early extinction, an indicator of cellular Fos expression (Barth et al., 2004), would also predict subsequent reactivations during extinction. To examine this, we compared the relative GFP intensity (Signal normalized to Background; Fig. 4A) of E1 activated neurons that were persistently reactivated during extinction (E1+| E3+ E7+) to neurons that were persistently dismissed (E1+| E3- E7-). We classified these neurons as 'High', 'Mid' or 'Low' Brightness (Fig. 4A) and quantified the number of neurons in each Brightness Category according to their Activation History and the behavioural Group (Fig. 4B). In both pyramidal cells and interneurons, there was a significant interaction between Brightness Category X Activation History (Pyramidal cells: $F_{2,14}$=73.97, P<0.001; Interneurons: $F_{2,14}$=17.45, P<0.001) but no effect of Group (Suppl. Table 7). Thus, high GFP intensity in E1 is a general predictor of neuronal reactivation during extinction training, irrespective of appetitive learning.

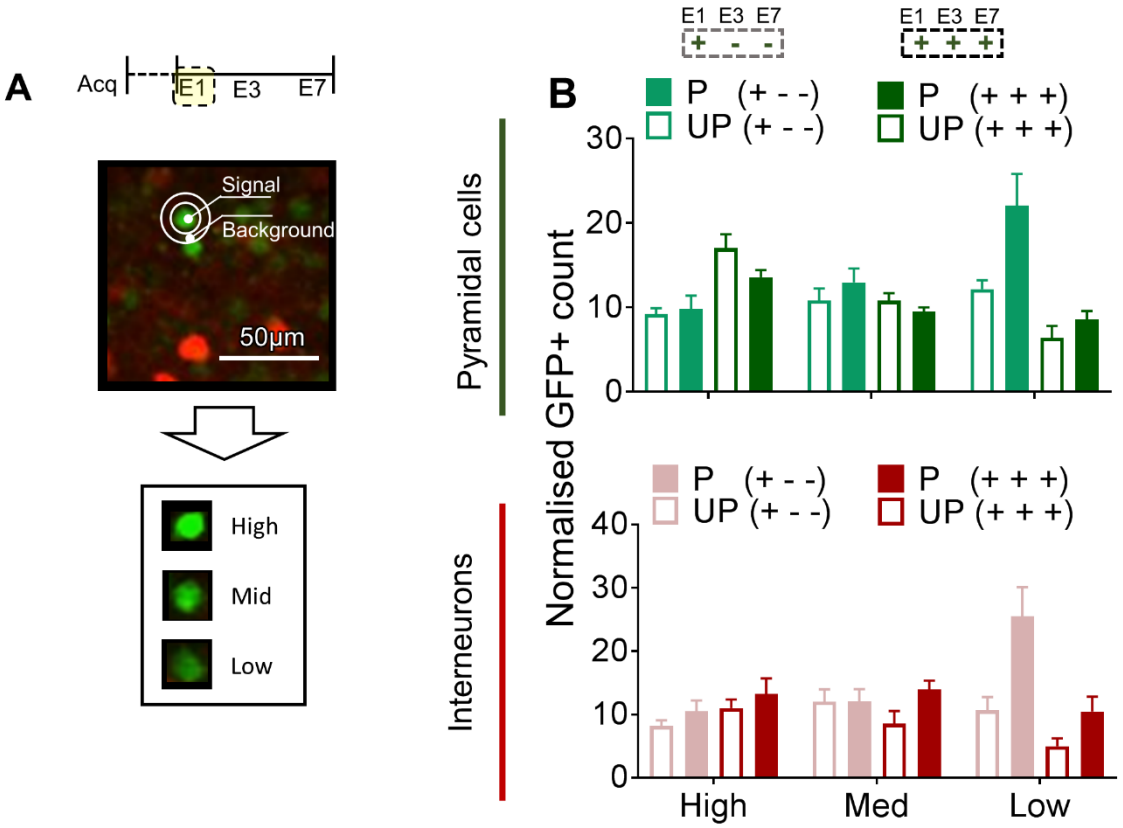


Figure 4: High GFP intensity in E1 predicts persistent reactivation, regardless of conditioning. (A) Relative GFP intensity was obtained by normalizing the signal of the cell to the surrounding background. Neurons were categorized as 'High', 'Mid' and 'Low' Brightness according to their relative intensity. **(B)** Normalized GFP+ counts in each Brightness Category for pyramidal cells (green) and interneurons (red) activated in E1 that show subsequent persistent reactivation (dark green/red) or dismissal (light green/red). Data on bar graphs is expressed as Mean±SEM. *Paired (P): n=5, Unpaired (UP): n=4.*

4.4 Discussion

We demonstrate that the weakening of CS-evoked responding following extinction learning was associated with the recruitment of a stable ensemble of interneurons in the dmPFC. This ensemble originated from a neuronal pool activated in early extinction and was then repeatedly reactivated during subsequent extinction sessions, suggesting it has a role in the reduction of the strength of the appetitive association. Furthermore, we find that activation patterns of neurons are modified in extinction learning such that the recruitment of the previously consolidated stable conditioning ensemble is generally reduced. Taken together, these results provide insights into the extinction of appetitive memories and the associated dmPFC inhibitory ensembles that underlie it.

4.4.1 Extinction recruits a repeatedly activated interneuron ensemble in the first extinction session

Interneuron activation has been detected in multiple forms of learning (Courtin et al., 2014; Doron and Rosenblum, 2010; Gaykema et al., 2014; Pinto and Dan, 2015; Stefanelli et al., 2016). Furthermore, many forms of learning recruit persistently activated neuronal ensembles (Cao et al., 2015; Czajkowski et al., 2014; Mattson et al., 2008; Tayler et al., 2013). Here, we bridge these findings in demonstrating that extinction recruits a persistently activated interneuron ensemble in the dmPFC. Crucially, this ensemble is preferentially recruited from interneurons activated in the first extinction session. Extinction is thought of as a form of inhibitory learning, where a new CS-no US association is formed (Bouton, 2004; Rescorla, 1993). From this perspective, the stable interneuron ensemble that is recruited in the dmPFC during extinction may be involved in encoding this new association.

Furthermore, we observed generally increased activation of interneurons throughout extinction sessions, supporting a number of studies suggesting a role for inhibitory signalling during extinction learning (Courtin et al., 2014; Sparta et al., 2014; Zou et al., 2016). In contrast, we did not detect any changes in pyramidal cell activation. These findings are consistent with previous observations that the dmPFC displays less excitatory activity in extinction than during the expression of associative memories (Burgos-Robles et al., 2009; Moorman et al., 2015; Nair et al., 2011; Sierra-Mercado et al., 2011).

Although our method allowed the differentiation between pyramidal cells and interneurons, we did not distinguish between the many interneuron subtypes. As such, we cannot determine precisely which interneuron subtypes may have been part of the persistently activated interneuron ensemble in extinction learning. In the cortex, neurons expressing Parvalbumin (PV+), Somatostatin (SOM+) and the Vaso-intestinal protein (VIP+) represent the majority of GABA-ergic interneurons (Rudy et al., 2011; Xu et al., 2010). Sparta et al. previously demonstrated that generalised activation of PV+ interneurons of the dmPFC would accelerate extinction of reward-seeking (Sparta et al., 2014). Furthermore, the inhibition of dmPFC PV+ interneurons following extinction has been shown to increase the reinstatement of fear conditioning (Courtin et al., 2014). Together, these findings suggest that PV+ interneuron activity in the dmPFC has a role in mediating extinction memories. However, both SOM+ and VIP+ mPFC interneurons have also been shown to be involved in reward-seeking behaviours with distinct roles to that of PV+ interneurons (Gaykema et al., 2014; Kim et al., 2016a; Kvitsiani et al., 2013; Pinto and Dan, 2015). Furthermore, interneuron subtypes are highly interconnected within the mPFC (Ährlund-Richter et al., 2019). Thus, we theorize that PV+ interneurons are a key component of the observed persistently reactivated inhibitory ensemble but that they are unlikely to be the only interneuron subtype recruited to this ensemble. As

such, further work will be necessary to determine the exact composition of the stable interneuron ensemble we observed.

4.4.2 Extinction learning alters neuronal recruitment

In the ventral region of the mPFC, extinction learning has previously been shown to activate a distinct ensemble to that activated in conditioning (Warren et al., 2016). Here, we extend those findings by demonstrating that neuronal activation of pyramidal cells and interneurons are also altered in the dmPFC, such that the recruitment of the previously established conditioning ensemble is less prominent.

More specifically, among interneurons activated by early extinction training, the proportion of neurons that were previously persistently activated in conditioning is decreased in Paired mice, suggesting the recruitment of a new pool of interneurons. Furthermore, we observed a general increased recruitment of interneurons in early extinction in Paired mice. Thus, we theorise that early extinction learning may have activated a large pool of interneurons, which, in turn, allowed the recruitment of a stable extinction ensemble that was distinct from the interneuron population that was stably activated in conditioning.

Pyramidal cells that are persistently activated in extinction are less likely to have been persistently activated in conditioning. Moreover, extinction learning was associated with a decreased likelihood of reactivation of the pyramidal cell pool activated by early extinction learning, where food-seeking responses had not yet been extinguished. Together, these findings suggest pyramidal cell activation may be regulated to reduce the recruitment of neurons activated during cue-evoked behaviour. Furthermore, previous evidence has demonstrated that both PV+ and SOM+ interneurons have a role in controlling ensemble size in the amygdala and hippocampus respectively (Morrison et

al., 2016; Stefanelli et al., 2016). Thus, we hypothesise that the stable interneuron ensemble may have had a role in regulating pyramidal cell activation, although further work will be necessary to determine the relationship between this inhibitory ensemble and the activation of excitatory neurons in extinction.

Of note, our current observations of the number of GFP+ neurons and their activation patterns following the first extinction session are different to those we made following a short recall test that was performed under extinction conditions (Chapter 2). Most notably, following this test, the level of interneuron activation was not significantly different between conditioned and control mice. Furthermore, among activated interneurons, an increased proportion had been persistently activated during associative learning in conditioned mice. This is in contrast with our current findings and may reflect the key difference between the recall test and extinction sessions; while the recall test in Chapter 2 contained 3 CS-presentations, here the extinction session contained 6 CS-presentations. Thus, we hypothesise that the large newly-recruited interneuron activation observed in conditioned mice may have originated following the latter 3 CS-presentations and, therefore, extinction specific neuronal activation may be more prominent following extended unrewarded CS presentations. Further work is necessary to fully determine the modulations in neuronal activation according to the length of extinction training sessions. However, in drawing the comparison between our recall test and first extinction session, we hope to provide guidance in the design of future tagging experiments, in particular in distinguishing between recall activated and extinction activated neurons.

4.4.3 Potential roles of increased inhibitory drive in extinction

During extinction, the previously established memory of the CS-US association is thought to be inhibited rather than forgotten (Bouton, 1993; Pavlov (1927), 2010; Pearce and Hall, 1980). We have suggested that pyramidal cell activation in extinction was

altered to reduce the recruitment of neurons that were activated during cue-evoked behaviour (i.e., conditioning sessions and early extinction), as a result of local inhibitory activity. Thus, in the dmPFC, the stable inhibitory ensemble that emerges may also be involved in inhibiting the previously established CS-US memory trace. Furthermore, the dmPFC is theorised to have a role in driving the expression of learnt behaviours (Moorman et al., 2015; Peters et al., 2009) and has also been shown to have a role in the reinstatement of food-seeking behaviours following extinction (Calu et al., 2013; Nair et al., 2011). These studies often do not account for cell type and therefore are likely predominantly observing excitatory neurons, as they represent the majority of neurons in the cortex (Beaulieu, 1993; DeFelipe et al., 2002). Thus, excitatory neurons activated in early extinction learning may be associated with the expression of the CS-US association. As such, we hypothesised that these alterations in pyramidal cell reactivation may serve to suppress CS-evoked behaviours.

This could occur through actions on downstream targets as altering pyramidal cell activations may result in pathways that were previously activated by the dmPFC in conditioning not being re-recruited. For example, this could affect outputs that may have had a role in promoting behavioural vigour in conditioning such as the projections to the nucleus accumbens (Otis et al., 2017; Parkinson et al., 2000). The dmPFC also has a role in attention (Bryden et al., 2011; Totah et al., 2009) and cue-response selectivity (Cardinal et al., 2002; Parkinson et al., 2000). In inhibiting and altering outputs to attentional processes, attentional and discriminatory networks that may have been consolidated during conditioning would have been prevented from becoming re-engaged during extinction. Thus, increased inhibitory activity in the dmPFC and decreased activation of previously strengthened networks targeted in conditioning may be crucial in promoting efficient extinction learning and adapting behavioural responses to new reward-cue contingencies.

4.4.4 Associative learning-independent activation mechanisms

While we demonstrated that extinction learning alters activation of neurons during and following early extinction, we also determined that, in control mice, more than a third (~40%) of stably activated neurons in extinction had also been stably activated in conditioning. This high proportion may be due to these neurons activating in response to stimuli common to both conditioning and extinction sessions (e.g., conditioning chamber, auditory cue, and handling). Both control and conditioned mice will have been repeatedly exposed to the context and cue prior to extinction learning and may have formed a context specific ensemble (Hyman et al., 2012). Thus, with this perspective, the high re-recruitment of stable neurons to the new extinction ensemble may reflect the existence of context-dependent ensembles. As such, although we cannot fully determine the role of associative learning-independent ensembles in our task, it is worth noting that deviation in recruitment patterns from our control mice may reflect a deviation from a stable memory trace rather than a randomly activated neuronal population.

Furthermore, similar to our findings in appetitive conditioning (Chapter 2), we observed a relationship between reactivation of neurons and GFP expression in early extinction that is independent of associative learning. Persistently reactivated pyramidal cells and interneurons were more likely to display high GFP intensity in early training, regardless of extinction learning. As GFP is highly co-expressed with Fos (Barth et al., 2004; Cifani et al., 2012; Koya et al., 2012), this suggests that following early extinction, levels of Fos expression within neurons may be a general predictor for subsequent high Fos expression in extinction training. As discussed previously in Chapter 2, Fos expression is involved in triggering plasticity mechanisms (Morgan and Curran, 1991) and the modulations of subsequent activity patterns (de Hoz et al., 2018; Jaeger et al., 2018). Thus, while further work is necessary to fully understand the relationship between Fos expression and repeated activation, these findings further support the existence of

mechanisms independent from associative learning which play a part in governing Fos-expressing ensembles.

4.4.5 Conclusion

Extinction of the original food-cue association is necessary for flexible adaptations to a dynamic environment when the cue no longer predicts food availability. Despite how crucial this behaviour is to survival, we still have not determined the precise role of dmPFC ensembles in meditating extinction learning. Moreover, the role of stably activated interneurons in learning has not yet received much attention. Here, we demonstrate that extinction learning recruits a stably activated interneuron ensemble in the dmPFC that emerges from a wider population recruited in the first extinction session. Furthermore, pyramidal cells show no significant changes in recruitment numbers for extinction but do demonstrate altered activation patterns. These mechanisms are distinct from those observed in appetitive conditioning, suggesting different processes are involved in the strengthening and weakening of food-cue associations.

Chapter 5: General Discussion

5.1 Summary of the Results

In this study, we provided new insights into the role of excitatory and inhibitory ensembles in layers 2/3 of the dmPFC during the acquisition and extinction of an appetitive association. We found that during appetitive conditioning, a stable, persistently activated pyramidal cell ensemble emerged from a pool of neurons recruited in early learning. Crucially, the performance of mice in early learning suggested that the association (CS-US) had not yet been established. This ensemble was then re-recruited for recall. Extinction learning, however, recruited a persistently activated interneuron ensemble from a pool of neurons recruited in early extinction. Furthermore, the recruitment patterns of both interneurons and pyramidal cells in extinction learning was altered. Most notably, pyramidal cells activated in the early extinction session had reduced likelihood of reactivation in subsequent sessions. Moreover, neurons that were repeatedly activated in extinction sessions were less likely to have also been repeatedly activated during conditioning in mice undergoing extinction learning compared to controls. Furthermore, our evidence suggested that the early learning pool may be task-specific as repeatedly enhancing the excitability of neurons activated in early conditioning across conditioning sessions disrupted learning. Finally, we determined that Fos expression is a predictor of reactivation but that this is independent of associative learning.

Together, these findings indicate that the dmPFC ensembles that encode associations are recruited from those neurons activated by the initial encounter with the association (CS-US or CS-no US), suggesting common mechanisms in ensemble formation during learning. However, they also highlight key differences in neuronal populations activated for acquisition and extinction of an appetitive association in the dmPFC. These

differences may originate from the specific role the dmPFC plays in mediating learned behaviours.

5.2 Common processes in progressive learning

5.2.1 Recruitment of a stable ensemble from early learning

We revealed common mechanisms in the dmPFC between two different forms of learning that require multiple training sessions to establish: appetitive conditioning and extinction. In both, a stable neuronal ensemble is recruited from neurons activated in the first training session. Furthermore, following conditioning, the stable ensemble is re-recruited for recall, supporting its role in encoding the association. Similar mechanisms have been observed previously in other cortical areas for various learning procedures (Cao et al., 2015; Czajkowski et al., 2014; Mattson et al., 2008; Milczarek et al., 2018; Peters et al., 2014). More specifically, Cao et al. also found that a stable ensemble is recruited from the first training session in the motor cortex for motor learning (Cao et al., 2015). Furthermore, both Czakowski et al. and Milczarek et al. observed repeated activation of a subset of neurons in spatial learning tasks (Czajkowski et al., 2014; Milczarek et al., 2018). Together, this suggests that the repeated recruitment of neurons that were robustly activated in the initial training session may be a common ensemble dynamic in progressive forms of learning.

We demonstrated chemogenetically that neurons recruited in the first conditioning session are likely relevant to the task (Chapter 3). One possibility is that this is due to pre-existing connectivity between neurons. In other words, the neuronal pool activated by early learning may have been part of a ‘dedicated’ pre-existing network that is utilised for tasks requiring processing of food and food-related stimuli (as conceptualised by Konorski (Konorski, 1948)). This hypothesis is in line with evidence suggesting specific neuronal pathways are preferentially recruited for appetitive learning (Martin-Soelch et

al., 2007; Petrovich, 2013; Petrovich and Gallagher, 2007) and that they are modulated according to the demands of learning (Keefer and Petrovich, 2017). With this perspective, the stable neuronal ensemble we observed would be preferentially formed of neurons that had received high input during training sessions through these pre-existing connections. Our own observation offer some support for this theory as neurons that were reactivated persistently in training also tended to express high Fos levels following early learning, suggesting they had received an robust input during the session (Cruz et al., 2013).

However, other studies suggest alternative mechanisms may be at work in allocating neurons to ensembles. In particular, multiple studies suggest a 'competitive' form of allocation where neurons compete to be part of the ensemble (Han et al., 2007; Josselyn and Frankland, 2018); for example, through increased excitability levels (Yiu et al., 2014) or by silencing surrounding neurons via inhibitory interneurons (Stefanelli et al., 2016). Thus, neurons activated in early learning may instead be recruited through these competitive mechanisms. If this is the case, a form of task-specificity may be acquired in the early learning activated population following training, rather than be pre-determined by connectivity. Fos expression is also an indicator of plasticity mechanisms (de Hoz et al., 2018; Jaeger et al., 2018; Morgan and Curran, 1991). As such, one possibility is that the early learning activated pool of neurons undergoes plasticity following the first training session to acquire task-specificity. In support, the relationship we observed between high Fos expression in early learning and neuronal reactivation in subsequent training suggests that plasticity mechanisms following early learning may have had a role in influencing neuronal reactivation in our task. In particular, high activation and Fos expression are thought to be involved in the remodelling of synaptic connections following learning (e.g., through CREB pathways) and, as a result, promote the consolidation of ensembles (Holtmaat and Caroni, 2016; Lisman et al., 2018).

We cannot determine here the relative contributions of both pre-existing connectivity and post-session plasticity in the specificity of the early learning neuronal pool. Our study suggests that the initial training session strongly activates neurons that are or will become specific for the learning task; whether this robust activation has a role in shaping this specificity will have to be investigated further. Of note, Cao et al. observed that the level of Arc-GFP expression in early motor learning also predicted reactivation in further training (Cao et al., 2015), further suggesting the relationship between the robustness of activation and subsequent reactivations may be generalizable to multiple forms of learning. Thus, we hypothesise that, in progressive forms of learning, a groundwork of a stable ensemble may be laid out during the very first training session.

5.2.2 Dynamic alterations across learning

While stable conditioning and extinction ensembles were recruited from the initial learning session, we and others (Cole et al., 2015; Warren et al., 2016; Whitaker et al., 2017; Ziminski et al., 2017) have observed that both appetitive conditioning and extinction require multiple training sessions to acquire. Therefore, while the stable ensembles we detected may be important to learning, this indicates that the initially activated neuronal population must be altered to fully establish an association (CS-US or CS-no US). Furthermore, we confirmed that alterations of the intrinsic neurophysiology of dmPFC neurons has an effect on expressed behaviour as repeatedly enhancing the excitability of early learning activated neurons impairs conditioning (Chapter 3). Thus, what alterations could be occurring to dmPFC neurons during learning?

One possibility is that neurons activated by the task undergo changes in intrinsic properties. In support, there were alterations in excitability in activated pyramidal cells from early to late appetitive conditioning in our task (Brebner et al, *in preparation*, Suppl. Fig 2 & 3). Furthermore, we found that chemogenetically interfering with excitability

modulations in conditioning impaired learning. While there were no similar intrinsic excitability modulations in extinction (*unpublished findings*), this suggested excitability alterations of activated neurons, and therefore of the stable ensemble, may have had a role in mediating appetitive conditioning. One possibility is that alterations of excitability may have served to adapt the fidelity of information transfer throughout conditioning, both within local and through the wider dmPFC network as increased excitability of neurons results in increased output. However, one limitation of these excitability recordings is that they occurred approximately an hour following training sessions (Suppl. Fig 2 & 3). Therefore, we cannot determine if this heightened excitability was present prior to, during, or following training. Increased excitability following high activation is thought to aid in long-term plastic changes (Hsiang et al., 2014). As such, the modulations in excitability observed may instead alter dmPFC output through the promotion of long-term changes in excitable neurons following learning (Lisman et al., 2018). Thus, while we cannot define the exact role of excitability modulations in conditioning, we hypothesise they have a role in altering the output of the dmPFC during the formation of a CS-US association.

Whether excitability contributes to plastic changes or not, our findings and those of others indicate that plasticity occurs during learning. As discussed previously, both Fos (Chapter 2 and 4) and Arc (Cao et al., 2015) expression levels in early learning predict reactivation in subsequent training. This suggests a role for plastic changes in reactivation of neurons as both these proteins are involved in mediating long-term plasticity (de Hoz et al., 2018; Jaeger et al., 2018; Morgan and Curran, 1991; Okuno et al., 2012). Furthermore, Baldwin et al. demonstrated that N-methyl-D-aspartate receptor dependent plasticity in the mPFC was necessary for operant appetitive conditioning (Baldwin et al., 2000). Moreover, Otis et al observed a progressive tuning of dmPFC neurons to a CS during a Pavlovian appetitive conditioning task (Otis et al., 2017). As neuronal tuning has been shown to be mediated in part by plasticity mechanisms (El-

Boustani et al., 2018), this further indicates a role for neuronal plasticity in the encoding of appetitive associations. This finding may seem in contrast with our own as the stable ensemble of neurons undergoes activation from the first learning session. However, it is worth noting that Otis et al. examined neuronal spiking as relating to specific events (e.g., the CS) with calcium imaging rather than tracking the overall activation of neurons as a consequence of entire training sessions. As such, to reconcile these findings, we suggest that progressive tuning of firing may occur preferentially in neurons that express high Fos following learning (e.g., the stable ensemble); although further studies combining Fos-based reporters and calcium indicators will have to be performed to test this hypothesis (see part 5.5.4).

Together, these various studies suggest a role for plasticity mechanisms in appetitive associative learning. However, it is worth noting that there were no conditioning-specific synaptic changes in pyramidal cells in conditioning (Brebner et al*. in preparation*; *unpublished findings*). It is possible that these synaptic changes were masked in these recordings. For example, they may have occurred only in the repeatedly activated, stable, populations we detected, which only represent approximately a third of early learning activated neurons and only a quarter of neurons activated by recall. Furthermore, a high proportion of neurons were persistently activated in control mice, suggesting there may also be neuronal mechanisms linked to associative learning-independent events (e.g., context exposure (Hyman et al., 2012), as discussed in chapters 2 and 4). Thus, associative learning-specific synaptic changes may have been masked by synaptic plasticity that was due to repeated sucrose and/or context exposure which will have been observed in both experimental and control mice.

Finally, while our evidence suggests that stable ensembles are likely involved in mediating associations, we cannot discount the role that other neurons with different activation patterns may have had in forming the associative memory. As suggested in Chapter 3, not reactivating certain early learning activated neurons may have been a key

factor in establishing proper cue discrimination. We hypothesised that disrupting this may have been a contributing factor in our chemogenetic impairment of appetitive conditioning. Furthermore, previous evidence has suggested different elements of an appetitive learning task (e.g., distinct stimuli, reward-seeking responses) may be encoded by different subsets of neurons (Otis et al., 2017; Suto et al., 2016), which supports the theory that some neurons recruited in the first learning session may have promoted CS-non selective behaviours (e.g., increased general investigations). However, to fully explore this, further investigation into the roles of reactivated and/or non-reactivated populations will have to be performed. These will require tools able to specifically target neuronal subsets according to their activation history over multiple sessions; to our knowledge, these have yet to be developed.

5.2.3 Summary

In combining different progressive forms of learning and longitudinal monitoring of Fos, we were able to observe patterns of robust activation in neurons across learning. As discussed above, our findings support the role of repeatedly activated ensembles recruited in early learning in the acquisition and extinction of appetitive memories. However, our work also demonstrated that the recruitment of this learning ensemble in the dmPFC is not in itself sufficient to the expression of learnt behaviour as discriminative behaviour is established several sessions after the ensemble is initially recruited. Here, we discussed possible mechanisms explaining this contradiction; in particular, the role of plastic changes on neuronal signalling as learning progressed. In drawing comparisons between our observations of ensemble activation during appetitive conditioning and extinction, we aimed to reveal common mechanisms in progressive forms of learning. However, though there are resemblances in dynamics of ensembles mediating appetitive conditioning and extinction, these two forms of learning are encoded differently within the dmPFC.

5.3 Appetitive conditioning and extinction are encoded differently in the dmPFC

5.3.1 Conditioning recruits an excitatory ensemble while extinction recruits an inhibitory ensemble in the dmPFC.

We observed in the dmPFC that a stable, persistently activated, pyramidal cell ensemble is recruited for conditioning. This ensemble may have led to increased and persistent activation of specific dmPFC output pathways. In contrast, we did not detect a pyramidal cell ensemble for extinction learning but instead a stable interneuron ensemble. Furthermore, while the different interneuron subtypes were not discernible in our experiment; we hypothesised that this ensemble was partially formed of PV+ interneurons (Chapter 4), as the activation of dmPFC PV+ interneuron has been shown to promote extinction learning (Sparta et al., 2014).

Cortical interneurons preferentially project locally (Kepecs and Fishell, 2014) and have been previously shown to control the recruitment of excitatory ensembles (Morrison et al., 2016; Stefanelli et al., 2016). This suggest that the persistently activated interneuron ensemble may be affecting the activation of local pyramidal cells during extinction. Our findings further support this theory as, while pyramidal cell activation numbers are not significantly altered by extinction learning, pyramidal cell recruitment patterns are (Chapter 4). Thus, with this perspective, we propose a model in which, in layers 2/3 of the dmPFC, a stable, excitatory pyramidal cell ensemble is recruited for conditioning. In extinction learning, a stable interneuron ensemble is recruited and partially silences the excitatory pyramidal cell ensemble, resulting in a reduction of the excitatory outputs bolstered in conditioning (Fig. 1). We hypothesise that these different encoding mechanisms in the dmPFC may affect food-seeking behaviours in conditioning and extinction.

How might the activation dynamics suggested above contribute to appetitive conditioning and extinction and in particular, how might they participate in the various functions mediated by the dmPFC?

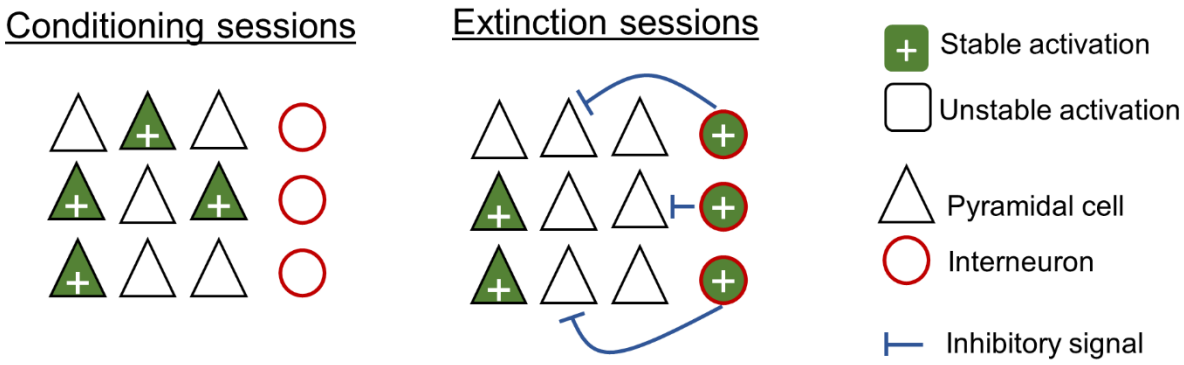


Figure 1: Schematic representation of the proposed model of excitatory and inhibitory ensemble activation in conditioning and extinction. In conditioning, a stable, repeatedly activated, excitatory pyramidal cell ensemble is recruited. In contrast, in extinction, a stable, inhibitory interneuron ensemble is recruited. This interneuron ensemble may have a role in inhibiting the pyramidal cell ensemble.

5.3.2 The role of the dmPFC in promoting the expression of learnt behaviours

Within the field of associative learning, the rodent dmPFC has been observed to have a role in promoting the expression of learnt behaviours for decades; both for aversive and rewarding associations (Moorman et al., 2015; Sotres-Bayon and Quirk, 2010). More specifically, there is evidence of the contribution of the dmPFC during recall of learnt behaviours (Corcoran and Quirk, 2007; Whitaker et al., 2017) and during their reinstatement following extinction (Calu et al., 2013). In further support, dmPFC connections to the nucleus accumbens (NAc) have been shown to be essential in stimulating behavioural responses in associative learning tasks (Parkinson et al., 2000). Similarly, dmPFC connections to the NAc core were observed to promote reinstatement

of cocaine seeking following extinction (Stefanik et al., 2013). Our observation of a stable pyramidal cell ensemble recruited for appetitive conditioning is in line with the theory that the dmPFC has a role in mediating the expression of learnt behaviour; in particular as this ensemble was re-recruited for recall. Moreover, our findings suggest this ensemble is less likely to be recruited in extinction learning, where cue-evoked behaviour is being suppressed. Thus, the stable interneuron ensemble may have been recruited to inhibit dmPFC signalling during extinction learning in order to reduce the expression of unnecessary cue induced food-seeking.

It is worth noting that this hypothesis regarding the role of the dmPFC in promoting learnt behaviours is often presented in conjunction with the hypothesis that the ventral mPFC is involved in inhibiting these behaviours during extinction (Moorman et al., 2015; Sotres-Bayon and Quirk, 2010). However, this functional dorsal/ventral dichotomy in the mPFC has been shown to be excessively simplified (Moorman et al., 2015). For example, there is evidence of the involvement of the dmPFC in the inhibition of inappropriate responses (Mihindou et al., 2013; Narayanan and Laubach, 2006). Crucially, these findings were linked to other known functions of the dmPFC. As such, we must also consider how other dmPFC functions may be involved in our appetitive learning task and how they might be mediated by the excitatory and inhibitory ensembles we observed.

5.3.3 The role of the dmPFC in mediating attention to specific cues

Modulations in attentional processes are thought to contribute to associative learning (Mackintosh, 1975; Pearce and Hall, 1980). This is particularly relevant to us as one of the most well-established functions of the mPFC is the control of attentional resources (for a review, Dalley et al., 2004) and the dmPFC has been suggested to be meditating attentional processes during associative learning (Han et al., 2003). One hypothesis that has been advanced is that the dmPFC directs attention to the most relevant stimuli in an

environment (Sharpe and Killcross, 2015). In support, Zhang et al. observed increased visual discrimination performance and increased firing within the visual cortex following activation of the dmPFC, suggesting a connections from the dmPFC to sensory areas mediate selective attention (Zhang et al., 2014). Thus, during appetitive conditioning, the dmPFC may be controlling attention towards the CS and increasing the efficiency of CS-selective responding. This is also in line with observations that the dmPFC mediates discrimination between environmental stimuli during learning tasks (Bussey et al., 1997; Cardinal et al., 2002).

With regards to our proposed model, this could suggest the stable pyramidal cell ensemble observed in appetitive conditioning has a role in mediating attention to the CS during learning and recall. In support, chemogenetic interference of dmPFC ensemble dynamics during appetitive learning decreased the efficiency of responses to the CS by increasing non-specific responses (Chapter 3). During extinction, the interneuron ensemble may be involved in reducing attention directed to the CS through the targeted inhibition of part of the pyramidal cell ensemble. In support, interneuron activity in the mPFC has been shown to contribute to altering attention (Kim et al., 2016b). Thus, we hypothesise that both pyramidal cell and interneuron ensembles in the dmPFC and the resulting alterations of the dmPFC output may be involved in adapting selective attention as conditioning and extinction learning progress.

5.3.4 The role of the dmPFC in mediating flexible behaviours

Another key role of the mPFC is to allow for flexible behaviours when rules in the environment change (Dalley et al., 2004). In support, dmPFC lesions hinder the ability of rodents to learn new reward seeking tasks (Seamans et al., 1995) and several studies have demonstrated that the dmPFC has a role in mediating the acquisition of new behavioural strategies following environmental changes (Durstewitz et al., 2010;

Karlsson et al., 2012; Laskowski et al., 2016). Furthermore, many studies have observed dmPFC signalling during the detection of prediction errors (Bryden et al., 2011; Hyman et al., 2017; Totah et al., 2009). Crucially, prediction errors events are thought to promote learning of new rules in associative learning and extinction paradigms (Li and McNally, 2014; Rescorla and Wagner, 1972). Thus, the dmPFC may contribute to detecting changes in the environment and mediating adaptive behaviours, through the promotion of adapted attentional resources (Kolling et al., 2016).

In our task, extinction training presents a change to the reward contingencies that mice have learnt in conditioning. Thus, this could suggest that interneuron recruitment in extinction learning may have a role in mediating behavioural adaptations in response to altered reward contingencies in the environment. We hypothesise that the interneuron ensemble may target the previously established excitatory ensemble in order to alter behaviours and allow flexible adaptations necessary to extinction learning. In support, dmPFC PV+ interneurons signalling has been linked to alteration in strategies during foraging (Kvitsiani et al., 2013). However, further studies will have to be performed to fully determine if the interneuron ensemble has a role in promoting flexible behaviours following a change in environmental contingencies.

5.3.5 Summary

Our work has demonstrated that appetitive conditioning and extinction recruit different populations of neurons; conditioning recruits a stable pyramidal cell ensemble while extinction learning recruits a stable interneuron ensemble. From these observations, we suggested a possible model in which the role of the interneuron ensemble in extinction is to suppress the pyramidal cell ensemble established in conditioning and through this, inhibit conditioning-specific behavioural mechanisms such as the promotion of food-seeking behaviours and attention to specific cues. We also hypothesised that the

interneuron ensemble may be involved in mediating behavioural flexibility in response to changes in the environment. However, the exact contribution selective attention and behavioural flexibility play in our task will have to be examined further as well as the role of the dmPFC in mediating them.

Of note, interneuron function has been of particular interest within the field clinical research, as disrupted excitation-inhibition balance within the brain is thought to be a contributing factor to autism spectrum disorders and interneuron dysfunction within the PFC has been linked to disorders such as schizophrenia (for a review, Marín, 2012). As such, our findings of the different roles of excitatory and inhibitory signalling in conditioning and extinction learning may have implications for clinical work and offer further perspectives into how excitation and inhibition work together to mediate different behaviours and the shifts between them.

5.4 Methodological limitations

Although we found compelling evidence of the role of different neuronal populations in appetitive conditioning and extinction, there are some methodological concerns that must be considered when interpreting these findings.

5.4.1 Using Fos as a marker of activation

As addressed in the introduction, Fos will reach peak expression at approximately 1h following high input, will rapidly decrease over the course of several hours (approximately 6h) and have will have returned to baseline by 24h (Bisler et al., 2002; Herdegen et al., 1991; Lin et al., 2018). Therefore, during our imaging sessions, there will be Fos expression triggered by events occurring outside of the learning session (e.g., in the home cage). This limitation is addressed by using our UP group as a control for

extraneous Fos signals that are not related to associative learning. However, Fos as a marker of activation can also only offer a limited amount of information and has been criticised in the past for not representing the complexity of the neuronal signals evoked by behaviour (Devan et al., 2018; Harris, 1998; Kovács, 2008; McReynolds et al., 2018). For example, Fos expression will not detect neurons that are inhibited during behaviour, which has been shown to occur in the dmPFC during appetitive learning (e.g., in response to the presentation of the CS (Otis et al., 2017)). Fos-based chemogenetics and optogenetics methods in particular target a range of neurons that may have been active independently during a task and alter their activity without considering the complexity of their normal patterns of signalling (Devan et al., 2018).

Furthermore, while Fos-expressing ensembles have been observed as necessary and sufficient in encoding associative memories (Cruz et al., 2013; Koya et al., 2009; Liu et al., 2012), surrounding neurons also likely have a role in mediating learnt behaviours. In support, Fos-expressing and Fos-non expressing neurons of the dmPFC display opposing intrinsic excitability modifications following training (Suppl. Fig 2 & 3; Brebner et al. *in preparation*; Whitaker et al., 2017), suggesting both populations contribute to the overall output of the dmPFC. Moreover, there is evidence of the contribution of widespread, coordinated firing of mPFC neurons in mediating new learning and behaviours in responses to alterations to the environment (Karlsson et al., 2012). Thus, the robustly activated populations of neurons we are observing in this study cannot be considered exclusively responsible for mediating learning and memory.

Finally, the expression of other immediate early genes (e.g., *Arc*, *zif268*), while also increased following high activation, is not always similar to the expression of Fos (Fanous et al., 2013; Guzowski et al., 2001), suggesting some neurons will have been highly activated but remained undetected in our study. In response to this issue, artificial activity-dependent promoters such as E-SARE (Kawashima et al., 2013) and RAM

(Sørensen et al., 2016) have been developed to improve targeting of strongly activated neurons.

Thus, we have to be aware that Fos expression as an activity marker can only provide an incomplete picture of neuronal activation and signalling during learning. It will highlight neurons that are or will become functionally relevant but it cannot capture the full complexity of dmPFC activation in appetitive conditioning and extinction.

Another concern with the use of Fos is that, as of now, the exact activity patterns and inputs necessary to induce Fos are unclear and may differ between cell types. In particular, there is evidence that suggests glutamatergic input will affect pyramidal cells and interneurons differently (Riebe et al., 2016). As such, we did not directly compare Fos expression patterns in pyramidal cells and interneurons here. However, we must also acknowledge that the varying neuronal phenotypes we observe under the broad category of GABA-ergic interneuron may also show differences in Fos expression, although this has yet to be studied to our knowledge.

5.4.2 Comparing 2P imaging and chemogenetics results

In using Fos-based 2-photon imaging, we were able to longitudinally examine Fos in mice trained under freely-moving conditions. This is crucial as it allowed us to use this technique alongside a chemogenetics method with a similar behavioural paradigm. However, we also must be aware that results obtained from our imaging and chemogenetics experiments are not directly comparable. Previous studies and our own (Chapter 2, Chapter 3 (NC tag experiment)) indicate that both *Fos-GFP* and *Fos-tTA* mice are able to learn associations similarly to wild-type mice (Yoshii et al., 2017; Ziminski et al., 2017). Thus, the different genotypes of the mice used in imaging and chemogenetics in themselves do not seem to alter performance in conditioning. Furthermore, neurophysiological properties of *Fos-GFP* mice have been observed to be

similar to that of WT lines (personal correspondence with Dr. Eisuke Koya). Together, this suggest the different mouse models are comparable.

However, one major difference between the imaging and chemogenetics experiments, which reduced their comparability, is the surgical procedures undergone by mice. Microprism implantation is a more invasive procedure than viral infusion surgeries and results in a long-term implant within the brain as well as head bars being fixed to the mouse's skull. Although we aimed to limit damage to the brain by placing the microprism between cortices rather than removing tissue to access the mPFC (Low et al., 2014), we cannot discount that there will have been damage to the underlying brain tissue. However, all our mice (including WT having not undergone surgery) learnt the appetitive task within similar timeframes, suggesting that damages incurred during surgeries do not significantly impair appetitive learning. Despite this, we must keep in mind that surgical procedures could still have affected neuronal signalling and plasticity in the dmPFC.

Another main concern in comparing these methods is whether or not we can target similar populations of neurons with both methods. While both models rely on the expression of Fos in some capacity, the threshold for 'Fos-expressing' we set in our imaging study may be different to the threshold necessary to drive the expression of the DREADD receptor in *Fos-tTa* mice. The nature of the imaging methods used (2 photon imaging on a live brain compared to immunofluorescence microscopy in fixed tissue) make it difficult to draw a direct comparison between the number of GFP+ neurons in our *Fos-GFP* mice to the hM3Dq+ neurons in *Fos-tTa* mice. Moreover, while Fos-GFP is nuclear, hM3Dq-mCherry is expressed within the whole cell. Furthermore, the relationship between mCherry intensity and the quantity of hM3Dq necessary to generate a significant effect within the cell cannot be determined post-fixation. However, chemogenetic methods rely on multiple factor for the hM3Dq-mCherry to be expressed: spread of the viral infusion (which we determined to be within the area imaged *in vivo*), integration of virus within neurons, as well as the threshold value of tTa for DREADD

expression. Together with our qualitative observations that the density of mCherry+ neurons in *Fos-tTa* mice is lower than GFP+ neurons in our 2P images, this suggests that the chemogenetics method will likely be restricted to a smaller population than those considered GFP+ *in vivo*. As both mouse models depend on Fos expression, it is likely that, if threshold differ, the population tagged by one method will be included within the population tagged by the other. Therefore, while we must assume that the populations tagged by these methods are not comparable in size, we can hypothesise that the population tagged within the chemogenetics experiment is included within the wider GFP+ pool of neurons detected with 2P imaging. Crucially, the manipulation of this population is sufficient to drive a behavioural effect. Thus, we must keep in mind that these methods likely do not target the exact same population; however, as both rely on the *Fos* promoter, we are able to use these models in a complementary manner.

5.5 Open questions and future directions

5.5.1 Role of activated populations in learning

While here we observed stable ensembles during the formation of associative memories, one limitation of this work is that we cannot determine here if the ensembles we detected and manipulated are involved in the expression of behaviour rather than learning. However, as previously discussed in our chemogenetics study (Chapter 3), discriminative performance was affected by our manipulation on sessions where we were not directly manipulating dmPFC neurons. We concluded that this might suggest the presence of long-term changes to neurons. Thus, this may indicate that the ensemble we detected is involved in learning rather than only in the expression of behaviour. However, our work raises a series of further questions as, while our experiments revealed that the population detected in early learning may be specific to the conditioning task and was likely *involved* in the formation of a CS-US association, we did not

determine if it was *necessary*. Moreover, we also did not determine what role the population recruited in late learning may have in encoding the CS-US association and how it may be involved in extinction.

One way to address these questions would be to perform a series of further chemogenetics experiments. For example, silencing either early learning or late learning activated neurons during recall with an hM4Di inhibitory DREADD receptor (Armbruster et al., 2007) would provide evidence to assess how necessary these populations are to the expression of learnt behaviours. Fear conditioning studies have previously suggested that dmPFC ensembles may only be necessary to mediating learnt behaviour during remote recall (Frankland et al., 2004; Restivo et al., 2009). However, this has yet to be fully examined in more progressive and incremental forms of learning such as appetitive conditioning. Furthermore, activating and/or inhibiting neurons activated by late learning during extinction may provide more insight into the role of this population during extinction. It would also test our theory that the previously established conditioning ensemble needs to be suppressed in extinction learning.

5.5.2 Interneuron subtypes

In our study, we demonstrated that a stable interneuron ensemble forms in the dmPFC during extinction. As we have already addressed, one limitation in our experiments is that we made no distinction between the various interneuron subtypes despite their phenotypical differences (Kepecs and Fishell, 2014). Furthermore, our population analysis (Chapter 2) as well as a number of previous studies (Gaykema et al., 2014; Kvitsiani et al., 2013) suggested that interneurons may be involved in food-seeking behaviours; however, in our study, the pooling of different interneuron subtypes may have masked any alterations in recruitment. Therefore, the next step would be to fully

investigate which interneuron subtypes may be active in conditioning and extinction of appetitive associations.

One way to address this would be to use transgenic mice models. In particular, Cre/lox systems expressed according to a specific promoter that correspond to specific interneurons subtypes (e.g., PV-Cre, SOM-Cre, VIP-Cre). These mouse models could be paired with other genetic constructs which allow fluorophore expression in Cre-expressing cells (e.g., *PV-Cre x DIO-tdTomato* mice (Ferguson et al., 2013) or viral injection of DIO-tdTomato into *SST-Cre* mice (Fu et al., 2014)). These models could then be crossbred with *Fos-GFP* mice to examine Fos expression within specific neuronal subtypes during learning and to detect repeated activation of specific interneuron subtypes. Another possibility is to use these Cre/lox models in conjunction with Cre-dependent calcium indicators to examine calcium signals during learning (e.g., Pinto and Dan, 2015). With these models, we would be able to identify different interneuron populations of the dmPFC separately during learning and assess their contributions and activity during appetitive conditioning and extinction. However, to our knowledge, a Cre-dependent *Fos-tTa* mouse model or viral construct has yet to be generated. As such, while the manipulation of general populations of dmPFC interneuron subtypes has been performed previously (e.g., Sparta et al., 2014), the specific manipulation of Fos-expressing interneuron subtypes would require the development of new tools. For example, the generation of a DIO-Fos-tTA viral construct which could be co-injected with the DREADD virus into a *PV-Cre* mouse. This model would theoretically allow DREADD expression in PV+, Fos-expressing neurons only (Fig. 2).

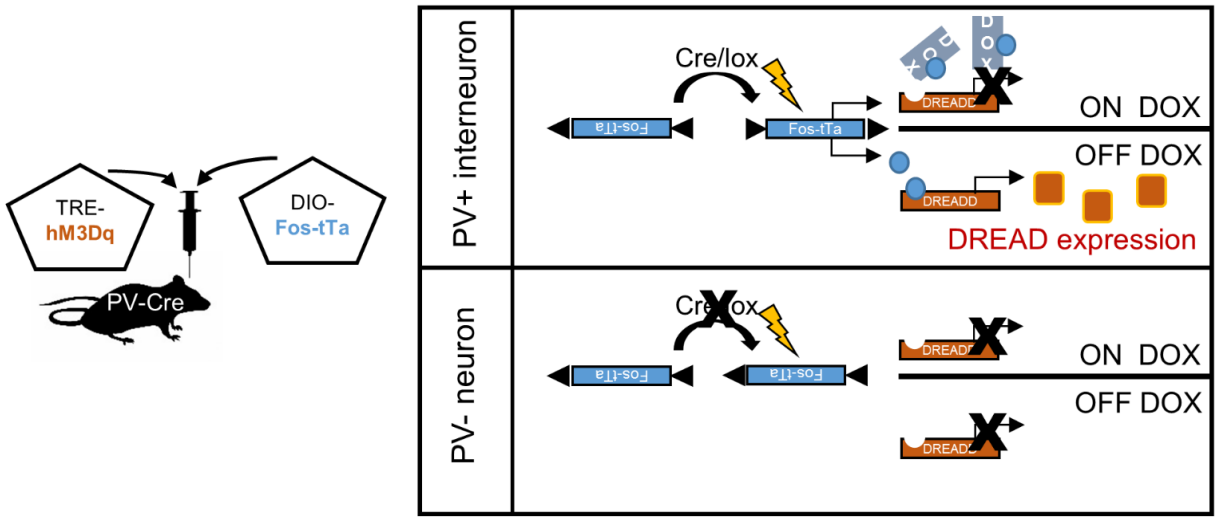


Figure 2: Schematic representation of suggested method to manipulate Fos-expressing PV+ interneurons of the dmPFC. A TRE-hM3Dq virus and a DIO-Fos-tTa virus are injected into a *PV-Cre* mouse in the dmPFC. The tTa protein (blue circle) will be expressed with Fos following robust activation in PV+ interneurons only. The DREADD receptor of interest (orange square) will be expressed in absence of Doxycycline (Dox) in Fos-expressing PV+ interneurons.

5.5.3 Connectivity of the ensemble

Furthermore, having identified ensembles during conditioning and extinction, a crucial question that emerges is: what other brain regions are these stable ensembles receiving input from and connecting to? Previous evidence suggests that dmPFC neurons that signal during an appetitive conditioning task connect to multiple areas (Otis et al., 2017) and we have already discussed what areas may be targeted by the dmPFC during our task and what role these connections may have in driving learnt behaviours (Experimental chapters). Together with our findings, this prompts the further investigation of the connections of the Fos-expressing ensembles we observed in conditioning and extinction. In particular, it would be valuable to examine what areas these neurons are projecting to during both types of learning and if these projections are

altered depending on the types of learning. Furthermore, it would also be crucial to examine where inputs to the dmPFC might be originating.

To address this, we could use viral constructs, similarly to the methods we suggested for the investigation of interneuron subtypes. One possibility would be to make use of retrograde viruses injected into known output areas of the dmPFC (e.g., NAc, Amygdala, Thalamus) and through them examine specific dmPFC neural populations according to their connectivity. If these viruses code for a fluorophore and are injected to *Fos-GFP* mice they could allow us to examine Fos-expression patterns within these specific populations in a longitudinal manner. Alternatively, these viruses could also code for calcium indicators to allow us to observe calcium signals within populations according to their connectivity (e.g., Otis et al., 2017). As such, we would be able to identify these populations separately during learning and assess their different contributions and activation during appetitive conditioning and extinction. Another possibility would be to make use of anterograde trans-synaptic viruses coding for a fluorophore injected to input areas of the dmPFC (e.g., Amygdala, Thalamus) (Beier et al., 2011). In pairing this method with *in vivo* Fos-GFP imaging, it would be possible to examine the inputs of dmPFC neurons according to their activation history in appetitive conditioning and extinction.

Finally, anterograde or retrograde viruses expressing the Cre/lox system could be used to manipulate neurons according to connectivity. Similarly to what we addressed when discussing interneuron subtypes, while the manipulation of general populations according to connectivity has been performed (e.g., Stefanik et al., 2013), ensemble specific manipulations according to connectivity have not as the Cre/lox system has yet to been used for conditional Fos-tTa expression. Thus, the development of a DIO-Fos-tTa viral construct would also benefit further research into how the contributions of Fos-expressing neurons may modulate according to connectivity.

5.5.4 Relating the different 'types' of ensemble

In our present study, we relied exclusively on the expression of Fos to mark recently activated ensembles. As discussed previously, this marker is valuable, as it has shown to be both necessary and sufficient to encoding associations (Cruz et al., 2013; Koya et al., 2009; Liu et al., 2012); however, it is unlikely that Fos-expressing neurons are the only ones contributing to learning and memory. Moreover, here we chose to focus our analysis further on neurons that were repeatedly activated as defined by Fos expression. This was driven by previous studies that suggested the importance of these repeatedly activated neurons (Cao et al., 2015; Czajkowski et al., 2014; Mattson et al., 2008; Milczarek et al., 2018) and by our own observations that there were no learning-specific changes in total activation numbers as conditioning and extinction learning progressed. However, our population analyses and chemogenetic studies also raised the intriguing possibility that the dismissal of certain populations may also be involved in encoding associations.

Furthermore, as addressed above, a major limitation of using Fos as a marker of recent activation is that the expression of Fos offers little detail as to what activity patterns (relating to both inputs and spiking) occurred during conditioning and extinction, in particular as relating to the different elements of the task (e.g., cue, sucrose, licking behaviour, etc.). In our introduction, we briefly raised the point that the definition of an 'ensemble' in literature has varied according to the methods used to observe neuronal populations (e.g., according to spiking patterns (Hyman et al., 2012), according to calcium signals (Cai et al., 2016) or according to Fos expression (Cruz et al., 2013)). In this study, we narrowed our definition of 'ensemble' further to a population of neurons that repeatedly displayed Fos expression following multiple learning sessions. However, there is little to no evidence truly linking ensembles defined by spiking patterns to our Fos-expressing ensembles. Recent findings comparing Fos expression following context exploration to place cell spiking activity within the hippocampus would even suggest that

these different types of ensembles may be distinct populations (Tanaka and McHugh, 2018; Tanaka et al., 2018). Moreover, to our knowledge, no studies have established the functional relevance of these 'spiking' and 'calcium' ensembles. As such, it is necessary to clarify how these different definitions of 'ensemble' might relate to each other.

In an appetitive conditioning task, Otis et al. demonstrated that dmPFC neurons would progressively tune to food predictive cues (Otis et al., 2017), prompting us to suggest above that these tuning neurons may overlap with our Fos-expressing ensemble. However, the existence and extent of this overlap has yet to be demonstrated.

To address this and to bridge the divide between Fos-expressing ensembles and those defined by neuronal spiking activity, we suggest a possible future experiment with a combined Fos and calcium *in vivo* imaging approach. Using *Fos-GFP* mice and a calcium indicator of a different colour (e.g., XCamP indicators (Inoue et al., 2019)) together, it would be possible to record calcium signals during head-fixed conditioning sessions and Fos expression following these sessions. This technique would also determine if spiking patterns displayed during learning correlate with Fos expression following the session and if past expression of Fos influences spiking activity.

5.6 Final summary and conclusion

While the strengthening and weakening of food-cue associations are crucial to survival as well as a key factor in a number of eating disorders, the neuronal mechanisms that underlie them are not as commonly examined when compared to other forms of conditioning with more salient stimuli (e.g., drugs and fear). Furthermore, neuronal ensembles of the dmPFC have been shown to be crucial in mediating learnt behaviours yet they are rarely examined during learning itself, prior to associations being formed. The appetitive conditioning and extinction paradigms we used provide an excellent framework to examine the activation of ensembles during the acquisition and extinction

of an appetitive association as these tasks occur over multiple sessions and across multiple days. Using a combination of techniques, we revealed that stable pyramidal cell and interneuron ensembles were recruited from the first session of conditioning and extinction respectively in the dmPFC and determined that the population of neurons activated in early appetitive conditioning must be specifically altered to establish appetitive learning. By using microprisms based *in vivo* imaging of a *Fos-GFP* mouse model, we were able to track the activation of individual neurons across multiple sessions of learning and examine patterns of activation in conditioning and extinction. Furthermore, we were able to observe these ensemble activation patterns in freely-moving animals which allowed us to use these findings in conjunction with those from our chemogenetics study as both were performed with a similar behavioural paradigm. In combining these approaches and comparing our findings to previous studies, we were able to provide unique insights, both into the function of the dmPFC in mediating appetitive associations but also into general dynamics of ensemble formation during progressive forms of learning. Our work also highlighted the importance of taking different cell types into account when examining the function of different brain areas in mediating learnt behaviours.

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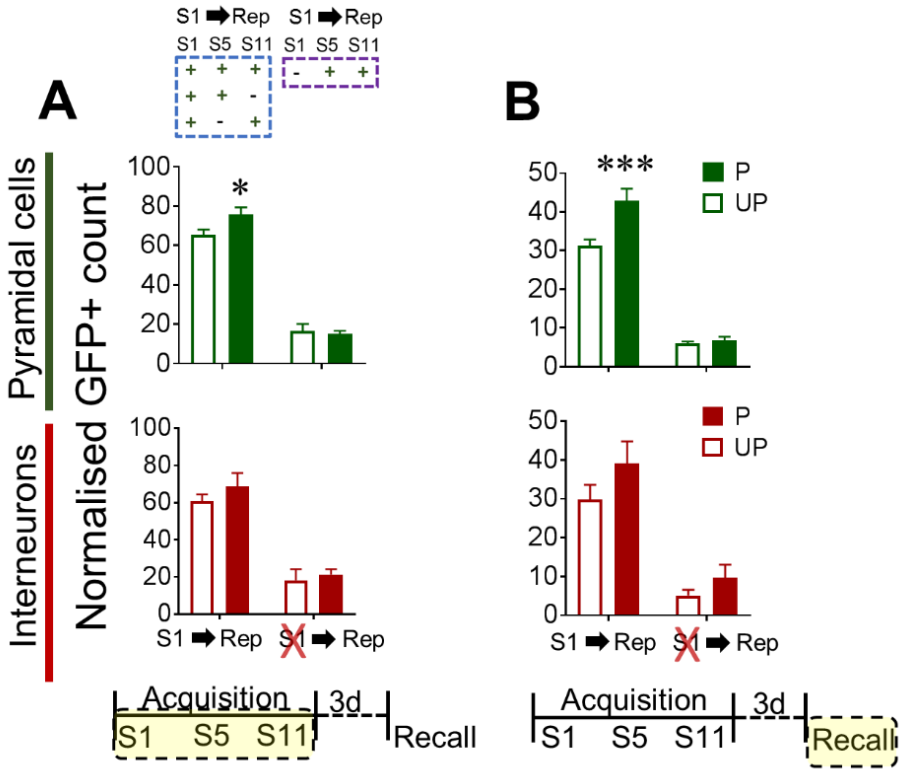
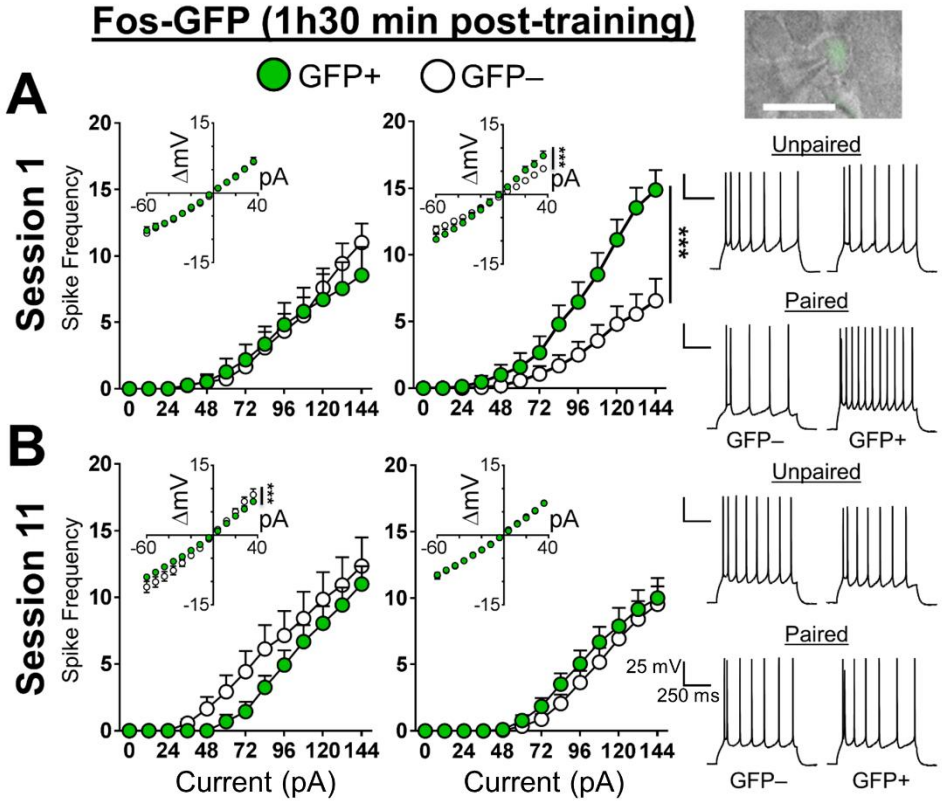
Annex:

Supplementary Material for Chapter 2:



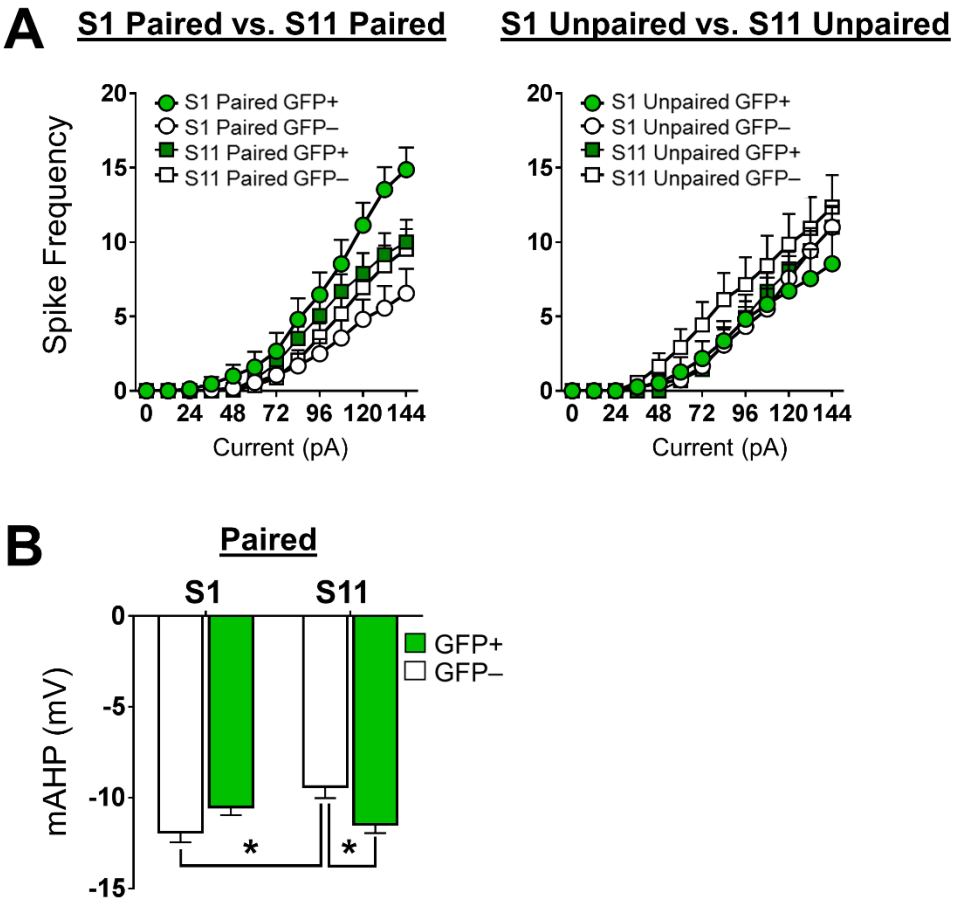
Supplemental Figure 1 (Associated with Chapter 2 Fig. 3):
Conditioning and memory recall recruit a pyramidal cell ensemble with a repeated activation history that includes the initial acquisition session.
(A) Normalised GFP+ counts of repeatedly activated ('Rep'; >1 activation in Acquisition) pyramidal cells and interneurons with a S1 (S1 → Rep) or no S1 (no S1 → Rep) activation history. In pyramidal cells, there was a significant interaction of Activation Category X Conditioning ($F_{1,17}$=5.19, P=0.036); Post-hoc testing revealed a significant increase in the number of S1→Rep in Paired compared to Unpaired mice (P<0.05). We detected no significant interaction in interneurons ($F_{1,17}$=0.19, P=0.667). **(B)** Normalised GFP+ counts for pyramidal cells and interneurons recruited in recall which had shown repeated activation (>1 activation in Acquisition) during training, according to their S1 activation history (S1 → Rep or no S1 → Rep). In pyramidal cells, there was a significant interaction of Activation Category X Conditioning ($F_{1,10}$=9.80, p=0.011). Post-hoc testing revealed a significant increase in the number of 'S1→Rep' neurons re-recruited in Recall in Paired mice compared to Unpaired mice (p<0.001). We detected no significant interaction in interneurons ($F_{1,10}$=0.73, p=0.413). Data expressed as Mean±SEM. *Post-hoc analysis: *** P<0.001, * P<0.05; P: n=10, UP n=9 for **(A)**, P n=6, UP n=6 for **(B)**.*



Supplemental Figure 2 (associated with Chapter 2 and 3):

The firing capacity of GFP+ pyramidal cells is enhanced following S1. This is not observed following S11.

(A) Following S1, GFP+ spike frequency is significantly higher than GFP– neurons in Paired, but not Unpaired mice (Paired; GFP+ n=5/15, GFP– n=5/16, Unpaired; GFP+ n=6/11, GFP– n=6/12). **(B)** Following S11, GFP+ and GFP– spike frequency is similar in both Paired and Unpaired mice (Paired; GFP+ n=6/19, GFP– n=6/17, Unpaired; GFP+ n=6/16, GFP– n=6/14)**.** Right top: Representative image of a patched GFP+ pyramidal cell in the mPFC of a Fos-GFP mouse, scale bar 20 µm. Right: Representative traces from GFP+ and GFP–pyramidal cells of Paired and Unpaired mice at 120 pA stimulation. *Inset:* Current/voltage (I/V) curves, scale bar 25 mV, 250 ms. All data are expressed as Mean±SEM; *n*= number of animals/number of cells total. *** *indicates Two-way mixed ANOVA Cell Type X Current p<0.001.*



Supplemental Figure 3 (Associated with Chap. 2 and 3):
Intrinsic Excitability Supplementary Analysis
A. Firing capacity in Paired GFP+ and GFP– neurons from Paired and Unpaired mice in S1 and S11 **B.** mAHP in Paired mice on S1 and S11.

Associated material for Supplementary Figure 2 and 3:

In parallel to our 2-photon investigation of conditioning (Chapter 2), Joseph Ziminski determined the physiological properties of pyramidal cells activated during early conditioning (Brebner et al. *in preparation*). We have included these results in order to provide further perspectives into our findings presented in Chapters 2 and 3.

Briefly, *Fos-GFP* Mice were randomly assigned to S1 and S11 groups. Mice in the S1 group received only a single acquisition session (Paired or Unpaired as described in *General Procedures*) following magazine training before being sacrificed for electrophysiology recordings. Unpaired mice received sucrose in their home cage 10 minutes before this session. Mice in the S11 group received 11 sessions of conditioning before being sacrificed for electrophysiology recordings. Unpaired mice received sucrose in their home cage 10 minutes before this session, for all other sessions it was delivered at a random time.

90 minutes following S1 or S11, *Fos-GFP* mice were anaesthetized and transcardially perfused with NMDG-HEPES recovery aCSF. The brain was quickly removed and sliced. Slices remained in standard recording aCSF for the remainder of the recording day. Whole-cell recordings on layer II-III mPFC GFP+ and GFP- pyramidal cells were performed using borosilicate capillary glass-pipettes. Pyramidal neurons were held at -65 mV for the duration of recording. The current clamp protocol consisted of 1000 ms positive current injections from -60 pA incrementing in 4 pA steps. Spike counts were conducted using Stimfit (Guzman et al., 2014) while spike kinetics were analyzed with MiniAnalysis software (MiniAnalysis, Synaptosoft).

We analysed the excitability of GFP+ and GFP– pyramidal cells in Paired and Unpaired *Fos-GFP* mice following S1 and observed significant alterations in firing capacity across groups (Suppl. Fig. 2A; Group X Cell Type X Current $F_{12,600}$=6.38, P<0.001). Further analysis revealed a revealed a significant difference in excitability between GFP+ and

GFP- neurons in Paired (Cell Type X Current, $F_{12,348}$=9.42, P<0.001), but not Unpaired mice (Suppl. Fig. 2A; Suppl. Table 5). We then examined the underlying intrinsic adaptations that may contribute to the increased firing capacity of GFP+ neurons (Suppl. Table 1, 5). In Paired mice only, the input resistance (Ri) of GFP+ neurons increased following S1 (Group X Cell Type X Current, $F_{25,1200}$=3.81, P<0.001; Paired: Cell Type X Current, $F_{25,700}$=6.85, P<0.001). Similarly, we observed a decrease in the rheobase or current necessary to elicit an action potential (Group x Cell Type, $F_{1,49}$=6.64, P<0.05). We observed no other interaction effects for the action potential peak, half width, threshold, or afterhyperpolarisation (both fast and medium).

We next determined the excitability properties of neurons activated in late conditioning following S11. We analysed the excitability of GFP+ and GFP– pyramidal cells following S11. We observed no alterations in firing capacity across groups (Suppl. Fig. 3B; Group X Cell Type X Current, $F_{12,744}$=1.21, P<0.27). We did observe a significant interaction effect for the Ri (Group X Cell Type X Current, $F_{25,1550}$=2.16, P<0.001), underpinned by a GFP– increase in Unpaired mice (Cell Type X Current, $F_{25,700}$=2.93, P<0.001) and a significant fAHP interaction (Group X Cell Type, $F_{1,61}$=4.73, P<0.05) driven by a GFP– decrease in Paired (P<0.05) but not Unpaired (P=0.50) mice, suggesting that some modulation of underlying parameters did occur (Suppl. Table 1, 5). We detected no other interaction effects in any other measured electrophysiological property. To confirm that the hyper-excitability of activated neurons we had observed in S1 was transient, we compared firing capacity in Paired mice at S1 and S11 (Suppl. Fig. 4A). As expected, we observed a significant change in firing capacity between S1 and S11 (Session X Cell Type X Current, $F_{12,756}$=4.38, P<0.001) and no such change in the Unpaired group (Suppl. Fig. 4B; Group X Cell Type, $F_{12,588}$=0.88, P=0.57); this was driven by an increase in the excitability of S1 GFP+ neurons ($F_{12,384}$=2.70, P<0.01) concurrent with a decrease in the excitability of S1 GFP– neurons ($F_{12,372}$=1.81, P<0.05). Underpinning this alteration was a change in the input resistance in Paired mice (Suppl. Fig. 2A and B (inset); Session

X Cell Type X Current, $F_{25,1550}$=5.02, P<0.001), due to an increase in GFP+ neurons ($F_{25,800}$=3.34, P<0.001) and a decrease in GFP– neurons ($F_{25,750}$=1.89, P<0.01). We also observed a significant change in the medium afterhyperpolarisation (mAHP) (Suppl. Fig. 3B; Session X Cell Type $F_{1,62}$=10.50, P<0.01) determined by a decrease in GFP– neurons (P<0.5) but not GFP+ neurons. In Unpaired mice, we did observe an interaction effect on S1 and S11 I/V curves (Suppl. Fig. 3A and B (inset); Session X Cell Type X Current $F_{25,1200}$=1.54, P<0.05), underpinned by an increase in GFP– ($F_{25,575}$=3.13, P<0.001) but not GFP+ ($F_{25,625}$=0.45, P=0.99) neurons and in the rheobase ($F_{1,62}$=4.79, P<0.05).

Supplemental Table 1

	Session 1 (S1)				**Session 11 (S11)**			
	Unpaired		Paired		Unpaired		Paired	
	GFP-	GFP+	GFP-	GFP+	GFP-	GFP+	GFP-	GFP+
Resting Vm (mV)	-69.35 ±0.96	-67.30 ±1.18	-68.73 ±0.69	-68.95 ±0.76	-66.75 ±0.71	-67.16 ±0.52	-68.42 ±0.82	-68.32 ±0.74
Rheobase (pA)	77.33 ±6.83	103.27 ±18.58	121.00 ±16.69	75.14***** ±8.24	82.57 ±14.37	79.20 ±5.36	86.71 ±8.46	91.37 ±10.26
Ri (MΩ)	160.38 ±9.20	151.10 ±14.11	138.48 ±13.81	193.82****** ±15.19	181.62 ±21.67	143.14 ±9.01	165.31 ±13.39	161.89 ±8.70
AP Peak (mV)	67.00 ±4.47	66.38 ±3.26	68.71 ±2.37	65.76 ±3.53	68.40 ±2.66	70.61 ±2.22	67.19 ±2.47	73.40 ±2.23
AP Half-Width (ms)	1.30 ±0.09	1.33 ±0.07	1.21 ±0.04	1.21 0.05	1.28 ±0.04	1.32 ±0.04	1.33 ±0.05	1.34 ±0.04
Threshold (mV)	-36.51 ±0.85	-36.06 ±1.47	-34.36 ±1.00	-36.57 ±0.94	-35.46 ±0.74	-36.43 ±0.77	-35.86 ±0.65	-38.39 ±0.54
fAHP (mV)	-3.01 ±0.40	-2.48 ±0.35	-3.33 ±0.42	-3.72 ±0.47	-4.10 ±0.52	-3.69 ±0.48	-2.01 ±0.30	-3.56***** ±0.42
mAHP (mV)	-11.12 ±0.68	-9.93 ±0.69	-11.91 ±0.54	-10.51 ±0.44	-11.54 ±0.86	-10.37 ±1.32	-9.39 ±0.63	-11.47 ±0.48

Electrophysiological properties of GFP+ and GFP– pyramidal cells from Paired and Unpaired mice across conditioning sessions. Data are expressed as mean±SEM. Liquid junction potential was − 13.7 mV and was not adjusted for. Spike characteristics were determined from a single action potential (AP); when a doublet was elicited the second

spike was analysed. Input resistance was calculated from the slope of the I/V curve measured in response to 4 pA current steps ranging from −60 to 40 pA. Spike threshold was measured using the third differential with Mini Analysis software. The AP peak was calculated as the difference between the AP peak and AP threshold. Half-width was measured as the AP width at half-maximal spike. Post-spike fAHPs and mAHPs were measured ~3 and ~40 ms following the AP threshold respectively, similar to Ishikawa et al. (Ishikawa et al., 2009). Sidak post-hoc tests between GFP+ and GFP– are indicated *p<0.05, **p<0.01.

Supplemental Table 2: Statistics table for Chapter 2, Figure 1 and 2

Analysis	Parameter	Test	Factors	n	Test Statistic & p
FGGT mice behaviour - Acquisition - Fig. 1B	Head entries	3-way mixed ANOVA	Cue, Session, Conditioning	P n=12; UP n=10	Conditioning: $F_{1,20}$=59.76, P<0.001; Session: $F_{11,220}$=1.17, P=0.307; Cue: $F_{1,20}$=23.63, P<0.001; Conditioning x Session: $F_{11,220}$=0.94, P=0.500 ; Conditioning x Cue: $F_{1,20}$=15.11, P=0.001; Cue x Session: $F_{11,220}$=5.61, P<0.001; Conditioning x Session x Cue: $F_{11,220}$=5.94, P<0.001
FGGT mice behaviour - Recall - Fig. 1C	Head entries	2-way mixed ANOVA	Cue, Conditioning	P n=7; UP n=6	Conditioning: $F_{1,11}$ = 9.02, P=0.012; Cue: $F_{1,11}$ = 17.29, P=0.002 ; Conditioning x Cue: $F_{1,11}$ = 15.46 P=0.002 ;
FGGT mice behaviour - Acquisition - Paired - Fig. 1D	Selectivity Index	1-way repeated measures ANOVA	Session	P n=12	Session: $F_{11,121}$=9.50, P<0.001
HC GFP+/mm3 - Pyramidal cells - Fig. 2D	GFP+ per mm3	2-way mixed ANOVA	Conditioning, Session	P=10, UP=9	Conditioning: $F_{1,17}$ = 0.55 P=0.469; Population: $F_{1,17}$ = 4.20, P=0.056; Conditioning x Population: $F_{1,17}$ = 0.02, P=0.888
HC GFP+/mm3 - Pyramidal cells - Fig. 2D	GFP+ per mm3	2-way mixed ANOVA	Conditioning, Session	P=10, UP=9	Conditioning: $F_{1,17}$ = 0.89, P=0.358; Population: $F_{1,17}$ = 0.00, P=0.991; Conditioning x Population: $F_{1,17}$ = 1.84, P=0.193

Supplemental Table 3: Statistics table for Chapter 2, Figure 3 and Supplemental Figure 1

Analysis	Parameter	Test	Factors	n	Test Statistic & p
Acquisition session – Pyramidal cells – Fig. 3A	Normalised GFP+ count	2-way mixed ANOVA	Session, Conditioning	P n=10; UP n=9	Conditioning: $F_{1,17}$ = 1.21 P=0.287; Session: $F_{2,34}$ = 3.59 P=0.038; Conditioning x Session: $F_{2,34}$= 0.0.20 P=0.821
Acquisition session – Interneurons – Fig. 3A	Normalised GFP+ count	2-way mixed ANOVA	Session, Conditioning	P n=10; UP n=9	Conditioning: $F_{1,17}$ = 0.79 P=0.388; Session: $F_{2,34}$ = 1.38 P=0.266; Conditioning x Session: $F_{2,34}$ = 0.06 P=0.945
Persistently activated neurons - Conditioning - Pyramidal cells - Fig. 3C	Normalised GFP+ count	2-way mixed ANOVA	Population, Conditioning	P n=10; UP n=9	Conditioning: $F_{1,17}$ =1.17; P=0.294; Population: $F_{1,17}$ = 61.75 P<0.001; Conditioning x Population: $F_{1,17}$=5.97, P=0.026
Persistently activated neurons – Conditioning – Interneurons – Fig. 3C	Normalised GFP+ count	2-way mixed ANOVA	Population, Conditioning	P n=10; UP n=9	Conditioning: $F_{1,17}$ = 1.10, P= 0.309; Population: $F_{1,17}$ = 81, P=0.011; Conditioning x Population: $F_{1, 17}$ = 0.17, P=0.681
Session 1 – Population analysis – Pyramidal cells – Fig. 3D	Proportions	Pearson's Chi-squared test	Category, Conditioning	P n=10; UP n=9	Category x Conditioning: X^2_3=58.98, P<0.001; Post-hoc, Bonferroni α=0.00625; (signif. for + + +, + + -, + - -)
Session 1 - Population analysis – Interneurons – Fig. 3D	Proportions	Pearson's Chi-squared test	Category, Conditioning	P n=10; UP n=9	Category x Conditioning: X^2_3=41.63, P<0.001; Post-hoc, Bonferroni α=0.00625; (signif. for + + +, + + -, + - -)
Recall - Pyramidal cells – Fig. 3E	Normalised GFP+ count	t-test	Conditioning	P n=6; UP n=6	t_{10}=2.40, P=0.037
Recall - Interneurons – Fig. 3E	Normalised GFP+ count	t-test	Conditioning	P n=6; UP n=6	t_{10}=0.67, P=0.516
Persistently activated neurons – Recall – Pyramidal cells – Fig. 3F	Normalised GFP+ count	2-way mixed ANOVA	Population, Conditioning	P n=6; UP n=6	Conditioning: $F_{1,10}$ = 5.90, P=0.036; Population: $F_{1,10}$ = 155.3, P<0.001; Conditioning x Population: $F_{1,10}$ =7.65, P=0.020
Persistently activated neurons – Recall – Pyramidal cells – Fig. 3F	Normalised GFP+ count	Post-Hoc t-test, Sidak correction	Conditioning	P n=6; UP n=6	S1+ | S5+ S11+: t_{20} = 3.56, Adj. P=0.004 S1-| S5+ S11+: t_{20}=0.46, Adj. P=0.878
Persistently activated neurons – Recall – Interneurons - Fig. 3F	Normalised GFP+ count	2-way mixed ANOVA	Population, Conditioning	P n=6; UP n=6	Conditioning: $F_{1,10}$ = 1.92, P=0.195; Population: $F_{1,10}$ = 44.69, P<0.001; Conditioning x Population: $F_{1,10}$ = 0.24, P=0.636
Recall - Population analysis – Pyramidal cells – Fig. 3G	Proportions	Pearson's Chi-squared test	Category, Conditioning	P n=6; UP n=6	Category x Conditioning: X^2_3= 77.512; P<0.001; Post-hoc, Bonferroni α=0.00625; (signif. for + + +, + + -, + - -)
Recall - Population analysis – Interneurons – Fig. 3G	Proportions	Pearson's Chi-squared test	Category, Conditioning	P n=6; UP n=6	Category x Conditioning: X^2_3= 13.537 ; P=0.004; Post-hoc, Bonferroni α=0.00625; (signif. for + + +, + - -)
Repeatedly activated neurons (>1 activation) – Conditioning - Pyramidal cells – Suppl. Fig. 1A	Normalised GFP+ count	2-way mixed ANOVA	Population, Conditioning	P n=10; UP n=9	Conditioning: $F_{1,17}$ = 1.68 P=0.212; Population: $F_{1,17}$ = 466.3 P<0.001; Conditioning x Population: $F_{1,17}$ = 5.19 P=0.036
Repeatedly activated neurons (>1 activation) – Conditioning – Interneurons – Suppl. Fig. 1A	Normalised GFP+ count	2-way mixed ANOVA	Population, Conditioning	P n=10; UP n=9	Conditioning: $F_{1,17}$ = 1.20 P=0.288; Population: $F_{1,17}$ = 68.93 P<0.001; Conditioning x Population: $F_{1,17}$ = 0.19 P=0.667
Repeatedly activated neurons (>1 activation) – Recall – Pyramidal cells – Suppl. Fig. 1B	Normalised GFP+ count	2-way mixed ANOVA	Population, Conditioning	P n=6; UP n=6	Conditioning: $F_{1,10}$ = 10.87, P=0.0080; Population: $F_{1,10}$ = 320.9, P<0.001; Conditioning x Population: $F_{1,10}$ = 9.80, P=0.012
Repeatedly activated neurons (>1 activation) – Recall – Interneurons – Suppl. Fig. 1B	Normalised GFP+ count	2-way mixed ANOVA	Population, Conditioning	P n=6; UP n=6	Conditioning: $F_{1,10}$ = 2.13, P=0.175; Population: $F_{1,10}$ = 97.16, P<0.001; Conditioning x Population: $F_{1,10}$ = 0.73, P=0.413

Supplemental Table 4: Statistics table for Chapter 2, Figure 4

S1 GFP intensity – (+ + +) vs (+ - -) – Pyramidal cells – Fig 4B	Normalised GFP+ count	3-way mixed ANOVA	Population, Brightness Conditioning	P n=10; UP n=9	Conditioning: $F_{1,17}$= 4.49 P=0.049; Population: $F_{1,17}$= 15.34, P=0.001; Brightness: $F_{2,34}$=2.45, P=0.102; Conditioning x Population: $F_{1,17}$= 2.54, P=0.129; Conditioning x Brightness: $F_{2,34}$=1.36, P=0.269; Population x Brightness: $F_{2,34}$=151.31, P<0.001; Conditioning x Population x Brightness: $F_{2,34}$=0.254, P=0.777
S1 GFP intensity – (+ + +) vs (+ - -) – Interneurons – Fig 4B	Normalised GFP+ count	3-way mixed ANOVA	Population, Brightness Conditioning	P n=10; UP n=9	Conditioning: $F_{1,17}$= 0.14 P=0.714; Population: $F_{1,17}$= 2.16, P=0.160; Brightness: $F_{2,34}$=2.24, P=0.122; Conditioning x Population: $F_{1,17}$= 1.23, P=0.283; Conditioning x Brightness: $F_{2,34}$=0.45, P=0.644; Population x Brightness: $F_{2,34}$=13.42, P<0.001; Conditioning x Population x Brightness: $F_{2,34}$=1.31, P=0.283

Normality testing:

For all data presented in the main text, the Shapiro-Wilk (SW) test was used to assess the distribution of independent samples alongside visual inspection of histograms to identify deviation in skew and kurtosis. For all repeated measures analyses, the unstandardized residuals were subject to SW testing. All samples subjected to t-tests were indicated as normally distributed. In all repeated measures mixed model analyses, the SW test indicated Gaussian distribution for the residuals of the vast majority of/all data sets within each analysis. In light of this and taking into account the robustness of ANOVAs to non-normal data (Lantz, 2013; Schmider et al., 2010), parametric tests were utilised for mixed-model analyses. However, in cases where non-normally distributed residuals represented 25% or more of the individual data sets within the analysis, non-parametric analyses were used to confirm findings (see below). In the main text, data was presented using parametric test analyses to correspond with submitted versions of the data.

Fig. 1C: UP CS and P ITI were not normally distributed, therefore non-parametric Wilcoxon signed rank tests were applied to P and UP groups (CS vs ITI). The Wilcoxon signed rank test revealed a significant difference between CS and ITI in Paired mice (Z=-2.37, P=0.018) but not in Unpaired mice (Z=-0.11, P=0.916), which is in line with the results of our parametric test.

Fig. 3C: '-++' UP was not normally distributed in pyramidal cells and interneurons, therefore non-parametric Mann-Whitney U (MWU) tests were applied to the '-++' activation category (P vs UP). The MWU test indicated no significant difference in the number of '-++' cells between P and UP in both pyramidal cells (U=40 , P=0.720) and interneurons (U=21 , P=0.056), which is in line with the results of our parametric test.

Fig. 3F: '+++R' P was not normally distributed in pyramidal cells, therefore a non-parametric Mann-Whitney U (MWU) test was applied to the '+++' activation category (P vs UP). The MWU test indicated a greater number of '+++R' neurons in Paired compared to Unpaired mice (U=5, P=0.041), further confirming the effect observed in our parametric test.

Supplementary Table 5: Statistics table for Supplemental Figures 2 & 3.

Session 1				
Analysis	**Parameter**	**Test**	**Factors**	**Test Statistic & p**
Ephys - Intrinsic	Spike Counts	3-Way Mixed ANOVA	Group, Cell Type, pA	Group X Cell Type X pA $F_{12,600}$=6.38, **p<0.001**
				Cell Type X Current $F_{12,600}$=2.58, **p=0.002**
				Group X Current $F_{12,600}$=0.30, p=0.990)
	Spike Counts	2-Way Mixed ANOVA	Paired: Cell Type, pA	Cell Type X Current, $F_{12,348}$=9.42, **p<0.001**
			Unpaired: Cell Type, pA	Cell Type X Current, $F_{12,252}$=0.69, p=0.760
Ephys - Intrinsic	I/V Curves	3-Way Mixed ANOVA	Group, Cell Type, pA	Group X Cell Type X Current $F_{25,1200}$=3.81, **p<0.001**
				Cell Type X Current $F_{25,1200}$=3.06, **p<0.001**
				Group X Current $F_{25,1200}$=0.19, p=1.000
			Paired: Cell Type, pA	Cell Type X Current $F_{25,700}$=6.85, **p<0.001**
			Unpaired: Cell Type, pA	Cell Type X Current $F_{25,500}$=0.11, p=1.000
	RMP	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,44}$=1.66, p=0.205, Group $F_{1,44}$=0.34, p=0.565, Cell Type $F_{1,44}$=1.07, p=0.308
	Rheobase	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,49}$=6.64, **p=0.013**, Group $F_{1,49}$=0.31, p=0.580, Cell Type $F_{1,49}$=0.51, p=0.478
	AP Peak	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,50}$=0.12, p=0.736, Group $F_{1,50}$=0.03, p=0.875, Cell Type $F_{1,50}$=0.27, p=0.605
	AP Half-With	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,49}$=0.07, p=0.782, Group $F_{1,49}$=2.86, p=0.097, Cell Type $F_{1,49}$=0.08, p=0.783
	AP Threshold	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,50}$=1.53, p=0.222, Group $F_{1,50}$=0.58, p=0.450, Cell Type $F_{1,50}$=0.66, p=0.419
	AP Decay	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,50}$=0.29, p=0.594, Group $F_{1,50}$=4.35, **p=0.042**, Cell Type $F_{1,50}$=0.003, p=0.954
	fAHP	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,49}$=1.61, p=0.286, Group $F_{1,49}$=3.31, p=0.075, Cell Type $F_{1,49}$=0.03, p=0.869
	mAHP	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,49}$=0.04, p=0.852, Group $F_{1,49}$=1.41, p=0.241, Cell Type $F_{1,49}$=4.97, **p=0.031**

Session 11				
Analysis	**Parameter**	**Test**	**Factors**	**Test Statistic & p**
Ephys - Intrinsic	Spike Counts	3-Way Mixed ANOVA	Group, Cell Type, pA	Group X Cell Type X Current $F_{12,744}$=1.21, p=0.269
				Cell Type X Current $F_{12,744}$=0.25, p=0.996
				Group X Current $F_{12,744}$=1.16, p=0.307
Ephys - Intrinsic	I/V Curves	3-Way Mixed ANOVA	Group, Cell Type, pA	Group X Cell Type X Current $F_{25,1550}$=2.16, **p=0.001**
				Cell Type X Current $F_{25,1550}$=2.72, **p<0.001**
				Group X Current $F_{25,1550}$=4.23, **p<0.001**
			Paired: Cell Type, pA	Cell Type X Current $F_{25,850}$=0.80, p=1.000
			Unpaired: Cell Type, pA	Cell Type X Current $F_{25,700}$=2.93, **p<0.001**
	RMP	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,58}$=0.13, p=0.721, Group $F_{1,58}$=3.94, p=0.052, Cell Type $F_{1,58}$=0.05, p=0.831
	Rheobase	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,61}$=0.16, p=0.691, Group $F_{1,61}$=0.66, p=0.421, Cell Type $F_{1,61}$=0.01, p=0.949
	AP Peak	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,62}$=0.69, p=0.409, Group $F_{1,62}$=0.11, p=0.744, Cell Type $F_{1,62}$=3.07, p=0.085
	AP Half-With	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,61}$=0.05, p=0.82, Group $F_{1,61}$=0.51, p=0.480, Cell Type $F_{1,61}$=0.22, p=0.637
	AP Threshold	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,61}$=1.34, p=0.252, Group $F_{1,61}$=3.06, p=0.085, Cell Type $F_{1,61}$=6.74, **p=0.012**
	AP Decay	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,61}$=1.64, p=0.205, Group $F_{1,61}$=0.98, p=0.326, Cell Type $F_{1,61}$=0.09, p=0.759
	fAHP	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,61}$=4.73, **p=0.033**, Group $F_{1,61}$=6.08, **p=0.017**, Cell Type $F_{1,61}$=1.52, p=0.222
	mAHP	2-Way ANOVA	Group, Cell Type	Group X Cell Type $F_{1,61}$=3.56, p=0.064, Group $F_{1,61}$=0.37, p=0.544, Cell Type $F_{1,61}$=0.27, p=0.602

Supplementary Material for Chapter 3

Supplementary Table 6: Statistics table for Chapter 3.

Analysis	Parameter	Test	Factors	n	Test Statistic & p
Repeated Cloz injection - Acquisition - Fig. 1B	Head entries	3-way mixed ANOVA	Cue, Session, Injection	Cloz n=5; WT n=5	Injection: $F_{1,8}$=1.53, P=0.251; Session: $F_{11,88}$=18.20, P<0.001; Cue: $F_{1,8}$=153.75, P<0.001; Injection x Session: $F_{1,8}$=0.68, P=0.756; Injection x Cue: $F_{1,8}$=5.10, P=0.056; Cue x Session: $F_{11,88}$=4.71, P<0.001; Injection x Session x Cue: $F_{11,88}$=0.51, P=0.894
Repeated Cloz injection - Recall - Fig. 1C	Head entries	2-way mixed ANOVA	Cue, Injection	Cloz n=5; WT n=5	Injection: $F_{1,8}$=0.26, P=0.627; Cue: $F_{1,8}$=10.23, P=0.013; Injection x Cue: $F_{1,8}$=0.19, P=0.677
Repeated Cloz injection - Acquisition - Fig. 1D	Selectivity index	2-way mixed ANOVA	Session, Injection	Cloz n=5; WT n=5	Injection: $F_{1,8}$=1.12, P=0.321; Session: $F_{11,88}$=15.34, P=<0.001; Injection x Cue: $F_{118,8}$=0.7943, P=0.6451
Repeated Cloz injection - Recall - Fig. 1E	Selectivity index	t-test	Injection	Cloz n=5; WT n=5	t_8=0.01, P=0.942
S1 tag - Chemogenetics behaviour - Acquisition - Suppl. Fig. 3B	Head entries	3-way mixed ANOVA	Cue, Session, Genotype	Fos-tTA n=6; WT n=12	Genotype:$F_{1,16}$=0.75, P=0.399; Session: $F_{11,176}$=15.06, P<0.001; Cue: $F_{1,16}$=248.65, P<0.001 ; Genotype x Session: $F_{11,176}$=8.27, P<0.001; Genotype x Cue: $F_{1,16}$=1.68 p=0.213; Cue x Session: $F_{11,176}$=6.94, P<0.001; Genotype x Session x Cue: $F_{11,176}$=2.00, P=0.031
S1 tag - Chemogenetics behaviour - Recall - Suppl. Fig. 3C	Head entries	2-way mixed ANOVA	Cue, Genotype	Fos-tTa n=6; WT n=12	Genotype: $F_{1,16}$= 2.82, P=0.112; Cue: $F_{1,16}$= 9.03, P=0.008; Genotype x Cue: $F_{1,16}$= 3.82, P=0.068
S1 tag - Chemogenetics behaviour - Acquisition - all mice - Fig. 3D	Selectivity index	2-way mixed ANOVA	Session, Genotype	Fos-tTA n=6; WT n=12	Genotype: $F_{1,16}$= 11.57, P=0.004; Session: $F_{11,176}$= 9.50, P<0.001 ; Genotype x Session: $F_{11,176}$= 3.81, P<0.001 ;
S1 tag - Chemogenetics behaviour - Acquisition - all mice - Fig. 6C	Selectivity index	Post-hoc, t-tests, Sidak correction	Genotype	Fos-tTA n=6; WT n=12	S5: t_{192}=3.125, Adj. P=0.024 S6: t_{192}=3.315, Adj. P=0.013 S12: t_{192}=3.352, Adj. P=0.012
S1 tag - Chemogenetics behaviour - Recall - Fig. 3E	Selectivity index	t-test	Genotype	Fos-tTA n=6; WT n=12	t_{16}=1.29, P=0.021
NC tag - Chemogenetics behaviour - Acquisition - Fig. 4B	Head entries	3-way mixed ANOVA	Cue, Session, Genotype	Fos-tTA n=8; WT n=10	Genotype: $F_{1,16}$=0.55, P=0.470; Session: $F_{11,176}$=14.89, P<0.001; Cue: $F_{1,16}$=131.92, P<0.001 ; Genotype x Session: $F_{11,176}$=0.38, P=0.964; Genotype x Cue: $F_{1,16}$=0.97 p=0.339; Cue x Session: $F_{11,176}$=5.19, P<0.001; Genotype x Session x Cue: $F_{11,176}$=1.13, P=0.338
NC tag - Chemogenetics behaviour - Recall - Fig. 4C	Head entries	2-way mixed ANOVA	Cue, Genotype	Fos-tTA n=8; WT n=10	Genotype: $F_{1,16}$= 0.20, P=0.659; Cue: $F_{1,16}$= 45.53, P<0.001; Genotype x Cue: $F_{1,16}$=0.32, P=0.881
NC tag - Chemogenetics behaviour - Acquisition - Fig. 4D	Selectivity index	2-way mixed ANOVA	Session, Genotype	Fos-tTA n=8; WT n=10	Genotype: $F_{1,16}$= 0.55, P=0.471; Session: $F_{11,176}$=9.74, P<0.001 ; Genotype x Session: $F_{11,176}$= 0.33, P=0.980 ;
NC tag - Chemogenetics behaviour - Recall - Fig. 4E	Selectivity index	t-test	Genotype	Fos-tTA n=8; WT n=10	t_{16}=0.45, P=0.656

Normality testing

For all data presented in the main text, the Shapiro-Wilk (SW) test was used to assess the distribution of independent samples alongside visual inspection of histograms to identify deviation in skew and kurtosis. For all repeated measures analyses, the unstandardized residuals were subject to SW testing. All samples subjected to t-tests were indicated as normally distributed. In all repeated measures mixed model analyses, the SW test indicated Gaussian distribution for the residuals of the vast majority of/all data sets within each analysis. In light of this and taking into account the robustness of ANOVAs to non-normal data (Lantz, 2013; Schmider et al., 2010), parametric tests were utilised for mixed-model analyses. However, in cases where non-normally distributed residuals represented 25% or more of the individual data sets within the analysis, non-parametric analyses were used to confirm findings (see below). In the main text, data was presented using parametric test analyses to correspond with submitted versions of the data.

Fig. 4C: P CS was not normally distributed, therefore non-parametric Mann-Whitney U (MWU) tests were applied to the CS groups (P vs UP). The MWU test indicated no significant difference in the number of CS head entries between P and UP mice (U=37, P=0.829), which is in line with the results of our parametric test.

Supplementary Material for Chapter 4:

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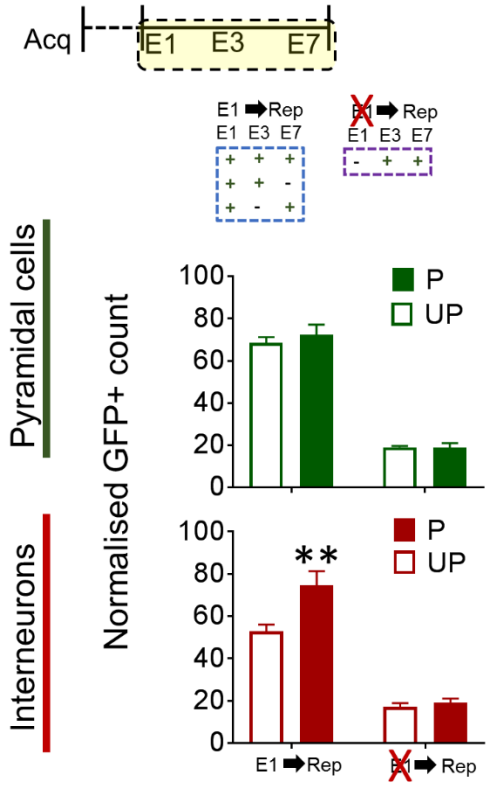

Supplemental Figure 4 (Associated with Chap. 4 Fig. 2):
Extinction learning recall recruits an interneuron ensemble with a repeated activation history that includes the initial extinction session.
Normalised GFP+ counts of repeatedly activated ('Rep'; >1 activation in Extinction) pyramidal cells and interneurons with a E1 (E1 → Rep) or no E1 (no E1 → Rep) activation history. In interneurons, there was a significant interaction of Activation Category X Conditioning ($F_{1,7}$=6.82, P=0.035); Post-hoc testing revealed a significant increase in the number of E1→Rep in Paired compared to Unpaired mice (P<0.01). We detected no significant interaction in pyramidal cells ($F_{1,17}$=0.42, P=0.538). Data expressed as Mean±SEM. *Post-hoc analysis: ** P<0.01; P: n=5, UP n=4.*

Supplementary Table 7: Statistics table for Chapter 4.

Analysis	Parameter	Test	Factors	n	Test Statistic & p
FGGT mice behaviour - Extinction - Fig. 1B	Head entries	3-way mixed ANOVA	Cue, Session, Group	P n=6; UP n=6	Group: $F_{1,10}$=18.829, P=0.001; Session: $F_{6,60}$=7.126, P<0.001; Cue: $F_{1,10}$=46.589, P<0.001; Group x Session: $F_{6,60}$=5.985, P<0.001; Group x Cue: $F_{1,10}$=13.561, P=0.004; Cue x Session: $F_{6,60}$=2.061, P=0.071; Group x Session x Cue: $F_{6,60}$=1.474, P=0.202
Extinction session - Pyramidal cells - Fig. 2D	Normalised GFP+ count	2-way mixed ANOVA	Session, Group	P n=5; UP n=4	Group: $F_{1,7}$=1.327, P=0.2871; Session: $F_{2,14}$=0.9814, P=0.3991; Group x Session: $F_{2,14}$=0.7006, P=0.5129
Extinction session - Interneurons - Fig. 2D	Normalised GFP+ count	2-way mixed ANOVA	Session, Group	P n=5; UP n=4	Group: $F_{1,7}$=7.913, P=0.0260; Session: $F_{2,14}$=0.4411, P=0.6519; Group x Session: $F_{2,14}$=0.5973, P=0.5637
Persistently activated neurons - Extinction - Pyramidal cells - Fig. 2E	Normalised GFP+ count	2-way mixed ANOVA	Population, Group	P n=5; UP n=4	Group: $F_{1,7}$=0.6502, P=0.5729; Population: $F_{2,14}$=53.93, P<0.001; Group x Population: $F_{2,14}$=0.4267, P=0.5345
Persistently activated neurons - Extinction - Interneurons - Fig. 2E	Normalised GFP+ count	2-way mixed ANOVA	Population, Group	P n=5; UP n=4	Group: $F_{1,7}$=9.07, P=0.0196; Population: $F_{2,14}$=35.17, P<0.001; Group x Population: $F_{2,14}$=6.589, P=0.0372
Persistently activated neurons – Extinction – Interneurons – Fig. 2E	Normalised GFP+ count	Post-Hoc t-test, Sidak correction	Group	P n=5; UP n=4	S1+ | S5+ S11+: t_{14}=3.96, Adj. P=0.003 S1-| S5+ S11+: t_{14}=0.59, Adj. P=0.808
Extinction session 1 - Population analysis - Pyramidal cells - Fig. 2F	Proportions	Pearson's Chi-squared test	Category, Group	P n=5; UP n=4	Group x Category: X^2_3=52.84, P<0.001 Bonferroni α=0.00625; (signif. for + + +, + - -, ++-)
Extinction session 1 - Population analysis - Interneurons - Fig. 2F	Proportions	Pearson's Chi-squared test	Category, Group	P n=5; UP n=4	Group x Category: X^2_3=3.12, P=0.375
Conditioning History in E1 - Population analysis - Pyramidal cells - Fig. 3	Proportions	Loglinear analysis	Activation History, Conditioning History, Group	P n=5; UP n=4	likelihood of model: X^2_0=0, P=1; Group X Activation History X Conditioning History: X^2_1= 9.68, P=0.021
Conditioning History in E1 - Population analysis - Pyramidal cells - Fig. 3	Proportions	Pearson's Chi-squared test	Conditioning History, Group	P n=5; UP n=4	+++': X^2_1=11.43, P=0.001 '++-': X^2_1=1.03, P=0.311 '+-+': X^2_1=0.00, P=0.987 '+--': X^2_1=0.20, P=0.887
Persistently activated neurons - Population analysis - Interneurons - Fig. 3	Proportions	Loglinear analysis	Activation History, Conditioning History, Group	P n=5; UP n=4	likelihood of model: X^2_1=10.402, P=0.109; Group X Activation History X Conditioning History: X^2_3=6.53, P=0.089; Partial associations: Activation History x Group: X^2_3=3.88, P=0.275 Conditioning History x Group X^2_1=4.94, P=0.026 Activation History x Conditioning History: X^2_3=90.62, P<0.001
E1 GFP intensity – (+ + +) vs (+ - -) – Pyramidal cells – Fig. 4B	Normalised GFP+ count	3-way mixed ANOVA	Activation History, Brightness, Group	P n=5; UP n=4	Activation History: $F_{1,7}$=1.82, P=0.219; Brightness: $F_{2,14}$=0.98, P=0.399; Group: $F_{1,7}$=0.01, P=0.934; Activation History x Group: $F_{1,7}$=0.16, P=0.700; Brightness x Group: $F_{2,14}$=1.40, P=0.278; Activation History x Brightness: $F_{2,14}$=73.97, P<0.001; Activation History x Brightness x Group: $F_{2,14}$=1.14, P=0.349
E1 GFP intensity – (+ + +) vs (+ - -) – Interneurons – Fig. 4B	Normalised GFP+ count	3-way mixed ANOVA	Activation History, Brightness, Group	P n=5; UP n=4	Activation History: $F_{1,7}$=4.49, P=0.072; Brightness: $F_{2,14}$=0.64, P=0.543; Group: $F_{1,7}$=3.70, P=0.096; Activation History x Group: $F_{1,7}$=0.10, P=0.763; Brightness x Group: $F_{2,14}$=0.21, P=0.814; Activation History x Brightness: $F_{2,14}$=17.45, P<0.001; Activation History x Brightness x Group: $F_{2,14}$=0.97, P=0.402
Repeatedly activated neurons - Extinction - Pyramidal cells - Suppl. Fig. 4	Normalised GFP+ count	2-way mixed ANOVA	Population, Group	P n=5; UP n=4	Group: $F_{1,7}$=0.33, P=0.585; Population: $F_{2,14}$=285.2, P<0.001; Group x Population: $F_{2,14}$=0.42, P=0.538
Repeatedly activated neurons - Extinction - Interneurons - Suppl. Fig. 4	Normalised GFP+ count	2-way mixed ANOVA	Population, Group	P n=5; UP n=4	Group: $F_{1,7}$=6.89, P=0.034; Population: $F_{2,14}$=144.1, P<0.001; Group x Population: $F_{2,14}$=6.82, P=0.035

Normality testing

For all data presented in the main text, the Shapiro-Wilk (SW) test was used to assess the distribution of independent samples alongside visual inspection of histograms to identify deviation in skew and kurtosis. For all repeated measures analyses, the unstandardized residuals were subject to SW testing. In all repeated measures mixed model analyses, the SW test indicated Gaussian distribution for the residuals of the vast majority of/all data sets within each analysis. In light of this and taking into account the robustness of ANOVAs to non-normal data (Lantz, 2013; Schmider et al., 2010), parametric tests were utilised for mixed-model analyses.

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