The smell of fear: Comparing the neural ensembles underlying odor fear memory conditioned by innate, inherent, and learned sources of danger

by

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Abstract

Memories can elicit strong emotions, both positive and aversive. Recognizing danger cues in an unfamiliar environment can make the difference between life and death. However, if aversive memory formation becomes dysregulated, safe cues can be misinterpreted as danger signals and lead to avoidance, fear reactions, and other maladaptive behaviours. Rats are utilized as model organisms in combination with classical conditioning to delineate mechanisms underlying aversive memories, but human memory is diverse and complicated. This thesis utilized two forms of "higher-order" learning, pheromone- and second-order conditioning, to recapitulate the diverse ways that humans employ associative learning. Alarm pheromones released from a stressed rat can act as an unconditioned stimulus when paired with a neutral odor to produce an odor fear memory in a conspecific. The basolateral (BLA) and central amygdala are consistently activated across shock- and pheromone-conditioned odor memory recall, but pheromone-conditioned and shock-conditioned memories elicit activation in the accessory olfactory bulb and main olfactory bulb, respectively. Rats can also learn that an odor signals danger when it is paired with a tone or context that was previously paired with shock. First-order conditioned and both forms of second-order conditioned odor memory recall elicit activation in the BLA, the dorsal and ventral hippocampus, and the olfactory cortex. Interestingly recall of an odor fear memory that was conditioned with a feared tone activates the lateral amygdala and auditory cortex. Overall, the results described in this thesis highlight that odor fear memory traces are present in some areas regardless of how the memory was conditioned, while other areas participate in memory traces differentially,

possibly depending on sensory features of the conditioned stimuli. Elucidating the mechanisms underlying higher-order conditioning are crucial and could provide valuable insight for treatment of disorders involving aberrant fear, such as post traumatic stress disorder, which currently focus on exposure to faulty danger cues to rewrite traumatic memories.

General Summary

Memory guides us as human beings to help us navigate the world we live in. Our emotions and perceptions of the external environment influence our memories. In turn, our memories and emotions influence the way in which we perceive the world. Memories are intimately tied to emotion; for example, the smell of a bonfire will elicit different reactions in people who have lost their home in a fire and those who have not. Post traumatic stress disorder (PTSD) incidence has increased over the last two years, with as high as 10% of patients citing trauma directly related to the pandemic. The sheer number of aversive stimuli we are exposed to by the media daily will likely cause problems with fear and anxiety circuitry in a very large percentage of people.

My thesis work aimed to create animal models for traumatic fear that more closely represent the ways in which humans learn negative associations. Humans and rats can both communicate specific danger signals to one another, so that any one individual does not need to experience a threat directly in order to learn about it. Additionally, many traumatic memories in humans are not directly associated with an aversive event; for example, if someone pointed a gun at you, the next time you see that person you will likely feel afraid because they have now been associated with a gun – a learned danger signal. Guns are not inherently dangerous as they are simply plastic and metal, however at some point in our lives we learn that guns are associated with violence or death. Rats utilize this “higher order” associative learning as well. For both models, the brain areas that were active

following recall of these memories were unique depending on how the memory was formed, with some areas showing consistent activity regardless of the type of memory. The results presented here will help to elucidate the ways in which the brain encodes traumatic memories so that we may open the doors to new therapeutic interventions in fear related psychiatric disorders such as PTSD.

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I would like to dedicate these pages to my grandmother, **Marion Goodman**. Sadly, she will not get to read this thesis, but it is her strength and determination that shine through me every day, and she is a huge reason why it exists.

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List of Abbreviations

aBLA	anterior basolateral amygdala
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ANOVA	analysis of variance
AOB	accessory olfactory bulb
aPC	anterior piriform cortex
Arc/Arg3.1	activity-regulated cytoskeletal-associated protein
ART	accelerated resolution therapy
ASR	acoustic startle response
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CaMKII	calmodulin kinase II
cAMP	cyclic adenosine monophosphate
catFISH	compartmental analysis of temporal activity by fluorescence in situ hybridization
CeA	central amygdala
ChR2	channelrhodopsin-2
CNS	central nervous system
CoA	cortical nucleus of the amygdala
CR	conditioned response
CREB	cAMP response element binding protein
CS	conditioned stimulus
CS1	first order conditioned stimulus
CS2	second order conditioned stimulus
D1R	type 1 dopamine receptors
D2R	type 2 dopamine receptors
D-AP5	(2R)-amino-5-phosphonovaleric acid (D-APV)
DAPI	4′,6-diamidino-2-phenylindole
DG	dentate gyrus
DH	dorsal hippocampus
DIG	digoxigenin
DNA	deoxyribonucleic acid
DOX	doxycycline
DSM-5	diagnostic and statistical manual of mental disorders, fifth edition
EC	entorhinal cortex
EEG	electroencephalogram
EMDR	eye movement desensitization and reprocessing
EMG	electromyography
EPSP	evoked postsynaptic potential
EPT	emotional processing therapy
ERK	extracellular signal-related kinase
FC	fear conditioning

fEPSP	field evoked postsynaptic potential
FLU	fluorescein
fMRI	functional magnetic resonance imaging
FOC	first order conditioning
GABA	gamma-aminobutyric acid
GAD	generalized anxiety disorder
GluA1/2	subunits of AMPAR
GluR1	AMPAR subunit glutamate receptor 1
H1a	Homer1a
H2O2	hydrogen peroxide
HRP	horseradish peroxidase
IEG	immediate early gene
IHC	immunohistochemistry
ISH	in situ hybridization
KO	knockout
LA	lateral amygdala
LC	locus coeruleus
LOT	lateral olfactory tract
LTD	long-term depression
LTP	long term potentiation
MAPK	mitogen-activated protein kinase
mCeA	medial central amygdala
MeA	medial amygdala
mEPSC	mini evoked postsynaptic currents
mGluR	metabotropic glutamate receptor
mGluR5	subtype of mGluR
MOB	main olfactory bulb
mRNA	messenger ribonucleic acid
MSN	medium spiny neurons
MWM	Morris water maze
NAc	nucleus accumbens
NE	norepinephrine
NET	narrative exposure therapy
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptors
NR2B	2B subunit of NMDARs
NS	neutral stimulus
OSN	olfactory sensory neuron
OT	olfactory tubercle
PAG	periaqueductal grey
PB	phosphate buffer
pBLA	posterior basolateral amygdala
PC	piriform cortex
PE	prolonged exposure therapy
PKA	protein kinase A

PKC	protein kinase C
POD	peroxidase
pPC	posterior piriform cortex
PSD	postsynaptic density
PTSD	post traumatic stress disorder
RNA	ribonucleic acid
SB	soiled bedding
SBT	2-sec-butyl-4,5-dihyrothiazole
SC	Schaffer collateral
SEM	standard error of the mean
SNS	sympathetic nervous system
SOC	second order conditioning
UR	unconditioned response
US	unconditioned stimulus
VH	ventral hippocampus
VNO	vomeronasal organ
VSN	vomeronasal sensory neuron

Co-Authorship Statement

I, Samantha Joy Carew, in collaboration with my supervisor, Qi Yuan, designed, performed, and analyzed all experiments presented herein and wrote all portions of this thesis unless specified below. Essential contributions to this thesis were provided by collaborators as listed below:

Behavioural procedures in the pheromone project were completed by Iain MacIntyre, Bandhan Mukherjee, and Abhinaba Ghosh. Cannula placement surgery and intra-BLA infusions were completed by Bandhan Mukherjee. All sacrifice procedures including odor exposure, brain extraction, and freezing was completed by Iain MacIntyre. Retrograde tracing experiments were completed by Sa Li and Gilbert Kirouac.

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Figures 4 – 13 and sections from the methods, results, and discussion from this thesis were adapted from my published paper titled “Pheromone-induced odor associative

fear learning in rats." Because this paper was published in the open access journal *Scientific Reports*, the authors retain IP rights as per Springer Nature policy.

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Lay outline

Learning and memory is objectively fascinating. It affects us all during every minute of every day; from what we eat, how we exercise, what we study, our careers, our thoughts and opinions on popular culture, literature, science, politics, religion, the list goes on. Our electric sacs of fat take it all in, sort through what they consider relevant based on previous experience and chuck the rest, sometimes without any conscious effort on our part at all. Humans have presumably gotten so far up the evolutional hierarchy in part due to our capability to learn, retain, relate, and integrate information to make decisions that will improve our experience as a species. Everything that we see, touch, taste, hear, smell, and think gets processed by our brain and shapes the way we see the world. Likewise, our emotions, memories, and perceptions are influenced by our external environment and surroundings.

One of the biggest problems in learning and memory research has, at the same time, been instrumental to the revolutions that have enabled the probing of these questions to begin with: the animal model. Animals and humans share physiological similarities in fear processing and expression, which implies that fear conditioning is relevant in the genesis of anxiety and fear disorders. The human brain is irrefutably more complicated and intricate than the rodent brain, yet the core idea underlying using rats to study memory is that the basic building blocks of memory are similar across species due to the inherent evolutionary advantage that learning and memory confers to any species. The issue rears its head when conceptualizing how to mimic the nuance and context involved in human memory

formation in an animal model. While controlling for variables is implicit in the scientific process so that one can conclude a specific cause and effect or correlation, it removes some relevance to the human condition. It is unlikely that every fear or triggering thought that is experienced by the human psyche was caused by classical conditioning – a direct association between an innately negative stimulus and a neutral one that can easily be traced to a single molecular target and "fixed" with some pharmacological intervention. So how do we design studies that minimize confounding variables while appreciating that memories should be more complex if the goal is to translate any of this research to the human condition? The answer, perhaps, lies in 'higher-order learning,' a frequently discussed topic in the previous century that has largely been dismissed or abandoned in the last two decades.

My PhD work explored two different forms of higher-order learning: pheromone-conditioning and second-order conditioning. The first is important to consider when thinking about the construct of social learning. As humans, we do not need to experience a dangerous or frightening situation ourselves to learn something; a friend recounting a scary first-hand experience is enough for us to avoid a certain place or person in the future. Rats, like humans, are social animals and have been shown to communicate via ultrasonic vocalizations and pheromones in a variety of situations. The first project in this thesis showed how a specific fear conditioned memory can be transferred to a conspecific in the absence of the same aversive conditioning stimulus. Classically conditioned rats were able to communicate information about the valence of an objectively neutral stimulus which led to a behavioural fear response and activation of the same neural fear pathway in the

companion rats, except a different olfactory structure mediated classical conditioning versus pheromonal. This reflects the importance of the method of learning and not simply the content.

The second project in this thesis explored if the same odor could cause physical changes in different structures of the brain and recruit regions to its engram depending on how it was encoded, i.e., directly paired with shock, paired with a learned aversive context, or paired with a learned aversive tone. It is logical to surmise that some negative or anxiety-inducing memories that we experience as human beings are a result of an associative chain of conditioned stimuli from traumatic or aversive events in our past. We are often unaware of our triggers, since they could be dissociated temporally from an actual traumatic event.

Importantly, with the second project I showed how the engram complex for an odor fear memory encompasses brain regions differentially depending on the sensory features of the first stimulus used to condition the second stimulus. When animals are trained to first associate an auditory stimulus with a shock and subsequently experience the same auditory stimulus paired with an olfactory stimulus, recall of this memory trace via re-exposure to the odor activates neurons in the lateral amygdala (LA) and auditory cortex. The LA and the auditory cortex do not get wired into the engram complex if the same odor is first order conditioned, or if the odor is second-order conditioned with a feared context. The rest of the engram complex is the same for all three types of memory and includes the posterior piriform cortex, the basolateral amygdala (BLA), and both the dorsal (DH) and ventral hippocampus (VH). This implies that a single conditioned stimulus in the associative chain

can reactivate the entire engram complex, including areas that process a sensory stimulus even when that stimulus is absent. The DH was unexpectedly activated in response to recall of each type of odor fear memory, implying a general or more complicated role in fear memory formation. Both observations could have implications for the way humans are treated for aberrant memories or anxiety disorders.

It is vital to elucidate the mechanisms by which the brain associates and integrates stimuli into existing fear networks. Pavlov himself showed us that classical conditioning can be extended to second-, third-, and even fourth- order associations, which means we could be (and likely are) going through our days being triggered by things that we do not consciously know are triggering to us. There is no shortage of traumatic experiences in the information age. Simply by being active on social media one runs the risk of reading an article detailing horrendous acts around the world, or even worse coming across a violent or triggering video with little or no warning as to its contents. The past two years alone we have lived through a pandemic, are feeling the effects of an incoming recession, and for the first time we are watching a massive war unfold through the lens of the people who are most affected by it. The issues that have been thrust upon us and the way in which social media curates the content we receive based on what will capture our interest has led to massive polarization of opinions. Perhaps the anxiety and trauma-based fear and stress disorders that are becoming more and more prevalent in society reflects the vast capability of the fear conditioning system. While trying to be helpful and steer us away from harm as is its evolutionary purpose, it may instead be bombarding us with “threat” signals to stimuli

that have no real chance of harming us, but still initiate a physiological stress response because, for instance, we can see them happening in real time online.

Chapter 1 – Introduction

1.1. Learning, memory, and synaptic plasticity

In the truest sense of the word, memory is essential for humans (and other animals to varying degrees) to live a complete, enriched life. Every perception, thought, and opinion we formulate during our lifetimes is contingent upon previously formed memories or information that has been retained by our brains. Often these memories have strong associational value; if your grandmother often made bread for you as a child the smell of baking bread may bring her to the forefront of your mind without any conscious thought at all. The way we react behaviourally is largely based upon sensory input and associational value that has been unconsciously ascribed to objectively nascent stimuli. Memories often flash in our minds without any sort of conscious warning, and with something as simple as a scent or a song we can be transported back in time, to a place that holds a special spot in our hearts to give us a moment of comfort. Unfortunately, memories do not always elicit positive emotions, and the same can be true for deeply traumatizing, fear inducing, or painful experiences we may have endured. Patients suffering from post traumatic stress disorder (PTSD) may be triggered by a simple (or complex) sensory input that had been associated in some way to their trauma, causing them to lapse into a flashback. A flashback is a very realistic sort of re-telling of the memory automatically played in the brain, it is often very disturbing to the patient, and can be resistant to interruption[1]. A person does not need to have a clinical diagnosis for PTSD to experience flashbacks, in fact aversive or negative memories affect even the healthiest and most self-aware people[2]. Adaptive memory is how we learn and if we did not experience anything negative, we would have

no basis for understanding dangers and making informed decisions. In this way stimuli, and by extension memories, are said to have positive or negative valence based on either innate or learned qualities of the stimulus that either cause an aversive (negative, avoidance) or appetitive (positive, approach) behavioural response[3]. Discussion in the current thesis will focus heavily on mechanisms relevant to aversive (fear) learning.

1.1.1. Classical conditioning – associative memory

Associative memory is complicated – researchers have been carefully crafting behavioural paradigms for decades to try and dissect the individual aspects of this broad concept. The problem stems partially from the need to control for all types of variability. While decreasing variability is necessary to pinpoint a single phenomenon and separate from other potential explanations, it results in a shift away from the conditions under which this would happen in "real life." Associative memory is involved in every conscious decision we make and practically everything that makes us who we are; context and nuance are important for meaningful, strong, and emotional memories. This is true for both fond and traumatic memories[4].

One of the first people to demonstrate associative learning as a behavioural phenomenon was Ivan Pavlov, a Russian physiologist who won a Nobel prize in 1904 for his work on canine gastric functions. Throughout his regular contact with dogs, he noticed that they would salivate prior to food being placed in their mouths and he decided to pursue what he called this "psychic secretion." Pavlov soon discovered that repeated paired

presentations of the food with another benign or neutral stimulus, in this case the auditory stimulus of the ticking of a metronome, such that the ticking preceded the food delivery, would eventually remove the need for the food to be present at all, and the dog would salivate upon hearing the metronome alone. He called these "conditioned reflexes"[5]. These types of experiments have been used extensively for the last century with many different adaptations. We have since come to call the salivation of Pavlov's dogs the unconditioned response (UR), because no conditioning is required to elicit this response – it is a physiological reaction that a dog has when food is near to prepare the gastric system to digest it. Because the food is the stimulus that elicits the UR, it is considered an unconditioned stimulus (US). Prior to pairing with food, the ticking of the metronome was considered a neutral stimulus (NS) but after the repeated pairings take place the metronome is a conditioned stimulus (CS), and because it is being induced by a conditioned stimulus, salivation is now considered a conditioned response (CR)[6]. The example described above is called first order classical conditioning and is outlined in Figure 1A.

Not only did Pavlov define first order classical conditioning (FOC), he took it a step further and introduced the concept of second order conditioning (SOC)[5]. Continuing from the original experiment the metronome sound is the first order conditioned stimulus (CS1), he found that repeated presentations of this CS1 paired with another NS, a black square, eventually led presentation of the black square alone to elicit the CR, effectively making the visual stimulus of the black square the second conditioned stimulus (CS2; see Figure 1B). As Pavlov's experiments utilized an inherently positive US (the food), this type of

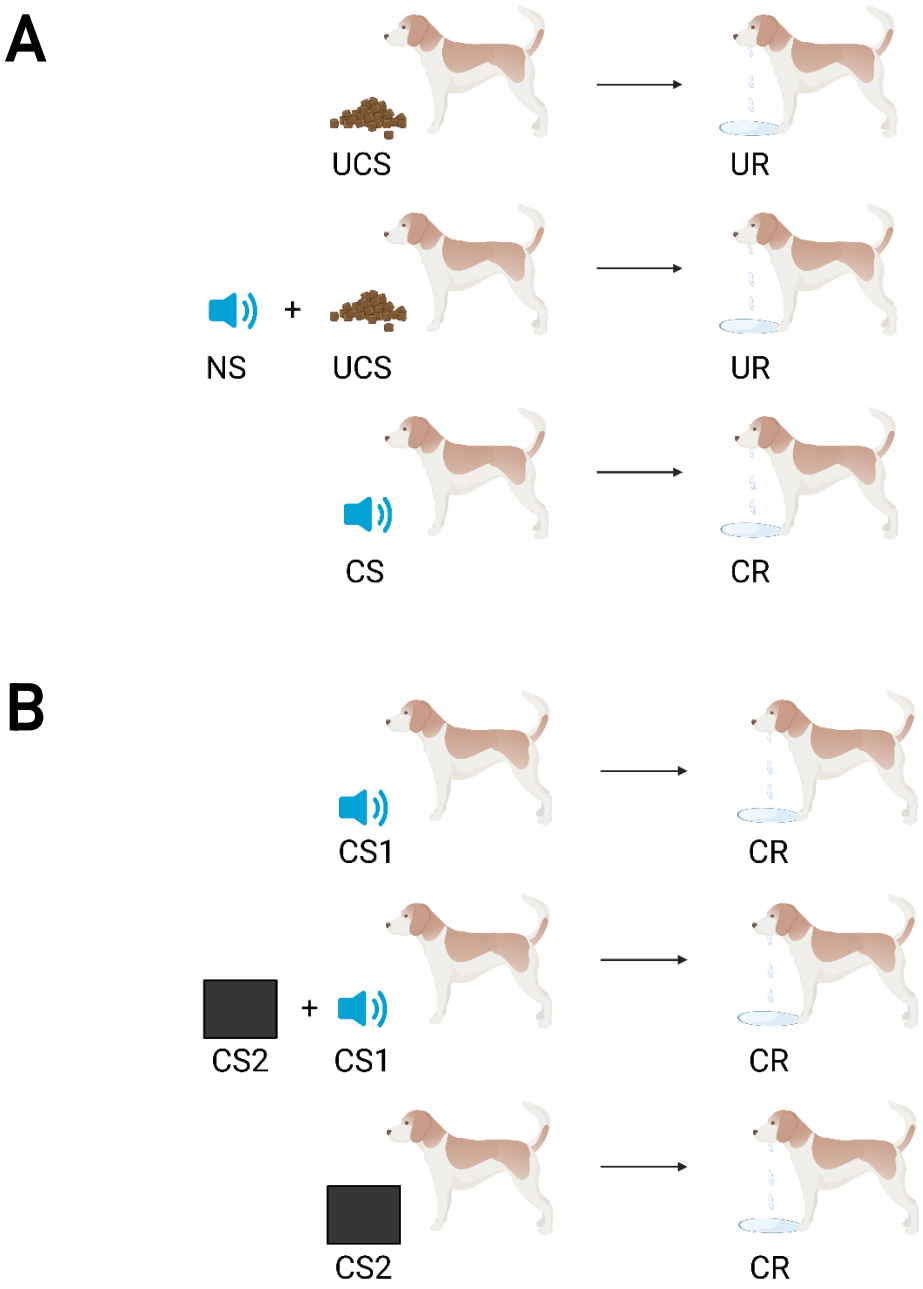


Figure 1. ***Classical (Pavlovian) conditioning.*** **A)** Food elicits the unconditioned response of salivation in dogs. If trained such that an auditory cue predicts food delivery, the previously neutral sound acquires the ability to evoke the now conditioned response of salivation. **B)** If the previously conditioned tone is paired with a visual stimulus, in this case the presentation of a black square, the now second conditioned stimulus (CS2) elicits the conditioned response of salivation. Image created in Biorender.

conditioning is called appetitive. Conversely if the US is inherently negative or painful (such as a foot shock) it is called aversive conditioning, also known as fear conditioning[6].

1.1.2. Fear conditioning

Pavlov's experiments yielded the behavioural response of salivation. What happens when the US used for training is not a pleasant thing like food, but painful? Aversive conditioning activates the sympathetic nervous system (SNS), more commonly known as "fight or flight." Another behavioural response to SNS activation, freezing, is reliably exhibited by rodents when they are physically incapable of escaping the foot shocks (i.e., the floor in the encased shock chamber). While other measures such as the acoustic startle response, heart rate, and blood corticosterone[7,8] are commonly used to measure fear, freezing is considered the gold standard behavioural response to fear conditioning as it is easily measured and reliably induced across several species and strains. The experiments in this thesis utilized Sprague Dawley rats who exhibit freezing behaviour in a stereotyped manner and are classified as "freezing" when there is absence of movement in the body except for breathing[9].

Fear conditioning has been used to study associative learning for decades, and often is chosen over appetitive paradigms to study memory in general because it can be completed quickly in comparison. Aversive memory lasting at least 24 hours can be induced in a single 30-minute training session[10]. Because of the short turnaround time for fear conditioning experiments, they are ideal for studies that aim to evaluate molecular or cellular correlates of learning. Of course, at this point it is known and well appreciated that

different circuits are responsible for mediating information with appetitive and aversive valence, and it is crucial to understand both forms of learning to truly comprehend how the brain processes, stores, and recalls information. I have personally been fascinated by aversive conditioning and think that the potential for a greater understanding of the human brain and solutions to trauma and problems we are facing in society lies in understanding the underlying mechanisms of aversive, over appetitive, memory.

Aversive conditioning was utilized regularly in the 1960's and was aimed towards untangling the mechanisms of memory in general. In fact, at this time the amygdala wasn't even considered to be important – it was thought that the hippocampus and cortical areas were responsible for encoding fear memory and the amygdala played some vague facilitatory role[11]. In recent decades it has been established and appreciated that several subnuclei of the amygdala are crucial for classical fear conditioning, which will be described in detail in Section 1.3.6. Rodents can learn to associate a variety of neutral stimuli with an unconditioned stimulus like a foot shock; auditory, visual, olfactory, and contextual cues can become classically conditioned to elicit freezing behaviour[11]. Typically, studies choose a single sensory modality (usually it is auditory) and tend to describe their results in the context of all sensory modalities as if the molecular mechanisms underlying the process of learning to fear a tone for example is representative of those underlying all fear memory. Apart from olfactory stimuli, sensory information enters the brain and travels to the thalamus before reaching its target cortical area. Sensory information can therefore reach the amygdala via direct "fast" thalamic projections or indirect "slow" cortical projections[12].

Since most fear conditioning studies utilize auditory and visual stimuli, many details of these types of fear learning are understood[13–19]. Even though the sense of olfaction has the highest association with emotional context[20] and the capacity to easily trigger highly emotional and therefore salient memories[20–24] the unique mechanisms underlying odor fear learning has been largely ignored in the scientific literature (reviewed by Hakim[25]). Even in humans, the olfactory component of memories is more resilient[26,27], able to persist despite degradation in cues belonging to other sensory systems[28], and tends to be longer-lasting than memories which utilize other sensory cues[29]. Olfactory fear conditioning is also appealing due to its relatively simplified circuitry; information about an odor does not get sent to the thalamus prior to the amygdala so there is one less step in the processing chain compared to other sensory modalities[30]. Every experiment that is contained in this thesis utilizes some form of odor fear conditioning, whether it be directly via shock, pheromones, or previously danger-associated CS. Specifically, I examined if the memory trace for an odor fear memory (to the exact same odor) could look different in the brain depending on how it was conditioned.

1.1.3. Alarm pheromones

Humans, like rats, are social creatures. While humans communicate predominantly through language and rely heavily on our visual and auditory systems, rodents have a highly developed and specialized olfactory system, since they tend to favor the sense of smell over visual or auditory inputs[31]. Despite this, most Pavlovian fear conditioning paradigms do not utilize odors as conditioned stimuli, turning instead to visual or auditory cues,

presumably to try and more closely match the human condition. Since rats have two primary methods of communication, pheromone signaling and ultrasonic vocalizations, I wanted to know if a rat could disseminate information about a specific fear memory or danger signal and cause a fear reaction in its naïve cage-mate. The associative nature of rat alarm pheromones has been shown in pups – pairing a novel peppermint odor with the scent from their fearful mother is sufficient to induce avoidance in pups to future peppermint exposures[7]. To our knowledge, it was not known whether an alarm pheromone could act as a UCS between adult rats. Therefore, I sought to identify a candidate rat pheromone with the potential to serve as an unconditioned stimulus during odor fear conditioning.

Alarm pheromones are substances that are secreted by an animal in danger and can act as a threat signal to alert conspecifics of impending doom. The term "alarm pheromone" originates from research on minnows. These were first reported as "alarm substances" which "communicate the presence of danger, provided that they are produced by members of the same species"[32]. While a great proportion of research on alarm pheromones has occurred in fish and insects, the fact that rodents and other mammals also utilize this communication system has been established. One of the first studies on alarm chemosignals in mice demonstrated that mice will avoid a pathway which contains an odor from a stressed conspecific[33]. Mice will also avoid odors released by conspecific foot shock or defeat in a fight[34]. Rats can distinguish between odors released from a stressed and a non-stressed rat when they are associated with an aversive or appetitive US[35] and the former presence of a stressed rat in the pool of the forced swim test alters the subsequent behaviour of a non-stressed rat to closely match the behavioural phenotype of the stressed rat[36]. Further,

exposure to an alarm pheromone can induce convulsions when combined with a drug that increases serotonin and norepinephrine (NE)[37] and alarm pheromone exposure can directly suppress rat T cell and B cell proliferation[38]. These early studies proved a similar type of alarm substance as found in minnows and insects also exists in rodents; the alarm pheromone induces a behavioural effect in a conspecific[36], release can be stimulated by a variety of stressors[33,34], and rodents can discriminate between stress-related and non-stress-related conspecific odors[35] .

An important distinction needs to be clarified regarding rat and mouse alarm pheromones. It is common to interpret behavioural data from rats and mice in the same fashion, but pheromones are part of a deeply ingrained, innate and evolutionarily conserved process and it is necessary to understand that mice and rat pheromones may not consist of the same odor components or produce the same behavioural responses (see Inagaki[39] and Brechbühl[40]). Caution must be applied when comparing results across these two model systems.

Dr. Yuki Mori's group has produced a vast amount of valuable information on the effect and composition of rat alarm pheromones. Stress-induced hyperthermia occurred in male rats when placed in a box that was recently used to foot-shock 2 odor-donor rats[41] and that these substances were released from the perianal region[42], like how a skunk sprays when threatened. A multitude of anxiolytics were found to reduce the stress-related odor effects on the acoustic startle response (ASR): benzodiazepines, non-selective monoamine oxidase inhibitors, ß-adrenergic receptor antagonists, α2-adrenergic receptor agonist, or

corticotropin-releasing hormone subtype 1 receptor antagonist[43,44]. The response of an animal to its own stress-related odor was no different than the response to a conspecific stress-odor, suggesting there was likely a specific molecule or combination of molecules responsible for the anxious behaviour[45]. Two short years later two molecules were identified by the same group as core components of rat alarm pheromones: 4-methylpentanal and hexanal. Exposure to this mixture increased the ASR and decreased time spent outside in a modified open field test while increasing "head-out" risk assessment behaviours, indicating heightened anxiety. As the vomeronasal organ was shown to be required for these stress-odor effects[46,47], it is unsurprising that increased expression of cFos was found in the accessory olfactory bulb (AOB) in response to presentation of the binary mixture or a stress-released odor[39]. Collectively, the work from Dr. Mori's lab has identified a rat alarm pheromone that causes a vomeronasal organ-dependent stress response in a naïve receiver rat and increased activation in the pheromonal detection system.

If these stress-related odors cause a behavioural response not unlike direct exposure to a stressful experience itself, and allow for threat signals to be effectively communicated among a species, they may have the potential to act as an unconditioned stimulus in a Pavlovian fear conditioning task. Because this paradigm involves communication and socialization between rats, reflecting their natural tendency, this may mimic evolutionarily relevant conditioning rather than a painful stimulus such as a foot shock which is unlikely to occur in a natural environment.

1.1.4. Second order conditioning

Despite knowing that SOC was an existing behavioural phenomenon nearly 100 years ago[5], the mechanisms underlying it have not yet been fully elucidated with surprisingly little current interest. A group at the University of New South Wales led by Fred Westbrook and Nathan Holmes has spent the last two decades studying the differences between SOC and sensory preconditioning. As described above SOC entails pairing of CS1+US and subsequent pairings of CS2+CS1, while sensory preconditioning (as the name suggests) involves pairing the neutral stimuli together first, as in CS2+CS1 and subsequently pairing the US+CS1. In both cases CS2 presentation at testing elicits the conditioned response. Although the two procedures sound practically identical and produce the same result, the underlying circuitry and brain areas involved are vastly different when the order of pairings is reversed. For the purposes of this thesis the discussion that follows will be limited to SOC and not sensory preconditioning.

Westbrook and Holmes model their experiments on SOC and sensory preconditioning after the classic experiments of Rizley and Rescorla[48]. Rescorla was so passionate about using these two paradigms to uncover the fundamentals of associative learning that he published a book in 1980 called *Pavlovian Second Order Conditioning*[49]*,* detailing the kinds of information researchers could gain from these types of studies. In this book, Rescorla has argued that Pavlovian conditioning is unlikely to reflect any type of real learning that humans or rats experience in the wild. He thought there was no translational benefit to this type of research, but was adamant that second-order and sensory

preconditioning paradigms hold the potential of uncovering hidden variables and overall enriching our understanding of associative learning. Rescorla beautifully outlines experimental evidence that the sensory modality of the CS itself influences the magnitude of the conditioned response, while most people at the time studying associative learning did not appreciate this fact and were more concerned about how the US and CR were related to one another. Specifically in SOC, the CS2 is more strongly conditioned by the CS1 if they are similar to one another[49]. This book also presented evidence supporting the idea that simultaneous presentation of cues can be just as, if not more, successful in producing a given CR. The striking uniqueness of SOC could not be stated more eloquently than by Rescorla himself,

> *"Second-order conditioning represents one case of such learning in which a particular stimulus serves as the significant event in place of the US. In most Pavlovian experiments the significant event, the reinforcer, has its power innately, without the organism having any special individual experiences. The distinguishing feature of SOC is that its reinforcer is not of that sort; instead, its reinforcer has that status only because of past learning experiences by the organism. Thus, in a typical second-order conditioning experiment the presentation of S2 signals that of S1, but S1 is of importance only because in the past it has signaled the occurrence of some US."*

The use of SOC in the current experiments allows the comparison of a US directing odor learning and a CS directing odor learning in terms of both freezing behaviour and

neural activity. Are the same regions of the brain activated by an odor memory if that memory is directly associated with either an innate or learned "unconditioned" stimulus? Are areas recruited differentially based on sensory features of the CS1 used to form the CS2 odor memory? Another important consideration on SOC goes back to the types of conditioned stimuli that are used. As mentioned above, auditory cues are commonly used for fear conditioning and in SOC literature the bias is towards auditory and visual stimuli. To the best of my knowledge, the SOC project in this thesis is the first to compare olfactory fear memories that have been formed by 3 different conditioning stimuli (i.e., shock, contextual CS1, auditory CS1). My first project reflects the importance of the olfactory system for pheromonal conspecific communication, and previous data from our lab has shown that an odor can be used as a CS1 or a CS2 with context or tone in any combination to produce robust freezing behaviour[50]. Clearly, odor is an extremely salient stimulus modality for rats. While odors are not thought to be quite as salient for humans as they are for rats, it is important to consider the model organism being used for the experiment. I would argue that it is important to choose cues that are relevant to the species in the experiment, and this will yield a better understanding of associative memory, which ultimately can later be adapted and translated to human research. At many points throughout his book, Rescorla emphasizes the importance of choosing conditioned stimuli carefully, as they are not equally efficacious and may not produce the same level of conditioned response. Hence, odor is a prime candidate and was chosen here as the final substrate with which to compare the different types of SOC.

Akin to the Mori lab with respect to alarm pheromones, research in the lab of Westbrook and Holmes has contributed a great deal to the current understanding of the mechanisms and brain regions involved in aversive SOC. Using experiments with auditory and visual cues, they have shown that the BLA (BLA) is required for both first- and second-order conditioned fear[51–53]. More specifically, consolidation of both FOC and SOC fear requires calmodulin kinase II (CaMKII) signaling, gene expression, and DNA methylation in this area, but SOC occurs independently of ERK/MAPK signaling, PKA/PKC signaling, and *de novo* protein synthesis[51,53,54]. This suggests that first-order conditioning somehow changes the BLA which is then exploited by the consolidation of second-order conditioned fear, removing the requirement for protein synthesis. However, when the two CS are both auditory in nature, protein synthesis in the BLA is required for SOC[55]. This has been explained in terms of the types of information being integrated. When S1 and S2 are of the same sensory modality, the association occurs between the sensory properties of the stimuli, but when they are of different sensory modalities the association occurs between CS2 and the "responses or motivational state elucidated by the CS1"[56]. Encoding of the SOC fear is dependent on NMDARs containing NR2B subunits, as ifenprodil reduced freezing to the CS2[57,58].

Essentially, second-order conditioning allows for the propagation of an experience (appetitive or aversive) across a network of associative connections. This has direct evolutionary advantage as it implies that animals can integrate novel and relevant stimuli which predict danger in real time, potentially leading to optimization of defense strategies. Of course, the potential for this system to be damaging to the organism if unregulated or

dysfunctional is apparent, and might contribute to the persistent withdrawal and avoidance behaviour seen in fear and anxiety disorders[56]. Considering the numerous studies outlined above that have been completed since Rescorla published his opinion, it is reasonable to question his conclusions about the relevance of SOC to the human condition. SOC could represent common associative learning in humans and at least in part reveal mechanisms underlying the ability to update memories in the brain with additional stimuli. This has clinical implications; if someone is experiencing a great deal of anxiety, fear, panic, and stress in response to increasing external triggers, aberrant processes in higher order conditioning could lead previously formed fear memories to integrate new stimuli into the engram complex and therefore become activated by more varied stimuli, causing the person to experience more stress and anxiety, and the cycle continues[59].

When Rescorla was studying second-order conditioning, the understanding of molecular mechanisms underlying associative learning was in its infancy. In the past several decades alongside technological advances, memory research has exploded. Many of the historically elusive and seemingly magical neural processes of memory formation, maintenance, and expression have been discovered and theories have built upon this information. Perhaps the most revolutionary was the discovery of long-term potentiation (LTP) in the 1960s. LTP is widely considered to be the molecular substrate of learning and memory, and it is important to understand this basic process of how associative connections form.

1.1.5. Long term potentiation and synaptic plasticity

Current neuroscience research revolves around the importance of finding molecular mechanisms in the brain that account for behavioural phenomena. The mechanism by which our experiences lead to changes in the brain was elusive for decades, and it was commonly believed that the brain was rigid and unchanging in structure once a person reached adulthood. We now know that synapses can become stronger or weaker, depending on the input. This concept is referred to as synaptic plasticity[60]. One of the most important and ground-breaking findings of the century was the discovery of LTP, a strengthening of synapses, in the hippocampus by Lomo and Bliss in 1966, and has since in essence been broadly accepted as the molecular substrate of learning and memory[61].

The phenomenon of LTP was observed in 1966 by Terje Lømo, a PhD student in the lab of Per Anderson at the University of Oslo. He did not immediately pursue the finding himself, as he was focused on finishing the arduous and relentless task that was his thesis. Tim Bliss, a recently minted PhD graduate (McGill, 1967) reached out to Per Anderson about his interest in learning and memory who pointed him in the direction of his former student, Terje Lømo[62]. Their collaborative efforts culminated in publishing the seminal paper, "*Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path*" in 1973. This paper was revolutionary to the field of learning and memory and as of 2022 has been cited on Google Scholar close to 10,000 times. In the paper an area of the hippocampus called the entorhinal cortex (EC) was stimulated in living rabbits and the response in the dentate gyrus

(DG) of the hippocampus was recorded, along a streamlined pathway called the perforant path. In this way they were able to measure evoked post synaptic potential (EPSP), in other words they could measure the potential of the post synaptic cells which they had evoked in the presynaptic cells. They found that when they gave a burst of tetanic stimulation to the extracellular field surrounding EC cells, the EPSPs of DG cells changed. Some main findings which are now considered fundamental are 1) the long-lasting increase in amplitude of EPSP was due to a strengthening of synaptic connections, 2) LTP is saturable, meaning there is a ceiling effect of synaptic strength, and 3) LTP results in increased coupling between the synaptic response and the firing of postsynaptic neurons[63]. It had been decades since Hebb postulated his theory of associativity – "neurons that fire together, wire together"[64] and yet had no way to definitively prove if he was correct. This single paper allowed researchers to finally pinpoint a substrate for the way in which the brain obtains knowledge; to say this paper was huge would be an enormous understatement. The field of electrophysiology was born in a single moment, when scientists around the world learned of this captivating discovery that originated from the mind of a very talented PhD student.

The types of experiments first performed by Lømo are called field recordings and are extremely commonplace today especially in the hippocampus. While perforant path experiments are still regularly performed, one of the most utilized circuits to measure and manipulate LTP today is the Schaffer collateral (SC) pathway, also in the hippocampus, where principal neurons in region CA3 project to those in region CA1. A stimulating electrode is placed inferior to the cell body layer near the superior border of CA3, closest

to CA1. A recording electrode is placed some distance away from the stimulating electrode in CA1, again inferior to the cell body layer. The stimulating electrode sends a positive charge into the extracellular space surrounding the axons of the CA3 cell bodies, causing voltage dependent channels along the axons to open and the generation of action potentials in these cells. Neurotransmitter (in this case, glutamate) is released by these cells at the axon terminals and post synaptic potentiation occurs in the dendrites of CA1 neurons. The recording electrode measures the change in electric potential in the extracellular space surrounding the dendrites of neurons in CA1, which will be greater or smaller depending on the availability of postsynaptic ion channels. This type of readout is called a field evoked post synaptic potential (fEPSP). As these are extracellular recordings, they essentially measure the flow of ions either rushing into or out of the neurons, and while it is an indirect measure to assess the general plasticity of the network, it has become one of the most used and easily accessible strategies to assess plasticity in various experimental contexts. If a larger inward flow of positive current is observed by the recording electrode in the CA1 dendritic layer following some experimental stimulation, postsynaptic ion channels must be more plentiful to allow more ions to flow into the cell. This is consistent with the established molecular mechanism by which LTP occurs in the SC pathway, and reflects a strengthening of the synapses between CA3 and CA1 neurons. Weakened synapses, having fewer postsynaptic ion channels, consistent with the established molecular mechanism for long term depression (LTD), would be reflected by less inward positive current flow (reviewed by Malenka[65]).

Since its pivotal discovery, the molecular mechanisms underlying LTP have been dissected by some of the most intelligent minds in history. The gold standard textbook written by Kandel, *Principles of Neuroscience*[30], outlines the process beautifully to inform graduate students interested in learning and memory around the world. For brevity and clarity, I will focus on the best understood type of LTP, NMDAR-dependent LTP. When a presynaptic neuron fires an action potential, it releases neurotransmitter into the extracellular space. If this neurotransmitter is glutamate, it binds to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on the postsynaptic membrane and opens them, allowing for Na^+ ions to rush into the postsynaptic dendrite and depolarize the cell membrane. High frequency stimulation (HFS) can induce intense, long-lasting depolarization in the seconds that follow it. When the postsynaptic membrane potential reaches 0mV in segments containing NMDA receptors (NMDARs), the magnesium (Mg^{2+}) ion blocking the pore of the NDMAR is removed via electrostatic repulsion, and calcium (Ca^{2+}) ions are now able to enter the cell. Ca^{2+} ions act as intracellular second messengers leading to a variety of effects, but most pertinent to LTP is the activation of CaMKII, which phosphorylates AMPA receptors (AMPARs; gating Na^+ ion channels), facilitating their movement into the postsynaptic membrane and allowing more Na^+ to enter the postsynaptic cell. Once this occurs, a single electrical pulse from the presynaptic terminal will elicit a stronger postsynaptic response than observed prior to HFS. If LTP can be easily induced, the system is thought to have a high degree of plasticity. Conversely, if LTP is reduced or entirely absent this indicates an impairment in plasticity.

LTP can be induced with many different protocols. Following one or several bursts of high frequency or tetanic stimulation, this increase in postsynaptic potential can be observed hours later. The discovery and adaptation of LTP to so many different paradigms blew the doors wide open to new (and old) questions regarding memory formation, maintenance, and recall that could now be probed and ultimately answered. There exist multiple forms of LTP in the hippocampus alone: NMDAR-dependent (described above), NMDAR-independent through a class I metabotropic glutamate receptor (mGluR) and NMDAR-independent/mGluR-independent. While the hippocampus may be historically the most studied with respect to LTP, it is important to clarify that LTP is not observed solely in this region. Many other cortical and subcortical structures contain plastic synapses, and it has even been proposed that LTP occurs in all excitatory synapses (see Malenka[66] for a comprehensive review on LTP and LTD). The amygdala[67], piriform cortex[68], olfactory bulbs[69,70], and auditory cortex[71], have all been shown to undergo LTP under certain conditions. The prevailing theory of associative memory is that in order to form an association between information from two parts of the brain, neurons carrying information from both sources must converge upon a common structure (a single neuron or group of neurons) and form stronger synaptic connections by utilizing LTP[72]. This explains the ability of a conditioned cue to elicit the behavioural response of freezing in the absence of direct danger, if sensory information encoding the cue has been wired into the memory trace, then areas of the brain involved in freezing behaviour are now automatically engaged by that sensory input. It all goes back to Hebb's classic line, "neurons that fire together, wire together." So where does all the information converge?

While the terms “fear” and “amygdala” seem almost synonymous in the literature at present day, it is not the only structure that holds a long-term memory trace for such an event. The existence of a physical memory trace or “engram complex” has been hotly contested for the past century. The next section of this introduction focuses on a theory of associative memory that has garnered renewed support in recent past, owing to exponential scientific and technological advances allowing this theory to be probed and dissected, known as the “engram theory.”

1.2. Engram theory

Around the turn of the twentieth century, a German scientist named Richard Wolfgang Semon was seemingly the only mind of the time with an endless curiosity regarding how memories were physically stored in the brain or body. In fact, when Semon coined the phrase “engram” he was largely ignored by his peers or even written off as delusional. The term was originally intended as “…the enduring through primarily latent modification in the irritable substance produced by a stimulus…”[73]. Nowadays, the term engram is specifically used to describe the neural cell population that is involved in the encoding or recall of a particular memory, colloquially known as a “memory trace,” and sometimes referred to as an ensemble. It is now understood that rather than a particular memory utilizing one engram of cells in a particular area, memories are supported by the activity of connected engrams in distinct brain areas, collectively known as an engram complex[74]. Each memory holds the potential to utilize a unique combination of brain areas for its engram complex. The engram theory has been picked up and fallen to the wayside a

few times throughout history and at some points it was even thought to have been definitively proven wrong. As such, the engram theory has received its fair share of criticism based on early lesion and/or inactivation studies whereby inactivation of a region of the brain thought to be crucial for memory formation could be circumvented and the memory formed regardless. Conclusions based on these kinds of experiments tended to either incorrectly reject the importance of the area in question for memory, or lean towards rejection of a physical substrate for memory entirely (reviewed by Josselyn[75]). Fortunately, there is currently a renewed interest and vigour directed towards engram investigation, owing to new discoveries like immediate early genes (IEGs) and technical advances in methods such as transgenic systems, chemogenetics, and optogenetics. These refined techniques enable the direct labelling of cells active during a learning experience or recall of a memory and have provided convincing support for the engram complex, which will be discussed in more detail below.

1.2.1. Immediate early genes

The discovery of IEGs has been instrumental in developing techniques to probe the properties of the engram complex. Transcription of IEGs is activated rapidly and transiently, generally on the timescale of minutes[76–79]. While IEGs are responsive to membrane electrical activity and trans-synaptic stimulation in neural cells, they were discovered outside of the central nervous system (CNS) following stimulation by growth factors[80,81]. Hundreds of IEGs have been identified since their discovery and this class of genes is often exploited to deduce if a cell has been recently active, depending on temporal

expression patterns of the IEG in question. Generally, IEGs share several characteristics: baseline or resting expression is low but transcription is rapidly induced within minutes of stimulation, transcription does not rely on new proteins and is transient, and they often have a short half life[82]. Importantly, many IEGs encode transcription factors and nuclear mediators that can control the expression of other genes potentially coupling external stimuli with long term changes in gene expression[82].

Common IEGs used to visualize active neurons include *cFos, Arc, Homer1A,* and *Zif268*[83]. IEG *in situ* hybridization (ISH) or immunohistochemistry (IHC) is employed on brain slices following behavioural testing or training to visualize neurons that were active during the task. The time required between completion of the task and sacrifice of the animal is determined by the expression pattern of that IEG's mRNA or protein product and which cellular compartment expression is being measured from. For example, *Arc* mRNA is expressed in the nucleus within 5 minutes of stimulation and moves to the cytoplasm within 30 minutes. As such, IEGs provide a way to discern which neurons are active to discrete stimuli in many brain areas simultaneously. The current thesis utilized three IEGs: cFos, Arc, and Homer1a (H1a).

1.2.1.1. cFos

cFos was among the first IEGs to be discovered in the 1980's[78–80,84]. Transcriptional activation is transient, occurs within minutes of stimulation, and *cFos* mRNA is undetectable after 30 minutes[80,85,86]. The protein product of *cFos* is detectable within 30

minutes but its expression peaks 90 minutes following stimulation of the cell[87]. Its importance in neural tissue is highlighted by the fact that mice with the *cFos* gene knocked out globally from birth are viable, but they have reduced brain and body weight and show impairments in differentiation and development of neural stem progenitor cells throughout the late embryonic period[88]. Its primary role is as a transcription factor, regulating genes in response to influences from external stimuli[89]. Relevant to the work in this thesis, cFos mediates the conversion of extracellular glutamate signals into changes in neuronal gene expression[82]. This is an important distinction between cFos and Arc/H1a whose activity does not directly influence gene expression and instead act locally at the synapse[90–96].

The earliest studies illustrating *cFos* expression found it can be induced in the central nervous system following seizures[84,97], direct electrical stimulation of the motor or sensory cortex[98], in dorsal horn neurons after peripheral sensory stimulation[99], and in areas which control thirst following water deprivation[98]. Following seizures, the DG of the hippocampus displays the most rapid and dramatic induction of *cFos* expression[84] and cortical areas follow soon after[100]. Not long after its discovery, scientists indulged in thoughts of potential value for *cFos* to be used as a tool for mapping functional pathways in the CNS[82,84].

cFos ISH and IHC is commonly used to visualize active ensembles in various brain regions following the acquisition or retrieval of fear memories. One group showed that cFos levels were increased in pyramidal CA1 neurons of the DH (DH) following recall of a contextual fear memory[101]. One year later the LA, BLA, and central amygdala (CeA)

were added to the list of structures showing enhanced levels of cFos protein following contextual fear memory recall[102]. While these kinds of cFos studies have enabled the discovery of many relevant brain areas in various types of memory, it is under acknowledged and underappreciated that *cFos* is expressed in several CNS cells other than neurons, including astrocytes, oligodendrocytes, and microglia (reviewed by Steward[103]).

1.2.1.2. Arc

Arc is short for activity-regulated cytoskeletal-associated protein, which also goes by the name activity-regulated gene 3.1 (*Arg3.1*). Expression in the nucleus is observable within 5 minutes of stimulation, while *Arc* mRNA are detectable in the cytoplasm within 30 minutes of neural activity[104].

Notably, both the mRNA and the protein product of *Arc* accumulate in dendrites and are enriched at sites of synaptic plasticity, suggesting that they are synthesized locally[89–92,105,106]. *Arc* knockout neurons show reduced AMPAR endocytosis and increased mini evoked postsynaptic currents (mEPSCs; reviewed in Shepherd[107]). Importantly, this AMPAR removal is dependent on CaMKIIβ, the inactive form of CaMKII which is a critical signal transducer for LTP. These results have led to the theory that *Arc* plays a role in homeostatic or non-Hebbian plasticity, where large scale changes in synaptic strength occur over a large region without changing individual synaptic strength relative to one another[108–110]. However, *Arc* also participates in NMDAR-dependent LTP, a form of Hebbian plasticity, which is also dependent on *de novo* dendritic protein synthesis[111,112].

Further complicating things, *Arc* mRNA in dendrites is critical for synapse-specific LTD[111].

Arc is intricately involved in LTP, making it a well suited IEG candidate for studying fear memory engrams. A number of these discoveries have been made possible by the *Arc* knockout (KO) mice, developed by Plath and colleagues[113]. These mice breed normally, are the same size, and live as long as their WT littermates. They show no observable differences in gross or histological brain morphology, with normal distribution of synapses and postsynaptic density (PSD) as well as glial cells. There are, however, a multitude of learning and plasticity related impairments in the *Arc* KO mouse: reduced precision in processing spatial information and flexibility in relearning the Morris Water Maze with no effects on swimming velocity or floating times, reduced contextual and auditory fear conditioning with no effects on anxiety, failure to learn conditioned taste aversion with no effects on taste processing, impaired long term but not short term object recognition memory, impaired acquisition and elimination of late phase LTD, and enhanced early phase LTP and impaired late phase LTP with no change in baseline synaptic transmission[113]. Together these results suggest that *Arc* is critical for the conversion of a memory from a short-term plasticity state into a lasting memory, but it is not involved in baseline synaptic transmission or the induction of synaptic plasticity.

Choosing an IEG as a marker of cellular activity with a known primary purpose of endocytosing AMPA receptors seems counterintuitive, considering the primary mechanism of strengthening active synapses is by the insertion of AMPA receptors into the PSD[30].

How can a molecule that removes the molecular substrate for learning be so critical for so many types of it? An elegant study published just months ago has provided a possible answer to this question. The authors found that *Arc* and PSD95 essentially compete for AMPAR binding in a highly *Arc* concentration sensitive manner, but the capacity of *Arc* to compete with PSD95 is completely blocked by specific phosphorylation patterns which occur on AMPAR binding proteins that indicate active synapses. In other words, *Arc* is only able to directly regulate AMPAR endocytosis in inactive synapses by out-competing PSD-95 for binding[114].

Clearly, *Arc* is involved in various forms of neural plasticity, and has been used in combination with another IEG, Homer1A, for the technique utilized in this thesis **c**ompartmental **a**nalysis of **t**emporal activity by **f**luorescence ***in situ*** **h**ybridization (**catFISH**).

1.2.1.3. Homer1a

Homer1a (*H1a*) is an IEG that comes from a family of proteins called the Homer family that are involved in PSD assembly via scaffolding of proteins. *H1a* mRNA is visible in the nucleus within 30 minutes of a stimulus and the cytoplasm within 60 minutes[115].

Like *Arc, H1a* selectively targets active regions of the dendritic branch[93], and has been implicated in homeostatic plasticity involving decreased expression of synaptic AMPA receptors, but through a class I mGluR-dependent pathway. When *H1a*

transcription is induced in a postsynaptic neuron, mGluRs are activated in the absence of glutamate[94,116,117], a mechanism that is central to the role of *H1a* in homeostatic plasticity. *H1a* KO mice are viable and embryonic cultures derived from these mice display elevated GluA1, GluA2, and mGluR5 surface expression with increased mEPSC amplitude. This study also found that while they produce the same effects, the action of *H1a* is not dependent on *Arc* which suggests that they function by independent pathways[95]. This data supports the hypothesis that a *H1a*-dependent dynamic reduction in mEPSC amplitude is evoked by in vivo neural activity, and is reversed by inhibition of group I mGluRs. It also supports the long-suspected role of *H1a* in modifying glutamatergic signaling pathways[96].

1.2.2. *Arc/H1a* catFISH

CatFISH is a technique that simply relies on the application of a FISH procedure, using one or more IEGs to map ensemble activation at two time points within the same animal. Inter-animal variability in gene expression can be large, and catFISH can be used to test hypotheses about brain areas involved in conditioned versus neutral stimuli, how the neural representation of a conditioned stimulus changes or remains stable over time, or even to compare appetitive and aversive engrams, all within the same animal. This can help to reveal complicated dynamics that are not necessarily obvious when the noise from inter-animal variation is not accounted for. This technique was first described in 1999 by Guzowski[104], and was specifically developed for *Arc* catFISH but subsequently for combined *Arc* and *H1a* to map an engram[115], illustrating how the basic principles of temporal gene expression patterns can be exploited to create paradigms which can integrate

multiple IEGs and map activity to a larger number of individual stimuli within the same animal, enabling the probing of more complicated questions.

The first studies to explore the temporal expression dynamics of *Arc* transcription with *Arc* catFISH found that *Arc* transcription is reliably activated in about 40% of DH CA1 neurons following exploration of a novel context[104,115,118]. This replicates data observed in electrophysiological studies that found 30-50% ensemble reactivation depending on the environment size[119,120], providing evidence that specific IEGs are expressed in pyramidal hippocampal neurons during the formation of an ensemble for a specific context. Citing evidence that both *Arc* gene expression and stabilization of hippocampal place fields are NMDAR-dependent[90,121], it has been proposed that *Arc* may be involved in long term engram stabilization[104]. Since then, evidence has accumulated in support of *Arc*'s role in long term memory, summarized above[113,114].

If *Arc* and *H1a* utilize independent intracellular mechanisms of action[95], it is important to ensure that they are both transcribed in response to the same stimuli. Vazdarjanova showed how *Arc* expression appears in the nucleus within 5 minutes and is present in the cytoplasm 30 minutes following exposure to a stimulus. In addition, exploration of a novel context induced coincident *Arc* cytoplasmic labeling and *H1a* nuclear stain 30 minutes later, rates of co-labelling were 95% in DH CA1, 94% for DH CA3, and 93% for the parietal cortex. This study indicated that these two IEGs were dynamically regulated by physiological activity in the same hippocampal and cortical neurons[115].

Arc/H1a catFISH has also been used to investigate which cells in the LA are active during fear conditioning and whether the same neurons are active during a rest period which immediately proceeded fear conditioning (FC)[122]. This FC/Rest group was compared to a Rest/FC group, where the rest period preceded fear conditioning. In this way, the overlap ratio of the two groups was compared to see if the same neurons are active during fear conditioning and during rest. A higher proportion of neurons expressed both nuclear *H1a* and nuclear *Arc* only if the rest period came after the fear conditioning period, perhaps reflecting long term consolidation processes ongoing in these neurons. Importantly, this effect is unlikely to simply result from prolonged continuous transcription or continuous neural activity, because the number of neurons active during rest were the same whether FC occurred before or after the rest period. The authors concluded that *Arc* is preferentially transcribed during rest following fear conditioning in the same neurons that were activated by the fear conditioning itself, and this mechanism may underlie memory consolidation[122].

The lab of Diano Marrone was the first to exploit both *H1a* and *Arc* cytoplasmic and nuclear stain to detect three separate episodes of activity[123]. They included three groups: explore-rest-explore (E-R-E), rats who explored a novel context followed by a rest period followed by exploration of the same context; rest-explore-explore (R-E-E), rats who explored the novel context twice following a rest period; and rest-rest-rest (R-R-R), rats who did not explore the novel context at all. In groups E-R-E and R-E-E, ~80-90% of cells activated by the first exploration were reactivated by the second one, proving the feasibility of this method to accurately detect activation to three separate episodes[123]. As the study above described, IEG expression is not increased during the rest period regardless if it

occurs before or after one of the exploration periods, but the probability of the same neurons being active during exploration and rest was significantly greater when the rest period occurred after the exploration period[123]. Importantly, the two studies described above show the feasibility and reproducibility of using *H1a* and *Arc* as IEGs to map engram complexes through *catFISH* across the brain.

1.2.3. Transgenic, chemogenetic, and optogenetic strategies

More recent studies have capitalized on transgenic rodent models to tag neurons during a task with fluorescent reporters using temporally inducible IEG promoters, causing cells that were active during a particular task to fluoresce in the living animal so that this cell population may be compared to the ensemble active during other tasks of interest within the same animal. In this way, some experiments have tagged neurons active during learning and then used IHC for a different IEG to tag the neurons active during testing in order to compare the overlap of the two cellular ensembles and test if the same areas are active during and following training. These studies have provided strong support for the engram theory of memory, at least in classical conditioning tasks. Dr. Mark Mayford's lab demonstrated this for auditory and contextual fear conditioning using genetic tagging of cFos (encoded by the IEG *cFos*) during learning then IHC for Zif protein (encoded by the IEG *Zif268*) an hour following testing. They found that for auditory fear conditioning, cells in the LA that were active during training were more likely to be re-activated during testing, while cells in the BLA that were active during contextual fear conditioning were more likely to be re-activated during testing. Auditory fear conditioning did not influence the

engram in the BLA and contextual fear conditioning did not influence the engram in the amygdala. This strongly supports the idea that the LA participates in the engram complex for an auditory fear memory while the BLA participates in the engram complex for contextual fear memory[124]. This kind of "observational evidence" of the existence of the engram, where the same cells are activated during both learning as well as recall has been shown in many other areas such as the hippocampus[125–128], the amygdala[127–130], and cortical areas[131–133] in a variety of conditioning tasks.

Dr. Sheena Josselyn's lab has contributed a great deal of evidence in support of the engram theory of learning and memory. In 2009, Han and colleagues capitalized on a previous finding that the level of expression of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), a transcription factor, is a predictive factor for whether a given neuron will be activated by either auditory fear conditioning or testing[134]. They infused a virus into the LA that caused overexpression of CREB-cre in about 15% of LA neurons, and demonstrated how this caused this subset of neurons to be preferentially recruited into the fear memory trace. After testing the animals' fear memory as a baseline measure, they selectively ablated the neurons by injecting a drug that targeted cre+ cells (and therefore only those neurons which were artificially allocated to the fear memory trace) to initiate apoptosis. Following deletion of these neurons, this memory was erased at every time point tested, up to 12 days later, and importantly these ablating a similar number of random cells in the LA of mice did not result in memory loss. Together, these results show that fear conditioning causes an ensemble of cells in the LA to express high levels of CREB and if these cells are deleted, so too is the memory, with no long-term impairments

in LA function[135]. Since then, reversible or permanent inactivation of tagged neurons using IEGs in several brain regions thought to be necessary for memory tasks and therefore participate in engram complexes, such as the hippocampus[126,136,137] and nucleus accumbens (NAc)[138,139], have produced comparable results. These "loss of function" studies demonstrate that deletion of an ensemble of cells in an area critical for fear conditioning causes long lasting memory loss specific to the CS, and are crucial pieces of evidence in support of the engram theory of learning and memory.

Dr. Susumu Tonegawa has dedicated much of his passion in research to proving the existence and revealing the properties of the engram complex. His lab used an elegant system whereby optogenetic channels were expressed in DG cells of the hippocampus that were active during fear conditioning. A virus which carries cFos-tTA-ChR2 inserts channelrhodopsin-2 (ChR2) optical channels that can be later activated by light, and is driven by the cFos promotor. Importantly, tTA transcription of ChR2 is blocked by doxycycline (DOX), so the animals fed a diet with DOX added cannot transcribe optogenetic channels. During a particular behavioural task, the DOX is removed and animals are fed regular chow so the expression of the channels can proceed. The virus is directed by a *cFos* promoter, so that neurons which are activated during the behavioural task will express optogenetic channels and can be artificially turned on by light stimulation. By re-instating the DOX diet immediately following FC, channels will not be inserted randomly unrelated to the task. When the virus was injected bilaterally, levels of freezing in response to light stimulation of engram cells reached almost as high as those induced by exposure to the context. This was the first study to show that direct activation of an

ensemble of neurons involved in the formation of a memory alone can induce its behavioural expression[140]. Other studies since have demonstrated the same phenomenon in a variety of brain areas for different behavioural tasks, such as the prelimbic cortex in remote fear memory retrieval[131], the retrosplenial cortex in contextual fear memory which was independent of the hippocampus[141], and the hippocampus in various forms of memory[142,143]. Evidence gleaned from "gain of function" studies, together with that from "observational" and "loss of function" studies, highlight the incredible leaps that engram research has been able to make in the past decade[75].

Many of the studies described thus far have examined engram formation in specific areas of interest, but it is generally accepted that an engram that supports a specific experience is likely distributed throughout the brain and engrams in certain areas may contribute to distinct aspects of the experience[75]. Optogenetic experiments are hugely important for investigating the role of a particular area in memory, but chemogenetic experiments can activate all areas involved in a fear memory at once. They lack the precise temporal and spatial control of optogenetics, but have been applied to engram research for their ability to reveal properties of the engram complex. A recent study identified 247 regions with activated neural ensembles corresponding to contextual fear conditioning by expressing an inducible cre-dependent activation of cFos with Fos-TRAP mice, which allows brain-wide labelling of active neurons in a short time window, in the scale of hours. Using a modified version of CLARITY, a tissue clearing technique that enables visualization of tagged neurons within the brain and therefore produces a visible map of connections for a specific behaviour, cross-comparison of these 247 areas devised an

engram index to rank which areas are most likely to participate in the engram complex. Brain-wide engram reactivation studies using recall of an actual memory, and targeted combinations of engram cell ensembles in different areas using chemogenetics showed many of the identified engram candidates were functionally connected to BLA or CA1 hippocampal engrams, while identifying engrams in previously unsuspected brain regions. They also manipulated individual engrams using optogenetics proving that activation of engram cells in one area induces activity in other regions which were identified during recall of the memory, specifically the activity pattern following optogenetic engram cell reactivation of both BLA and CA1 was significantly correlated with that observed during recall of the memory. When ensembles from 3 putative engram regions were activated (CA1, BLA, and anteromedial thalamus), memory was enhanced compared to 2 regions (CA1 and BLA), and further enhanced compared to a single engram in one area (CA1), but interestingly no further enhancement was seen following the additional activation of a fourth region (nucleus reunions of the thalamus). The authors suggest that this may reflect a mechanism of homeostatic memory recall, as if the brain adjusts the strength of memory recall by recruiting more engrams based on how important the memory is for guiding decisions[74]. This elaborate set of experiments has demonstrated that activity of multiple ensembles which contribute to the engram complex can enhance memory expression to levels as high as induced by the CS itself.

The very existence of the engram complex itself has been elusive since its introduction by Richard Semon back in 1904. Fortunately, brilliant minds have devised

strategies based on technological advances that have demonstrated how an engram encodes a memory and that the typical behavioural response to recall of a memory can either be eliminated by impairing the engram or it can be induced by activation of the engram without causing any impairments in future learning. It is important to clarify that none of the experiments in this thesis provide cause and effect support for inclusion of any areas into a "true" engram complex (see Mayford[144]). Discussion in this thesis does however, use the terminology of engrams and engram complexes to describe cells that were active during recall of a particular odor memory although we do not know if they were active during the original learning experience, to provide clarity for the reader. In the following section specific brain regions relevant in either sensory processing or fear circuitry and therefore potentially participating in engram complexes are described.

1.3. Engram complex candidates

A variety of areas were chosen as potential contributors to the engram complexes. Some were included because of their proven relevance to fear conditioning while others were included for their roles in sensory processing. Areas were chosen that were deemed most likely to be important depending on the major question being asked in each project.

1.3.1. Olfactory bulbs

Rats possess multiple parallel odor processing pathways; of relevance to this thesis, olfactory sensory neurons project from the main olfactory epithelium (MOE) to the main

olfactory bulb (MOB), while neurons in the vomeronasal organ (VNO) project to the AOB. Both sets of nerves travel through the cribriform plate to synapse onto olfactory bulb tufted and mitral cells (see Figure 2A and 2B).

1.3.1.1. Main olfactory bulb

Olfactory sensory neurons (OSN) reside in the main olfactory epithelium. OSNs only express one receptor type each which recognize chemical features of the odorant[145]. For example, an early study tracked individual neural response to a variety of similar odors and found that OSN A was more likely to respond to para-isomers of disubstituted benzenes while OSN B responded only to di-substituted benzenes with short side chains[146]. OSNs project their axons through the cribriform plate of the skull and synapse with mitral and tufted cells of the main olfactory bulb in the glomerular layer. The main olfactory bulb is organized in a very stereotyped manner; the outermost layer is where the first synaptic connection occurs in odor processing. The glomeruli of the olfactory bulb are discrete functional processing units, and are organized into odorant-specific functional maps that remain relatively consistent across a species. As the name suggests, peri-glomerular interneurons surround the glomeruli and mediate inter-glomerular interactions to help provide the high degree of specificity in signaling required for accurate odor detection and recognition[147,148]. The cell bodies of the mitral and tufted cells reside in the mitral cell later,

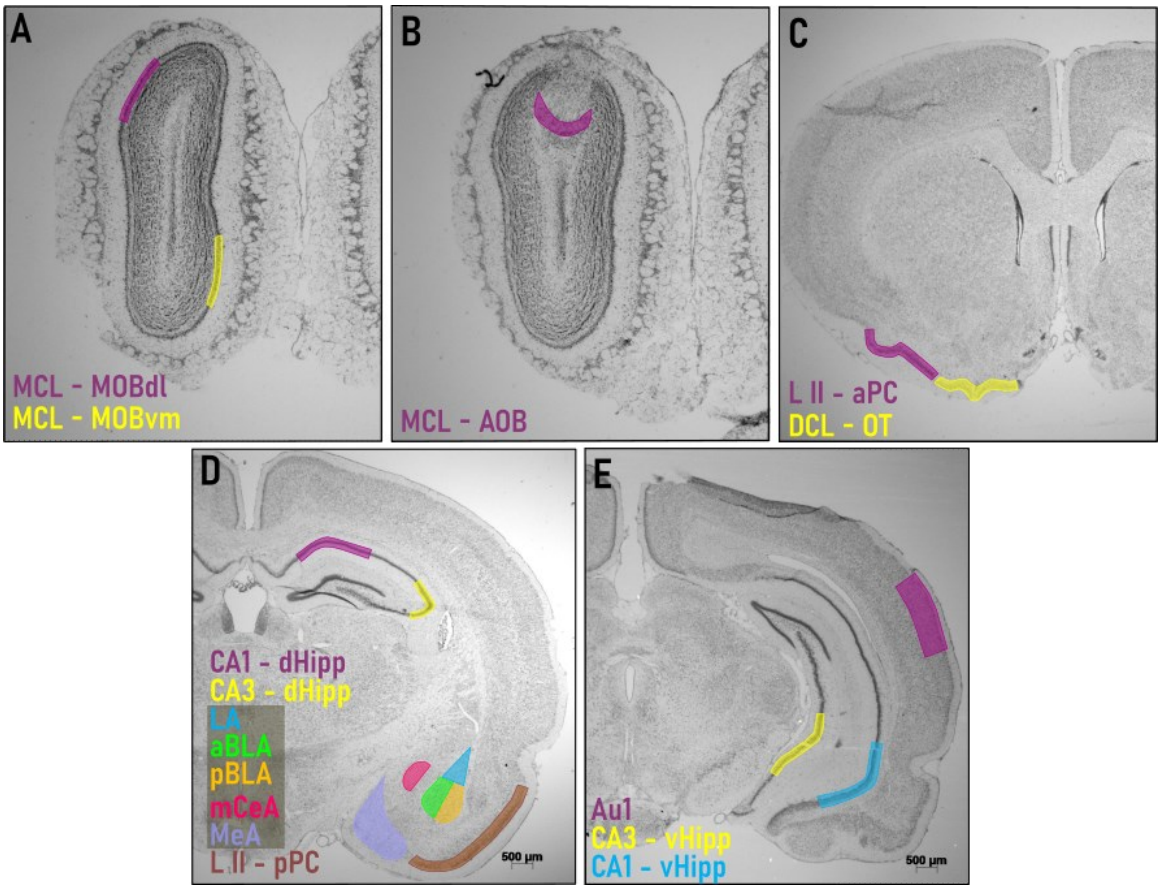


Figure 2. ***Anatomical regions of study.*** Nissl-stained sections reveal cell bodies across the antero-posterior axis of the rat brain. All images were taken with a 4X objective on a Zeiss Stemi-2000C dissecting microscope with an attached Zeiss Axiocam Mrm Rev3 camera (Carl Zeiss) system. **A)** The dorsolateral (dl; purple), ventromedial (vm; yellow) mitral cell later (MCL) of the main olfactory bub (MOB); section from bregma +6.70 mm. **B)** The MCL of the accessory olfactory bulb (AOB; purple); section from bregma +5.70 mm. **C)** Layer II neural cell layer (L II) of the anterior piriform cortex (aPC; purple), dense cell layer (DCL) of the olfactory tubercle (OT; yellow); section from bregma +1.7 mm. **D)** CA1 cell body layer of the dorsal hippocampus (DH; purple), CA3 cell body layer of the dorsal hippocampus (yellow), lateral amygdala (LA; blue), aBLA (green), posterior BLA (pBLA; orange), medial division of the central nucleus of the amygdala (mCeA; pink), medial amygdala MeA; light purple), layer II (LII) of the posterior piriform cortex (pPC; brown); section from bregma -3.14 mm. **E)** Primary auditory cortex (Au1; purple), CA3 cell body layer of the ventral hippocampus (VH; yellow), CA1 cell body layer of the ventral hippocampus (blue); section from bregma -4.8 mm; scale bar 500 µm.

and since these are the main output cells of the MOB (Figure 2A), they were chosen in the first project to compare shock-mediated odor memories with those formed by using pheromone as the US. Deep in the olfactory bulbs are the granule cells, and odor discrimination and memory are regulated by a complicated milieu of excitement and inhibition in these regions[149–152]. The MOB is conferred additional plasticity by the frequent turnover and regeneration of interneurons[153–155], and this area is also modulated further by input from regions carrying information about arousal, valence, and learning[156,157].

1.3.1.2. Accessory olfactory bulb

The AOB has a very similar circuitry to that of the MOB with some obvious differences. The vomeronasal organ (VNO) is a chemosensory apparatus which detects a wide range of volatile and non-volatile molecules like peptides and small proteins, either pheromones secreted from conspecifics or non-pheromonal predator odors[158]. Substances that activate VNO neurons can induce mating[159], aggressive or fearful behaviour[160,161], they can influence hormone levels[162], and can also serve as cues for recognizing conspecifics[163]. Vomeronasal sensory neurons (VSN) are located in the VNO which is located inside the anterior part of the nasal cavity near the septum[164]. VSN axons travel through the cribriform plate with axons from OSNs and synapse with mitral cells of the AOB in the glomerular layer to relay pheromonal information[164]. In contrast to the MOB, the spatial map of glomeruli corresponds to VNO receptor subtype activation and not particular odors[165]. The Mori group has shown that recognition of their identified rat alarm pheromone by conspecifics is dependent on the VNO[46].

The AOB is embedded in the dorsal portion of the MOB, has smaller glomeruli than the MOB, but a larger mitral cell layer[166]. The AOB forms reciprocal connections with the amygdala, projecting to the medial and posteromedial nuclei[167] and receiving input through the stria terminalis[168]. The bed nucleus of the stria terminalis (BNST) projects GABAergic axons to the mitral layer of the AOB, while the amygdala projects glutamatergic axons to the granule cell layer[168]. MOB and AOB neurons can converge onto single neurons in the hamster amygdala[169]. Pheromonal blends from urine induce increased *cFos* mRNA production in the AOB compared to the MOB of conspecific mice[170]. *cFos* reactivity in the AOB has also been observed in response to soiled bedding from opposite sex conspecifics[171]. If the alarm pheromone was able to act as an unconditioned stimulus, it would be logical to expect the AOB to participate in the engram complex, specifically in the "Companion" group. Because they are the AOB's main output cells, the mitral cell layer was analyzed to compare pheromone-mediated odor memories with those formed by using shock as the US (Figure 2B).

1.3.2. Piriform cortex

The piriform cortex (PC) is a tri-laminar paleocortex located on the ventrolateral rodent brain and is the largest of one of few brain areas to receive direct signals from the OB, via the lateral olfactory tract (LOT). Olfaction is the only sense which does not send information through the thalamus prior to reaching its sensory cortex. In this way, piriform cortical neurons are only two synapses removed from the external environment. The LOT sends signals to the sparsely populated layer I, while layer II consists of densely packed

cell bodies of glutamatergic principal neurons, and layer III contains a lower density of principal neurons[172]. Feedforward and feedback interneurons shape odor representations in the piriform cortex[173,174], and these are scattered throughout the three layers. In addition to interneurons, semilunar and superficial pyramidal principal neurons make up the heterogenous mix of neural cells which reside here, and the pyramidal neurons are divided further into molecularly distinct subgroups depending upon where they project[175].

In general, the piriform cortex utilizes population coding to represent odor objects such that neural representations of different odors have elements that overlap with representations of other odors. In contrast to the highly stereotyped spatial order of odorant-dependent responses in the OBs, it is more accurate to conceptualize this area in the context of the coordinated activity of groups of neurons rather than on the basis of individual neurons[176]. When individual PC neurons are recorded over repeated presentation of a given odor it is apparent that the activity of an individual neuron does not reliably report the presence of that odor on any trial. Instead, a subset of neurons respond to individual odors reliably about half the time. This is true across neutral, appetitive, and aversive odorants, and the magnitude of response of individual PC neurons is not correlated with odor valence[176].

It is a common conception among olfactory researchers that the anterior and posterior piriform cortex (aPC, pPC) differentially process odor information. The aPC receives a larger degree of afferent OB inputs compared to the pPC, which receives more associational inputs, consistent with the idea that the aPC encodes odor identity while pPC

activity reflects quality of odors, including valence[177–179]. A recent study using retrograde trans-synaptic tracers to differentiate the inputs to aPC and pPC confirmed that aPC receives a higher proportion of inputs from olfactory structures, while the pPC receives a higher proportion of inputs from the hippocampal formation and amygdala[180]. The percentages of PC neurons that are unresponsive to odor increases along the anterior-posterior axis, again highlighting the differential features of this large cortical area[178].

The piriform cortex is often thought to be a hub of learning and memory for odor associations. It has been proposed to utilize sharp waves like the hippocampus[181], which may contribute to the enormous amount of plasticity in the piriform cortex and in turn odor memory[182]. Another similarity to the hippocampus is that the synapses interconnecting PC neurons are plastic into adulthood[68,183]. A more recent study found that chemogenetic activation or inhibition of PC cells that were tagged during olfactory fear conditioning (engram cells) either evoked the behavioural fear response in the absence of the CS or suppressed the fear response in the presence of the CS, respectively[184]. Plasticity in this region is also evidenced by the fact that associative odor learning induces changes in odor-evoked activity patterns[185].

Synaptic plasticity, specifically NMDAR-dependent LTP, has been demonstrated to be a mechanism involved in odor learning within the PC[68,186–190]. Plasticity can occur between neurons projecting to different areas or within associative connections of the PC itself. Previous work with *Arc* catFISH in our lab has shown dynamic plasticity in odor representations following an appetitive learning paradigm. Specifically, after associative

learning encoding-pyramidal PC neurons are more likely to respond to a given odorant, which was indexed by the higher overlap ratio of *Arc* expression to two consecutive presentations of the trained odor. Importantly, the total number of cells expressing *Arc* in response to presentation of this trained odor was decreased compared to controls, largely due to the reduction of "noise." If a mixture of two highly dissimilar odors is rewarded and the two odors are presented sequentially prior to sacrifice, the overlap ratio is significantly higher than if the two odors were presented randomly and not associated with reward. However, if rats are trained to distinguish highly similar odors, one with reward and one without, the overlap ratio between the two odors becomes considerably reduced. The first experiments show how an engram is reduced in size but is more reliable, or is "sharpened" following associative learning. The experiments on odor mixtures exemplify the phenomenon of pattern completion and pattern separation, which has also been shown to occur in the hippocampus (reviewed by Yassa[191]), and suggest that the PC might be involved in these processes for odors[192].

PC plasticity is complicated, highly dynamic, and still not completely understood. For both projects I included the aPC and pPC (Figure 2C-D) as potential components of the engram complex. Another region that receives a large input from olfactory related structures and has been hypothesized to participate in odor valence representation is the olfactory tubercle (OT), described next.

1.3.3. Olfactory tubercle

The olfactory tubercle has several functions and is perhaps the least studied of the olfactory areas. It receives information from the OBs predominantly via tufted cells[193] which has been modulated by mitral cell input from OB, and has been proposed to play a role in determining the source of an odor as well as contributing to odor identification[194]. It is essentially a cortex-like structure with three layers similar to the pPC, but has been broken further down to include domains bound by cap compartments, which contain densely packed small sized medium spiny neurons (MSNs) at the lateral surface[195,196], and Islands of Calleja made of densely packed granule cells from local interneurons distributed across the OT[197]. Like the pPC, it receives projections from the amygdala, but in contrast it is connected reciprocally to the NAc, the ventral tegmental area, and the basal ganglia[194,198] and lacks an associative fiber network[199]. Despite being unable to utilize associative fibers in odor coding like the PC, its interconnectivity with reward and fear circuitry makes the OT a likely candidate to participate in odor valence coding.

A study evaluating the OT's relationship to appetitive CS revealed that single neurons in the OT preferentially fire for rewarded over non-rewarded odors[200]. Murata and colleagues mapped cFos activation in the MSNs of the OT following exposure to learned odor cues. They broke the OT down into four domains, based on the spatial arrangement of the cap compartment and the Islands of Calleja. Using sucrose solution to produce appetitive odor conditioning and foot shocks for aversive learning increased cFos in the anteromedial domain of the OT and the lateral domain was observed for each type,

respectively. In both these areas, MSNs expressing type 1 dopamine receptors (D1Rs) were preferentially activated over those expressing type 2 dopamine receptors (D2Rs). The authors proposed that the OT acts as a rudimentary switchboard depending on which domains are activated by incoming stimuli, ascribing valence information among the functionally connected network of cells responding to odors[201].

The OT was included as a candidate for engram inclusion in the pheromone study to evaluate whether an odor conditioned with shock will differ from an odor conditioned with a pheromone in terms of their representation in an area proposed to play a role in coding odor valence. The lateral aspect of the dense cell layer of the cap was analyzed (Figure 2C) for *H1a* and *Arc* expression following exposure to the control and conditioned odor, respectively.

1.3.4. Auditory cortex

The auditory cortex is considered a neocortical structure and consists of 6 layers. It receives information about the frequency of sounds from the ventral medial geniculate nucleus. Each of these projections is narrowly tuned to a small band of frequencies and in this way, neurons are tuned to overlapping frequency bands[202].

The question of whether learned information about sounds is stored in the auditory cortex has been evaluated from different angles for the past several decades. One strategy, described by Weinberger[202], was to evaluate the receptive field, which is essentially the

response of a single cell to a whole range of stimulus values. The first study to utilize this idea in the auditory cortex found that classical fear conditioning induces a specific change in receptive fields in the adult guinea pig. Responses to a conditioned tone were significantly increased while responses to untrained CS frequencies were either reduced or unchanged[203]. These changes were dependent on stimulus pairing, and sensitization training resulted in only a general increase in response[204], supporting the idea that this type of plasticity is associative.

Fear conditioned tones induce a larger evoked potential response in auditory cortical neurons[205–207]. Imaging auditory cortex longitudinally with two-photon Ca^{2+} imaging revealed that CS- representations are selectively reduced while CS+ representations are maintained following fear conditioning. Additionally, the decline for CS- responses in this study were accounted for by habituation, leading the authors to conclude that the auditory cortex prevents habituation to CS following fear conditioning[208]. In fact, a disinhibitory circuit in the auditory cortex gates the acquisition of conditioned fear responses[209]. In this area the size of the ensemble recruited to represent a particular CS is positively correlated with the level of importance of that CS[210].

The auditory cortex projects reciprocally to the lateral amygdala (LA) and enhanced plasticity has been observed in both areas following auditory fear conditioning[205,211]. Interestingly, about one third of cells recorded in the piriform cortex respond to tones which suggests that incoming olfactory information from the OBs is subject to modification by auditory sensory input which may influence processing of or responses to odors[212]. The

evolutionary significance of a polysensory role in the cortex is clear; rodents in the wild need to remain vigilant of both predator sounds and odors and integration of features from both sensory modalities would give the animal the highest chance of recognizing the predator in the future and therefore surviving and passing on its genes to the next generation. However, PC neurons do not project to the auditory cortex, suggesting that neurons in the auditory cortex do not participate in odor processing[213]. The question of whether cells in the auditory cortex can be activated in response to an odor CS2 trained with a tone CS1 has yet to be answered.

The auditory cortex (Figure 2E) was chosen in the set of SOC experiments as a candidate for engram complex inclusion as one of the behavioural paradigms tied together a tone and an odor as CS1 and CS2.

1.3.5. Hippocampus

The hippocampus is widely accepted as the central hub of plasticity, owing to its relatively simple yet robustly critical circuitry. In general odor and other sensory information enters the hippocampal formation via the superficial layers of the EC. The EC projects to cells in the DG via a pathway called the perforant path, which in turn project to large pyramidal neurons in region CA3 via the mossy fiber pathway. Next, CA3 neurons project their axons along the SC pathway to small pyramidal neurons in CA1. From here, information travels to deep layers of the EC, which send signals to targets elsewhere in the brain. The EC is therefore referred to as the interface between the hippocampus and the

neocortex. Importantly, the circuitry within the hippocampal formation is consistent along the longitudinal axis[214–217], enabling this highly plastic structure to receive and send signals all over the brain. The tight cell and dendritic layering in the hippocampus allows researchers to exploit the power of deductive logic to infer large scale changes in synaptic strength. As described in detail in Section 1.1.5, the SC pathway is traditionally used to record fEPSPs, which measure the external change in ion concentration, revealing if the neurons are undergoing LTP or LTD following a stimulus protocol.

The hippocampus has been implicated in learning and memory since the historic case of H.M., a patient who underwent bilateral medial temporal lobe excision to cure his uncontrollable seizures and lost most of his memory as a result. The relationship between the hippocampus and memory is complicated, and all the details have not yet been fully elucidated. It is known that the hippocampus utilizes NMDAR-dependent LTP to integrate contextual information during the exploration of the new context prior to the context/shock pairing in contextual fear conditioning[218–220].

The suggestion that the hippocampus may not act as a unitary structure and instead play distinct roles along the dorsal-ventral axis was made over twenty years ago[221]. This hypothesis was built on existing findings that DH and VH have distinct input/output connections[222], spatial memory is dependent on DH but not VH[223], and stress responses and emotion-evoked behaviour are dependent on VH but not DH[224]. Because the VH is strongly associated with aversive memory and the DH is involved with encoding contextual

cues, both regions were included (Fig 2D, E) as separate candidates for engram complex inclusion in the SOC project.

1.3.5.1. Dorsal hippocampus

Visuospatial information is relayed mostly to the DH through the caudolateral band of the EC[216]. This region of the hippocampus contains the most place cells coding spatial location in terms of both density and selectivity[225,226]. Further evidence implicating the DH in spatial orientation comes from its projections; the DH sends information to regions involved in visuospatial information and memory processing[214,227–229], environmental exploration[230], and navigational information[229,231–233].

Importance of the DH in spatial memory is evidenced by a number of early studies utilizing the Morris water maze (MWM), a task where animals must find the location of a hidden platform based on external cues and landmarks outside of the pool[234]. Lesions affecting as little as 25% of the DH were able to disrupt learning in the MWM, while VH lesions had no effect[223]. The radial arm maze is another spatial cognition test, forcing animals to rely on their memory and visit arms they had not previously explored to obtain a food reward. Again, DH but not VH lesions impaired this type of learning[235].

More recently, it has been shown that optogenetic reactivation of a neural engram formed during contextual fear conditioning induces freezing in a novel context[140]. Retention of contextual but not cued fear is impaired when the DH is lesioned[236], and that this may have more to do with CA1 than CA3[237]. Cells of the EC convey discrete sensory

information about the context[238], cells in DH CA3 are responsible for storing a unified representation of the context, including multisensory cues[239], and signals from cells in both of these areas can induce plasticity in DH CA1 output cells[240–242]. Spike timing of these CA1 output neurons is regulated by specialized local inhibitory interneurons[243,244], which is like that which is observed in the auditory cortex following auditory fear conditioning, described above.

Standard models of context fear conditioning posit that the DH encodes features of the contextual CS and sends this information to the amygdala where plasticity mechanisms cause the CS-US association[72,227,236,245,246]. This implies that separate anatomical pathways carry information from the CS and the US and that the DH does not receive any information about the US. This view has been challenged, as one group found that during US exposure the entorhinal cortex sends an excitatory input to CA1, illustrating that the DH does indeed receive an overlap of information about CS and US prior to the amygdala[247]. Notably, lesions made to DH prior to contextual fear conditioning do not necessarily impair the process[248–250], suggesting that all the individual elements of the stimulus are capable of reaching the amygdala independently of the hippocampus and this is sufficient for learning to occur. However, the efficacy of contextual fear conditioning is positively correlated with the amount of time the animal is given to explore the context prior to receiving shock; that is, animals with hippocampal damage do not encode contextual cues as efficiently as animals with intact hippocampi[251,252]. This may explain why some early lesion studies incorrectly concluded that the DH is not involved in contextual fear conditioning.

Overall, in terms of contextual fear conditioning, the DH seems to enable the inclusion of elements of the environment (e.g. contingencies, spatial locations, temporal dynamics) to the memory trace, leading to a richer and more salient memory[253]. Given that only one of the experimental paradigms in the SOC project directly utilized context as a CS (Experiment 4), higher activation in CA1 and DH CA3 was expected in this paradigm only (Figure 2D).

1.3.5.2. Ventral hippocampus

Olfactory, visceral, and gustatory signals are sent preferentially to VH via the medial band of the EC[216]. In contrast to the DH, the VH sends direct projections to both the MOB and AOB with significantly denser terminals in the AOB, along with the PC and other olfactory areas[214]. Ventral CA1 also sends direct projections to the central nucleus of the amygdala, and the VH receives dense inputs from both LA and BLA[254,255]. The VH has been implicated in anxiety[8,256–259] and anxiety-like behaviour is actually mediated by a monosynaptic glutamatergic projection from BLA to VH[260,261], making VH a likely target to play a role in fear learning[262].

Lesions in the most ventral quarter of the hippocampus caused a decrease in anxiety-related behaviours and a decreased release of corticosterone in response to a confinement stress[8]. Interestingly, temporary or permanent inactivation of VH neurons block tone fear but have varied effects on contextual fear[237,262,263], while NMDAR antagonists in the VH block the acquisition of a context fear but not fear to an aversively

conditioned tone[264]. Using a temporal-order discrimination task, Hunsaker found that VH lesions produced more pronounced effects if the conditioned stimulus was an odor instead of an auditory or visual stimulus[237]. The amygdala receives direct hippocampal input from VH, not from DH, which is evidenced by their anatomical proximity, and it has been suggested that this may account for the varied results in fear conditioning studies, as VH lesions cut off input to the amygdala from both the DH and VH[265].

Historically the DH has received more attention than the VH. Given the evidence described above regarding the role of the VH in fear learning, VH participation in the engram complex was expected across all three experimental paradigms in the SOC project.

1.3.6. Amygdala

The amygdala has been implicated in assigning emotional significance or valence to sensory stimuli for over half a century primarily based on lesion studies. In particular, the classic studies of Klüver and Bucy indicated that lesions to the medial temporal lobe of monkeys caused significant changes in emotional behaviour[266,267], and this technique was further refined to include just the amygdala and avoid other medial temporal lobe structures such as the hippocampus and surrounding cortical areas[268].

Heterogeneity exists in the accepted nomenclature for some amygdalar subnuclei. Many studies refer to the BLA complex as a combination of the lateral, basal (sometimes called basolateral), and basal medial amygdala. This thesis does not use any collective

terms to refer to these, instead it describes two distinct sub-nuclei – the LA and the BLA (LA and BLA; see Figure 2D), the latter is broken down further in the pheromone project into anterior and posterior BLA (aBLA, pBLA; Figure 2D), but only aBLA was measured in the SOC project.

1.3.6.1. Lateral amygdala

The LA and BLA contain a mix of two non-overlapping cell populations, glutamatergic pyramidal-like neurons and GABAergic interneurons which constitute 85% and 15% of cells in these areas, respectively[269,270]. The LA receives information from sensory cortical areas, such as the auditory and piriform cortex as well as thalamic nuclei related to sensory processing. It sends information to the BLA as well as the CeA which constitute integral parts of the fear conditioning circuit (Reviewed by Maren[11]).

The lateral amygdala has been the focus of fear conditioning studies for a very long time, and results for auditory conditioning are often generalized and are reported as mechanisms underlying Pavlovian fear conditioning. This is reflected by the fact that both *in vivo* studies and IEG studies have shown that between 10 and 40% of LA neurons are activated by fear conditioning and are reactivated when the memory is recalled[135,211,271,272], but this is true specifically for auditory fear conditioning. Many highly cited review articles on the topic of fear conditioning published in prestigious journals report these findings as "fear conditioning" instead of "auditory fear conditioning" which is misleading as the BLA is heavily involved in both odor and contextual fear conditioning[124,273,274]. In fact, a recent

study showed that auditory cortex neurons synapse onto LA interneurons, but not BLA interneurons, highlighting the importance of the LA in auditory conditioning as well as the diversity between these two areas[275].

Nonetheless, the role of the LA in auditory fear conditioning has been cemented in the literature as the site at which auditory CS information and US information converge, evidenced by enhanced plasticity following fear conditioning[205,211,276] and suppression of auditory fear conditioning when NMDARs are blocked here[277,278]. Specifically, GluN2B containing NMDARs are involved in the acquisition of fear learning, but not memory consolidation[279]. The same group showed that CaMKII phosphorylation, a step downstream from NMDAR activation, is crucial for the acquisition but not expression of fear memory[280]. Another downstream effect of Ca^{2+} entry through NMDARs is phosphorylation of cyclic AMP-dependent protein kinase A (PKA) via activation of CaMKII which has shown to be crucial for auditory fear memory consolidation[281]. Importantly, Arc protein is increased in the LA following recall of an auditory fear memory and knockdown of this protein impairs fear memory reconsolidation processes[282]. Many structures in the fear conditioning and olfactory processing network utilize GABAergic interneurons to modulate neural firing and gate learning or consolidation, and the LA is no different[283–286]. Most LA interneurons receive input from both auditory thalamus and cortex, reflecting again the tight control of this region by inhibitory networks which utilize both feed-forward inhibition and disinhibitory control[287,288].

Since the LA had been highly cited as a required structure for CS-US associations, it was included in both projects as a candidate for engram inclusion. Given their high degree of inter-connectedness it was also of interest to separate the contribution of the LA and BLA in different types of odor fear learning, hence them being treated as separate structures and not simply as the BLA complex (see Figure 2D).

1.3.6.2. Basolateral amygdala

Like the LA, the BLA contains a similar mix of glutamatergic pyramidal neurons and GABAergic interneurons[269,270]. The BLA contains reciprocal projections with VH CA1[274], the LA, and the CeA[289]. It also receives projections from the PC, MOB, and the AOB. It has already been pointed out that caution needs to be exercised when interpreting results from the BLA, as differences in nomenclature and classification as well as a failure to accurately name the type of learning being probed can lead to improper conclusions about the homogeneity of LA and BLA. Research discussed in the following section was therefore chosen from authors who explicitly defined the LA and BLA subnuclei (regardless of specific nomenclature) rather than referring to the "BLA complex."

Importantly, lesions of the BLA have no effect on fear conditioning to a tone, meaning that the projection from LA to CeA must be sufficient to encode an auditory fear memory[290] while the BLA participates in both odor and contextual fear conditioning[291]. Colloquially, the amygdala tends to reflexively produce thoughts of aversive conditioning but the BLA participates in both aversive and appetitive learning. An elegant study used an

optogenetic strategy whereby neurons expressing *cFos* a short time after contextual fear conditioning were tagged with light sensitive channels so that these engram cells could later be activated or inhibited experimentally. They examined engram cells in the DG and the BLA and reactivated engram cells in the presence of a female mouse, which is typically rewarding for a male conspecific. Artificial activation of the DG engram during reward training was able to elicit a switch in the conditioned response from aversive to appetitive, while the same process in the BLA did not produce any change in aversive behaviour, suggesting that different neurons in the BLA encode appetitive and aversive memory[143]. Indeed, two years later the same group reported two spatially segregated and genetically distinct subpopulations of excitatory BLA neurons that are connected through mutual inhibition and reflect opposite states of valence. The study found that one subset of neurons were preferentially located in the aBLA and participated in the encoding of aversive contextual and odor memories, while the other subset of neurons were found in the pBLA and were involved in learned appetitive behaviours[292]. Importantly, optogenetic activation of each of these genetically distinct neuronal populations in the presence of a stimulus was sufficient to generate a conditioned response with its respective valence. The importance of lateral inhibition is once again highlighted in this fear conditioning region; aBLA neural activation in turn inhibits neurons in the pBLA directly through interneurons. This intra-BLA circuitry enables stimuli to be encoded with respect to valence in a binary fashion, either positive or negative[292].

Odor valence assignment is also dependent on firing patterns of the locus coeruleus (LC), a brainstem structure which sends the majority of NE to many areas of the brain

including the BLA. NE plays a large role in learning and memory, and produces differential effects depending on which receptor subtype is activated. Tonic and phasic firing were associated with activation in BLA-CeA pathway and the BLA-NAc pathway and correlated with aversive and appetitive behaviours respectively[293]. Both tonic LC activation and aversive conditioning were dependent on β-adrenoceptor activation in the BLA, while phasic LC activation and appetitive conditioning were dependent on activation of both β-adrenoceptors and α_1-adrenoceptors[294]. Considering the β-adrenoceptors have the lowest affinity for NE and tonic stimulation would likely cause a large degree of NE release, this suggests that aversive conditioning either requires a larger amount of NE or that aversive conditioning is preferentially engaged when a large amount of NE is present in the system.

Given that the BLA receives contextual, sensory, and pheromonal cues, and its anatomy supports physical separation of information from opposing valences, it is well suited as a site of convergence for the association of CS and US in odor fear conditioning, possibly mirroring the role of the LA in terms of auditory conditioning.

1.3.6.3. Central amygdala

In contrast to the LA and BLA, the CeA contains predominantly GABAergic medium spiny neurons, and while there are clearly defined subdivisions and stereotyped activity within them, the amount of inhibition involved to allow this crucial nucleus to perform its function can make understanding the circuitry difficult. The CeA is further

divided into four main regions: the lateral, medial, capsular, and intermediate (CeL, CeM, CeC, CeI).

Specific inactivation of the CeM interferes with the expression of fear memory, but not its formation[295], leading to the idea that CeL but not CeM activity was crucial for fear learning. This notion has been challenged by evidence showing that fear conditioning increased CeM responsiveness to a CS with no observable increases in CeL firing[296]. However, when CeL neurons were evaluated individually it was revealed that equal proportions actually changed their intensity of firing but in opposite directions; CeL+ cells (also called CeL_{ON}) exhibited excitatory CS-evoked responses while CeL- cells (also called CeL_{OFF}) were more inhibited in response to the CS. Interestingly, the following day the proportion of CeL- cells tripled with no change in the number of CeL+ cells, pointing towards overnight synaptic plasticity in an inhibitory projection to CeL- cells. Finally, the authors propose a model by which the suppression of CeL- neural firing from an unidentified inhibitory circuit leads to disinhibition of CeM neurons and activation of areas in the brain which control the physical aspects of freezing behaviour[296]. Within the CeL_{ON} and CeL_{OFF} cell populations, an extreme amount of heterogeneity exists and is not completely understood (reviewed by Fadok[297]).

The CeL receives varied inputs from various amygdalar nuclei, VH, thalamus, and cortex and sends inhibitory projections to the CeM. If the delicate balance of activity in the CeL leads to disinhibition in the CeM which projects to the periaqueductal grey (PAG) this could reasonably gate freezing behaviour (reviewed in Li[298]). The CeM was chosen as a

candidate for engram inclusion in the pheromone study as it is the final output structure of the amygdala in aversive fear conditioning and its intrinsic circuitry is simpler in comparison to CeL. If alarm pheromones represent highly biologically relevant and salient aversive unconditioned stimuli and the behavioural response is the same in animals trained this way or with shock, it is possible that the brain could utilize distinct circuitry to encode these two types of memory depending on the US.

1.3.6.4. Medial amygdala

The medial amygdala (MeA) is a structure with a large input from the VNO via the AOB and possesses a high degree of synaptic plasticity[299]. Early studies, based on the evidence that the MeA is critical in processing pheromonal signals[300,301], illustrated that AOB lesions can cause a reorganization of synaptic connections in the MeA[171,302–304]. Additionally, the Mori lab measured cFos immunoreactivity to direct exposure to a rat alarm pheromone and showed increased expression in the LA, BLA, and MeA[305]. It has yet to be determined if the MeA would participate in an odor fear memory engram if the alarm pheromone acts as an unconditioned stimulus, hence its inclusion as an engram candidate in the pheromone project.

1.4. Why is finding the engram complex so important?

Intellectual and technological advances alike seem to have put memory mapping perfectly within our grasp, at least within laboratory animals. What benefit does it confer to understand these systems? Typical species-centric humans may ask, how is this going to help ME?

1.4.1. Post Traumatic Stress Disorder (PTSD)

Earlier this year, it was reported that while 5% of Canadians have PTSD diagnosed by a medical professional, 8% meet the criteria for probable PTSD based on symptoms they have experienced in the month preceding the survey[306]. Almost twice as many women meet the criteria for probable PTSD than men (10% vs 6%). Interestingly, young adults between ages 18-24 are four times more likely to meet the criteria for probable PTSD than seniors aged 65 or older[306]. In terms of a diagnosis, the criteria for PTSD have changed over the years owing partially to the fact that its pathophysiological mechanisms have not yet been identified. A general consensus of four major symptom clusters categorizes PTSD (as described by the DSM-5): *experiencing*, which includes repeatedly re-experienced memories, dreams, or flashbacks associated with the trauma; *avoidant*, which includes avoiding cues involved with the trauma to avoid discomfort; *arousal,* which includes displaying excess arousal in response to cues or in other ways like disordered sleep; and *negative cognitions and mood*, including feelings of blame, estrangement from family, and inability to remember key aspects of the traumatic event[307]. One of the most replicated scientific findings in PTSD is that the NE system is dysregulated, NE is increased in

serum[308] and cerebrospinal fluid[309] following exposure to trauma cues. Altered functioning of the stress axis despite normal or even decreased levels of circulating stress hormones[310] has also been demonstrated. PTSD patients are thought to be excessively reactive to stimuli, situations, and stressors that are linked to a traumatic event or series of traumatic events.

The past two and a half years have been referred to as a "collective trauma," a term that refers to the psychological responses to a traumatic event that affects entire groups of people, societies, or communities[311]. Pandemics are among the causes of collective trauma[312], and we have witnessed first hand the emotional, financial, social, economical, racial, and political problems that surfaced throughout the course and as a result of the current Covid-19 pandemic. In September 2020, a few months into the covid-19 pandemic, a population of adults in Bangladesh displayed anxiety at levels 10 times those reported as the national anxiety rate in 2019[313]. Social media grants us access to anything happening at any given time anywhere in the world, as well as a platform for every person on earth to say or do anything they want. Just opening Twitter puts you at risk of reading about or seeing an image or video of war, climate disaster, political scandal, nonsensical laws with severe implications for fellow human beings, mass shootings at elementary schools, hate crimes, police brutality, etc. The sheer number of aversive stimuli we are exposed to daily could plausibly lead to problems with fear circuitry in a very large percentage of people.

Repeated exposure to upsetting news causes a phenomenon called "vicarious traumatization[314]." Self assessments of acute stress and posttraumatic stress symptoms increased in severity with repeated exposure to graphic images following the Boston

Marathon bombings; specifically, fear for future terrorism and functional impairment in areas of social- and work-related functioning were positively correlated with frequency of exposure to bloody images[54]. About 15% of psychologists who were repeatedly exposed to covid-19 related stimuli by their clients experienced high levels of vicarious traumatization[314]. It is startling to see the effect in a population of psychologists, considering their profession inherently deals with trauma and they are experts. One in five Canadians who met the criteria for probable PTSD reported that the worst event they experienced happened in the last two years and one in ten stated that it was directly pandemic-related[306]. It is well documented that symptoms of PTSD can develop weeks, months, or even years following a traumatic event. Collective trauma research regarding the covid-19 pandemic is still accumulating, and naturally will become more understood over the coming years[306]. Considering the steadily rising rates of PTSD[306] and our ever-increasing access to information combined with the fact that we are still living through a pandemic, now more than ever it is imperative to understand how complex, aversive, traumatic memories are encoded so that we may open the doors to effective therapeutic interventions.

1.4.1.1. Current PTSD treatments

Exposure therapies are considered the gold standard for the treatment of PTSD, and they are based on the notion that memories become 'labile' or subject to modification following their recall, a process termed reconsolidation[315]. Two major therapeutical exposure techniques have been employed in PTSD treatment: narrative exposure therapy

(NET) and prolonged exposure therapy (PE). PE is the most studied psychotherapy for PTSD[316]. Its main goal is to provide safe confrontation with trauma cues (e.g., thoughts, memories, places, activities, people, etc.) that have been avoided since the experience of the trauma[317]. PE prevents negative reinforcement of the traumatic cues by avoidance, and forces confrontation of traumatic memories. The main goal is to enhance the emotional processing of these traumatic memories in order to reduce PTSD symptoms[318]. Despite the recorded success of more than a dozen randomized controlled trials, 25-45% of PTSD patients still met diagnostic criteria after treatment[319]. NET, on the other hand, emphasizes that PTSD is a consequence of memory and memory storage alteration from a traumatic event[320], and that there is impaired distinction between declarative and non-declarative memories[321], and between semantic and episodic memories[322]. Non-declarative memories are difficult to verbalize, but may be triggered by sensory cues or internal emotional and psychological states. In NET, the main goal is to integrate traumatic events into the patient's whole life story, creating a narrative that is coherent and importantly includes their trauma. A recent paper which has compared the two techniques suggest that PE may be more efficacious in treating PTSD which has occurred from a single traumatic event, while NET is best suited for victims of complex trauma who have developed PTSD[318].

Eye movement desensitization and reprocessing (EMDR) is another therapy that has been applied to PTSD, but this technique has been widely mocked and criticized by the scientific community. Understandably the skepticism mostly arose from the original rationale, that rapid horizontal eye movements can catalyse a rebalancing of the nervous system, enabling the dysfunctionally "locked" information to be released from the nervous

system[323]. Even more skepticism focuses around the lack of understood mechanism of why this therapy works, although it certainly does and is among the most effective treatments for PTSD[324]. The essential technique of EMDR entails the patient describing details of traumatic experiences to their therapist while following a circle on a screen with their eyes which can move either horizontally or vertically. The main mysticism surrounding EMDR comes from the fundamental question: do eye movements matter? Conflicting observations have been reconciled by meta-analyses, and it has been suggested that eye movements have an additive effect in reported symptom relief[325]. How does eye movement during recall of a traumatic memory have the capacity to change the memory while simple recall does not? Perhaps because the eye movement itself is not important, but engaging the hippocampus with working memory is the crucial component. Recalling an emotional memory and tracking a point on a computer screen are both tasks which require working memory, and when two tasks which require working memory are completed simultaneously, they compete for its limited capacity[326]. The act of a person creating a vivid and detailed image in order to recall it to someone else can influence the original memory and in turn make it more vivid and realistic, called the 'imagination effect'[327] which is a notorious problem in police interrogation. The inflation of the imagination of the event can affect the next recall. In terms of working memory, EMDR could therefore cause imagination deflation and cause memories to become less vivid and realistic. It seems that if any task which engages working memory (and by extension, the hippocampus) is performed at the same time as recall of a traumatic memory, the memory can be modified if the task is not too easy or too hard[328]. A systematic review has recently stated that growing evidence supports the use of EMDR to treat trauma in both children and adults[329]. There are, of course, downsides to

EMDR as well. It is emotionally taxing, and involves patients describing horrific traumatic experiences in detail over long periods of time. Patients need to have a great deal of trust in their therapist to complete the therapy the full way though, which is evidenced by the large degree of variation in treatment length seen in many of the randomized controlled trials[329]. EMDR is in essence an exposure therapy, which integrates working memory tasks to modify the memory and reduce its potency for eliciting a fear response.

Overall, it seems like the most efficacious treatments for PTSD are those which: 1) expose the patient to trauma-relevant cues in order to reduce the negative reinforcement of avoidance, 2) allow for integration of traumatic experiences into the context of a person's life, and 3) tax working memory during recall of traumatic memories to impair reconsolidation processes, thereby reducing their vividness and potentially replacing them with other stimuli entirely. If a new therapy could be developed which enabled activation of these traumatic memories without conscious effort to remember unclear details, perhaps those memories could be altered if they were "reactivated" by sensory cues, instead of the retelling of the experience itself.

1.4.2. Higher order conditioning holds potential to inform human pathological fear learning

In order to translate results from animal behaviour into meaningful discoveries in humans, the behavioural paradigm must be as representative as possible to the types of learning that humans themselves participate in. The idea that SOC could represent a common avenue of human memory contributing to disorders was posited two decades ago,

when Gewirtz and Davis proposed that SOC may provide an animal model for human panic disorder[6]. Panic attacks may become linked to the contexts in which they occur and can lead to agoraphobic behaviours to avoid the uncomfortable symptoms of panic attacks. The authors highlight the potential value of higher order conditioning, citing that human learning likely does not regularly involve the direct pairing of objects with unconditioned reinforcers or punishments, and it is more likely that reinforcement or punishment of a behaviour is likely contingent upon something that has previously been learned rather than something that has intrinsic positive or negative valence[6]. For example, a gun can act as a stimulus that induces a fear behaviour in humans, but a gun in and of itself is not inherently negative, it is just plastic and metal; the salience of the stimulus is so high because of the learned association between guns and death and/or injury and is hence already a conditioned stimulus. Further, humans do not ever need to be in the room with a gun to know it is dangerous; we can learn directly through communication with other humans who have directly experienced a situation with a gun, or even vicariously through sources of media. Therefore, social communication of fear and second-order conditioning of fear likely represent common sources of human fear memory.

Aversive SOC studies in humans, specifically in PTSD patients are extremely rare, but one group did just that by using a trauma-specific image as the "acquired" CS1[59]. The idea here is that the punishing stimulus is a learned one, and is directly associated with the traumatic event in question. Therefore, participants can form an association between the CS1 and a neutral CS2. They used three groups; trauma-exposed subjects who were diagnosed with PTSD, trauma-exposed subjects who did not have PTSD, and healthy

controls who had not experienced any trauma nor were diagnosed with PTSD. Subjective rating of the CS, electroencephalogram (EEG) from 30 channels, skin conductance responses, heart rate, and electromyography (EMG) measuring startle response were recorded throughout the experiment. It was revealed that both groups who had experienced trauma displayed enhanced conditionability to trauma-relevant cues compared to controls. The study also revealed that compared to trauma survivors without PTSD, those with a formal diagnosis demonstrated a stronger predictive relationship with the CS2 as a danger cue, meaning that PTSD patients are very good at integrating previously neutral cues in an aversive associative memory chain as they are more likely to predict a threat. An additional aspect of aberrant fear processing in PTSD was shown by their inability to extinguish the conditioned response to the CS2 during repeated exposures in the absence of trauma-reminder CS1[59]. Collectively these results show that people who suffer from PTSD display increased conditioned responses to a higher-order conditioned cue and that these responses are resistant to extinction.

Understanding the fundamental aspects of this type of higher-order conditioning, whereby a fear association is created between a new conditioned stimulus and a previously learned conditioned stimulus is critical to understand the extent to which this occurs in humans and the implications it could have on aberrant fear processing. Depending on the severity and nature of the initial unconditioned stimulus (the traumatic event), this could potentially present as PTSD, social anxiety, or even generalized anxiety disorder (GAD). If a person keeps experiencing triggers without consciously being able to trace them back to a specific traumatic event or moment in time, attempts to predict and mitigate these

triggers are largely in vain. Animal models of higher order memory, such as those produced by second-order and social learning, are potentially valuable tools for elucidating the mechanisms of this type of memory, so that future treatment strategies can be directed in the least traumatic and most efficient way possible. Discovering how the engram complex for a specific odor (terpinene) changes depending on if it was conditioned with an inherently negative stimulus (shock/pain), an innately negative stimulus (alarm pheromone), or two different learned negative stimuli (context or tone), will provide important insight on how the fear conditioning systems in the brain encode different types of fear that elicit the same conditioned response (in other words, the responses look the same), depending on how they were conditioned.

Chapter 2 – Methods

2.1. Pheromone conditioning

2.1.1. Animals

Sprague Dawley rats (8–10 weeks old, weight 200–300 g, in good health) of both sexes (n = 110 total) were assigned randomly to groups. Rats were housed in polycarbonate cages on a 12hr light/dark cycle, given ad libitum access to food and water, with all behavioural manipulation completed during the light phase of the light cycle. Odor + shock conditioned rats (O^+/S^+) were housed with same sex companion rats one week before the experiments and for the duration of the experiments, while other groups were housed alone to exclude any social interaction effects in control groups. All procedures were approved by the Memorial University Institutional Animal Care Committee and carried out in compliance with the guidelines of the Canadian Council on Animal Care.

2.1.2. Experiment 1: Stressed cage mate-induced odor associative learning

2.1.2.1. Behavioral study

2.1.2.1.1. Odorants

Odorants were diluted with mineral oil to specific concentrations. Odorants used were terpinene (6.63%) and octanol (2.67%). These odorants were chosen as they are neither innately appetitive nor aversive to adult rats, and the concentrations were chosen so that the odors would emit a vapor-phase partial pressure of 1 Pascal[330].

2.1.2.1.2. Apparatus

All behavioural training and testing was completed with a custom-made olfactometer for air and odorant delivery attached to the shock chamber: a plexiglass chamber that sits on top of an electrified grid, connected to a shock generator/scrambler (Muromachi Kikai Model SGS-003DX). Polyvinyl carbonate bottles were used for each odor and connected to the olfactometer by C-flex tubing pinched shut when not in use. Evacuation tubing with a fan was attached to the top lid of the shock chamber to promote odor removal.

2.1.2.1.3. Odor conditioning and testing

All rats were habituated to the shock chamber for one 30 min session each on two consecutive days with clean air pumped through the shock chamber. On the third day, rats were trained individually with four separate exposures to either odor, shock, or odor and shock, depending on their respective groups at 5, 15, 20, and 30 min during a 30 min training session. Odorant (terpinene) was delivered for 1 min at each time point. Shock was delivered at the last sec of the odor delivery (0.5 mA for 1 sec) such that they co-terminated. Between each experiment, the shock chamber and grids were thoroughly cleaned with 70% ethanol and clean paper towels. There was a 15 min interval between the chamber cleaning and next experiment, where residual smell and ethanol were removed via the evacuation tubing. The fourth day consisted of a 30 min behavioural testing session in the same conditioning chamber. Medical air was delivered in the first half of the session and an odorant was delivered in the second half. Rats were tested with terpinene and octanol (a control odor) on the same day, and the order of the odorant testing was randomized and

counterbalanced. The percentage of freezing time in response to the terpinene and octanol exposure was measured in real time with a stopwatch and calculated as a percentage of the total first 5 minutes of both odor delivery and medical air exposure.

2.1.2.1.4. Groups

O^-/S^+, rats were housed alone and received shock only, no odor during the experiment; O^+/S^-, rats were housed alone and received terpinene odor but no shock during the training; O^+/S^+, rats received both terpinene odor and shock and were pair-housed with O^+/Comp (companion) rats exposed to terpinene only during the training. O^+/S^+ rats were returned to the cages with O^+/Comp rats immediately following the odor/shock conditioning. Ten minutes later, O^+/Comp rats were subjected to the odor only training. O^-/Comp rats were housed with the O^+/S^+ rats in the same way as the O^+/Comp animals described above, but were not subjected to the odor only training. A subset of the O^+/Comp rats received saline (50 µl, i.p.) during the habituation and 40 min before the training. O^+/Comp + Prop rats received saline during the habituation and propranolol (20 mg/kg, i.p.) 40 min before the training.

2.1.2.1.5. Anxiety testing

Anxiety was tested with a wooden elevated plus maze comprised of four 50 cm long and 10 cm wide sections (two open and two closed) merging in a 10 cm square piece at the center. Closed arms were surrounded by a 40 cm wall. The maze was placed 50 cm above the floor and a video camera was fixed above the maze to record movements for analysis. Rats were positioned in a closed arm and allowed to roam freely for 5 min. The number of

arm entries and time spent in the open vs. closed arms were calculated. Time in the closed arms was used as an index of anxiety-like behaviour.

2.1.2.2. Neural circuit mapping

A separate cohort trained identically to groups 1–4 as described above underwent tissue collection for *Arc* and *H1a* mRNA visualization on the 4th day. Animals were given a final odor exposure in lieu of behavioural testing. Rats were placed in a sealed plexiglass container ventilated with a continuous flow of charcoal-filtered air for 1.5 hrs. Rats were then given a 5 min exposure to octanol, another 20 min of charcoal-filtered air, then a 5 min exposure to the conditioned odor, terpinene, followed by immediate isoflurane anesthesia and decapitation. Brains were collected and flash frozen in 2-methylbutane immersed in an ethanol/dry ice slurry and kept at −80°C.

2.1.2.2.1. Tissue processing

Brains were trimmed so that the cerebellum was discarded, and only the right hemisphere was analyzed. The right hemispheres of rats from each behavioural group were arranged so that the rostral end of their olfactory bulbs touched a razor blade to align them at the same rostral-caudal level. Brains were then arranged in a custom-made plastic box filled with Optimal Cutting Temperature (OCT) medium (Tissue-Tek) at −20 °C in a cryostat and frozen in a block (Figure 3). Coronal sections of 20 μm were collected on 2% 3-aminopropyltriethoxysilane treated slides. Five representative slides over the rostral-caudal range of each of the MOB, AOB, aPC/tubercle, and pPC/amygdala were chosen for FISH and stored at −20 °C.

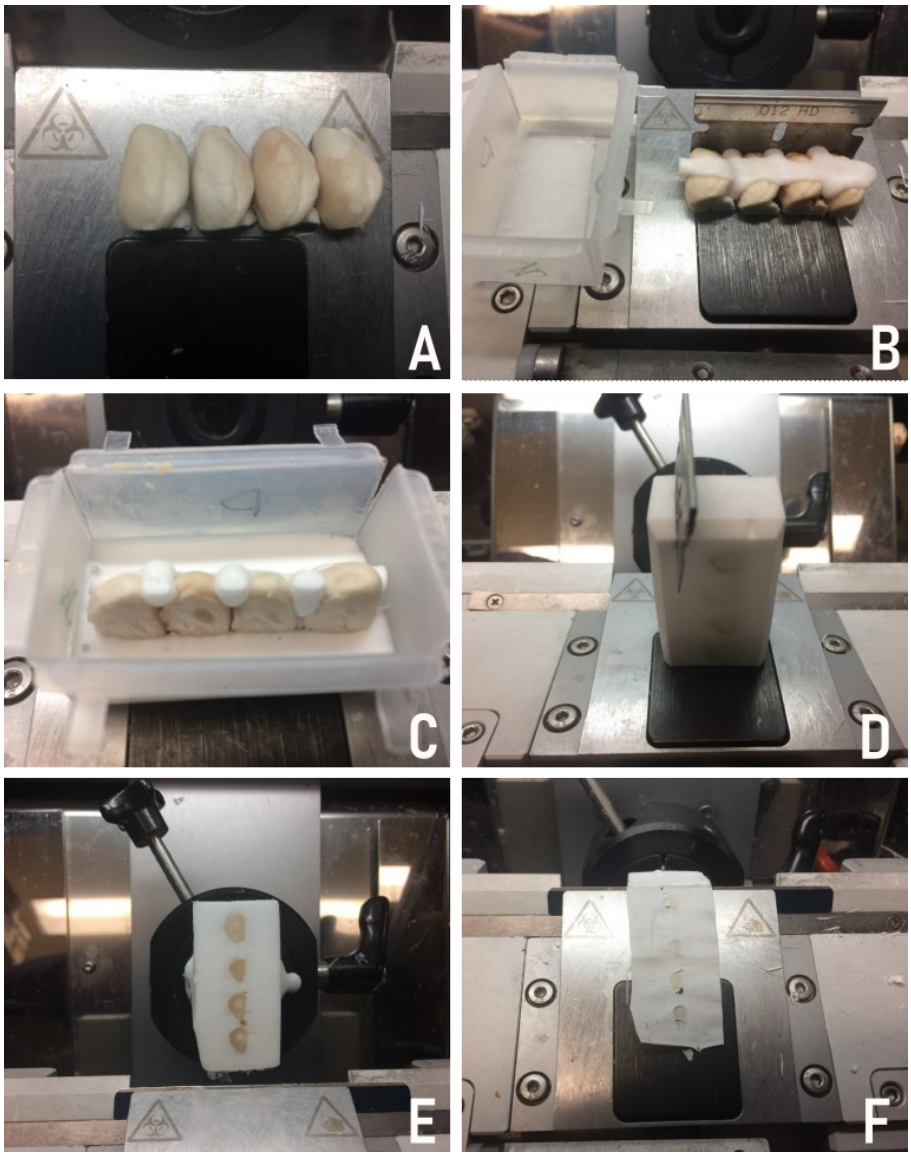


Figure 3. ***Preparation of slides for Arc+H1a catFISH.*** **A)** Right hemispheres from 4 different behavioural conditions were trimmed to include areas of interest (in this case, olfactory bulbs and cerebellum were removed). **B)** Brains are aligned using the edge of a razor blade and a layer of Tissue-Tek is added to lock them in the correct position. **C)** A thin layer of Tissue-Tek is added to the small plastic rectangular container and the hemispheres are aligned such that when the block is sliced the sections will be coronal. The entire box is filled with Tissue-Tek and allowed to freeze into a solid block. **D)** The frozen block is trimmed to minimize the size and ensure fit in the cryostat. **E)** The block is mounted to the cryostat specimen holder and the angle is adjusted to attempt similar antero-posterior levels for each hemisphere. **F)** Slices are applied directly to pre-chilled coated slides and kept inside the cryostat until sectioning is complete, at which point they are moved to the -80^{O}C freezer.

2.1.2.2.2. Fluorescence *in situ* hybridization

The double FISH protocol was established previously[115]. Briefly, full length *Arc* riboprobes conjugated to digoxigenin and *H1a* riboprobes conjugated to fluorescein were obtained using commercial transcription kits (Maxiscript) and RNA labeling mixes (Roche). Riboprobes were purified using RNA mini quick spin columns (Roche) and verified via agarose gel. Slides were thawed for 30 min at room temperature, fixed in 4% paraformaldehyde at 4°C, bathed in acetic anhydride and 50/50 acetone/methanol (Fisher Scientific), and treated with pre-hybridization buffer and hybridization buffer (Sigma-Aldrich) containing *Arc* and *H1a* probes. Slides were hybridized overnight in a 56°C oven. All steps until this point were performed in the absence of RNAse. Slides were washed in a series of sodium citrate solutions followed by cleavage of any remaining single-stranded RNA using RNAse A. Endogenous peroxidases were quenched with H_2O_2 and slides blocked with 5% sheep serum (Sigma-Aldrich). *Arc* riboprobe was detected with anti-digoxigenin-peroxidase (DIG-POD; Roche) and a TSA cyanine-3 substrate kit (Perkin Elmer). Following *Arc* detection slides were dipped in 2% H_2O_2 solution to quench any residual HRP activity. *H1a* riboprobe was detected with anti-fluorescein-POD (FLU-POD; Roche) and a TSA Fluorescein Tyramide substrate kit (Perkin Elmer). Nuclei were counterstained with 1:1000 4′,6-diamidino-2-phenylindole for 30 minutes (DAPI; Sigma-Aldrich). Slides were coverslipped with Vectashield antifade medium (Vector Laboratories), sealed with clear nail polish, and kept at 4 °C before confocal microscopy scanning.

2.1.2.2.3. Image acquisition and analysis

All slides were scanned on a Fluoview FV1000 confocal microscope (Olympus). All images were taken at 20X magnification. The photomultiplier tube assignments, confocal aperture size, and contrast remained constant for each slide. The z-stacks (optical thickness: 1.0μm) were taken throughout the thickness of the section and were acquired from 3–4 slides for each animal. The mitral cell layer was analyzed in the olfactory bulbs, including the dorsolateral and ventral medial regions in the MOB. Layer II was analyzed in the PC, and the dense cell layer was analyzed in the OT. Images were analyzed from the center of each of the amygdala subdivisions.

ImageJ software was used for counting cells in the scanned images. In all areas except the OBs total cell counting was done automatically for the DAPI stained nuclei; images were cropped to include only the area of analysis, transformed to binary images (black and white), and cells were counted using the "Analyze Particles" function in ImageJ. For the *H1a*+ and *Arc*+ cells, counting was done manually over 20% of the mid-range of the stack that comprised each cell. Average cell counts of *Arc*+ cells were divided by the average cell counts of *H1a*+ cells to compute a ratio of cells active to the conditioned odor versus cells active to the control odor for each animal.

2.1.2.3. Protocol optimization

2.1.2.3.1. Eliminating behavioural testing prior to sacrifice

Preliminary experiments included behavioural testing on the day prior to sacrifice, but since the animals received an additional exposure to the CS with no US, I hypothesized that extinction of the memory could occur when exposed to the CS prior to sacrifice. I decided to implement an additional cohort of animals that were not tested for freezing behaviour in response to the CS. The vast majority of optimization of the staining occurred in brains from the original cohort, for which behavioural results were recorded.

2.1.2.3.2. Brain processing

Brains were immediately removed following sacrifice, flash frozen in a cold 2-methylbutane solution, and stored in a -80ºC freezer until they were processed. Prior to cryosectioning the brains were trimmed and cut to separate the cerebral hemispheres. When brains were taken directly from the -80ºC freezer they would either crack during trimming or the sections would tear on the cryostat blade. After confirming that it would not affect the staining quality, brains were removed from the -80ºC freezer and placed in the -20ºC freezer the night before they would be cut on the cryostat. Allowing the brains to gradually come up to the temperature of the cryostat drastically improved tissue quality and this was maintained throughout all experiments.

To be able to compare conditions directly to one another, it was imperative that all groups were represented on a single slide, as each group on the slide would be subject to

the exact experimental conditions (e.g., solution volume, concentration, wash time). Since the brains were flash frozen, it was not possible to slice each brain separately – either the mRNA was destroyed by heat from the slides being kept out of the cryostat, or if slides were kept in the cryostat they need to be warmed to collect each section so the other sections already on the slide experienced repeated freeze/thaw cycles which severely damaged tissue quality. Therefore, it was imperative to slice the brains simultaneously. This posed a unique challenge: to compare groups in the same brain area, the brains being cut together must be at the same (or very similar) antero-posterior position. This obstacle was overcome by developing the protocol depicted in Figure 3. A detailed map was recorded to ensure the identity of each brain was maintained throughout the cutting process. Brains were blocked to remove the cerebellum, hemisected, right hemispheres lined up along the edge of a razor blade to ensure the cortex of each brain was aligned (during freezing olfactory bulbs end up in a much more variable position than the cortex, so the standard was always the beginning of the cortex. This is depicted in Figure 3A with one discrepancy: brains were blocked to exclude OBs in the image, but all brains sliced for Experiment 1 did include OBs as reflected in the text. Tissue-Tek was poured on the lateral surface to lock the brains in the correct position, and this was left to freeze for about 3 minutes (Figure 3B). Tissue-Tek was applied liberally to the bottom surface of a small, flexible, plastic rectangular box deep enough to fully encase all of the brains. The brains were placed inside the box and adhered to the Tissue-Tek on the bottom (Figure 3C), and Tissue-Tek was poured into the box gradually, freezing one layer at a time, until the brains were fully encased in a rectangular block of frozen Tissue-Tek. To remove excess Tissue-Tek and to ensure the block fit on the brain, and that each pass of the cryostat would yield a slice with each brain

hemisphere at the appropriate size to fit on a glass microscope slide, the edges of the brain block were trimmed (Figure 3D). The block was then mounted to the circular cryostat tissue holder with Tissue-Tek (Figure 3E), and a large heavy weight was applied during freezing as the weight of the brain block would often cause it to fall off the mount if it was not adhered tightly. The most challenging aspect of slicing the brain block was choosing the correct angle so the brains were sliced simultaneously, and each hemisphere was sliced at a similar neuroanatomical level for direct comparison. If angles had to be adjusted after slicing had commenced, a thin layer of Tissue-Tek was added to the brain block to preserve as much tissue as possible. Sections of 20μm thickness were collected on 2% 3-aminopropyltriethoxysilane treated slides and stored at -20ºC until used for catF*ISH.*

2.1.2.3.3. Establishment of *H1a* and *Arc* double labelled catF*ISH*

Assays that utilize *ISH* pose unique challenges compared to IHC, as they target mRNA instead of more stable proteins. Prior to the current project, catfish experiments in the lab targeted *Arc* only – and cytoplasmic versus nuclear staining were analyzed, as the staining protocol and potential errors were minimized. However, comparing cytoplasmic versus nuclear staining is difficult when analyzing areas with very densely packed neurons (e.g., MOB), and it is tricky to discern between background and cytoplasmic staining. We therefore sought to add an additional gene target with different temporal dynamics in order to evaluate nuclear staining only, as it is much more clearly defined and can be stained with an alternate color. *H1a* interacts with proteins at the postsynaptic density[331] and is dramatically upregulated following exploration of a novel environment[115]. *Arc* and *H1a* display coincident expression in the same neurons depending on experience and are

localized in the PSD[115]. The temporal dynamics of these two genes are staggered in that *Arc* is expressed in the nucleus within 5 minutes of cell activity and within 30 minutes it translocates to the cytoplasm; *H1a* is expressed in the nucleus within 30 minutes of cell activity and takes over an hour to move to the cytoplasm[115]. Given that two mRNA probes (which are notoriously difficult to work with) are needed instead of one, it was necessary to troubleshoot the protocol to obtain ideal staining parameters.

2.1.2.3.4. Probe synthesis

Compared to protein, the nature of RNA is that it needs to be degraded in a timely manner as it is directing cellular machinery to manufacture protein, and the signals for protein synthesis need to be tightly controlled. This makes working with RNA inherently difficult and much troubleshooting was involved in the manufacturing of these RNA probes from plasmid. First, the DNA templates for *Arc* and *H1a* needed to be extracted from plasmid and linearized. This protocol worked best if restriction enzyme and probe were mixed and allowed to sit at room temperature overnight, but otherwise the linearization protocol should be carried out exactly how it is written.

2.1.2.3.5. Switching order of *Arc* and *H1a*

The catFISH paradigm works so well based on the two gene targets having different temporal expression profiles, owing partly to the size of each mRNA. Homer is a larger transcript than *Arc*, and therefore takes a longer amount of time to synthesize. When the probes are added to the tissue they are added simultaneously, *Arc* nucleotides are tagged

with DIG, and *H1a* nucleotides are tagged with FLU. Using the original protocol where the *Arc* mRNA was processed (i.e., primary antibody and fluorescent tag adhered) prior to *H1a*, the signal for *H1a* was always of higher intensity than that for *Arc*. In separate experiments, several variables were manipulated to attempt to minimize the discrepancy between intensity of *Arc* and *H1a*:

1) Increase anti-DIG concentration from 1:100 to 1:50
2) Decrease anti-FLU concentration from 1:100 to 1:200
3) Increase cy3 concentration from 1:50 to 1:25
4) Decrease fluorescein concentration from 1:50 to 1:100
5) Process *H1a* first and *Arc* second

While the discrepancy between *Arc* and *H1a* was improved, changing variables 1-4 decreased the signal/noise ratio in each case. However, when *Arc* was processed after *H1a*, the staining intensity of each gene was comparable to the other without sacrificing the integrity of the signal to noise ratio. This key change to the protocol was maintained for all the experiments in this thesis.

2.1.2.3.6. Coverslips and optimization of staining quality versus amount of reagents

Coverslips are useful during incubation of tissue because the amount of liquid needed for an antibody solution to penetrate the tissue evenly is dramatically reduced when a coverslip is applied. In the original protocol, coverslips were used for each step of the staining process (i.e., pre-hybridization buffer, probe application, primary antibody

incubation, fluorescent dye incubation, DAPI incubation) which minimized the amount of reagent needed for each step in those processes, but often would damage the tissue during removal. Even working as carefully as possible, the tissue could still sustain irreversible damage during processing due to the repeated contact between tissue and coverslip. For the experiments described in the thesis, coverslips were only used for incubation of the probe, which occurred in a humid chamber at 56 degrees for 16 hours. It was necessary to minimize the probe volume as much as possible (110 μL per slide) but all other steps utilized a hydrophobic barrier pen to outline the sections and 500 μL per slide of all other critical solutions was used.

2.1.3. Experiment 2: Pheromone-induced odor associative learning

2.1.3.1. Behavioral study

2.1.3.1.1. Groups

O^+/S^- (terpinene odor only) and Ph-T (pheromone paired with terpinene) rats were housed alone. Ph-T rats were exposed to the clean bedding with a piece of filter paper soaked with 0.75 mL 4-methylpentanal (1.3 × 10−6 M) and hexanal (8.7 × 10−6 M) binary mixture (dissolved in purified water)[39] on top of the bedding and received terpinene as the conditioned odor. SB-T (soiled-bedding conditioned with terpinene) and SB-Oc (soiled-bedding conditioned with octanol) were also housed alone.

2.1.3.1.2. Odor conditioning and testing

Rats were housed alone and exposed to the soiled bedding. A donor rat was shocked to release pheromone in the shock chamber (4 shocks during 30 min). The soiled bedding was woodchip bedding placed underneath the shock chamber during the donor rat shock and was subsequently left untouched for the conditioning of the soiled bedding (SB) rat. Habituation, odor delivery during the training, and testing were carried out in the same manner as in Experiment 3, except testing lasted 10 min (5 min in clean air, 5 min in an odorant), instead of 30 min. Additionally, Experiments 1 and 2 were carried out in two different rooms with different experimenters.

2.1.3.1.3. Drug infusion

To study the role of NMDA receptors in the BLA, a separate cohort underwent cannular implantations. Cannular surgeries were performed 1 week before the behavioral experiments. During surgeries, rats were anesthetized with isoflurane gas and secured in a stereotaxic apparatus. Two holes were drilled 2.5 mm posterior, and 4.9 mm bilateral relative to bregma for the BLA. Guide cannulas were inserted 7.8 mm ventral to the skull surface. Guide cannulas were secured by dental cement to two skull screws. The skin was sutured and the rats were returned to their cages for recovery. O^+/S^+ and pheromone molecule conditioned (O^+/Ph) rats were infused with either saline or (2R)-amino-5-phosphonovaleric acid (D-APV; 5mM, 1µl) bilaterally into the BLAs 30 min before the conditioning experiments. Infusion tubing and cannular attachment were performed during habituation for animals to become acclimated to the attachment of the infusion tubing.

2.1.3.1.4. Retrograde tracing from unilateral BLA of a rat

Animals (n=2) were anesthetized with 2-3% isoflurane and given meloxicam (2 mg/kg, subcutaneous) for post-surgery pain management. Pressure injections of AF-CTb-488 (Invitrogen, USA) were made in the BLA (2.5 mm posterior, 4.9 mm lateral, 7.8 mm ventral relative to bregma and the Dural surface of the brain) using glass pipettes with outer diameter of approximately 37 to 40 mm as done previously[332]. After a 7- to 9-day postoperative survival, animals were deeply anesthetized with 10% chloral hydrate (600mg/kg i.p.) and transcardially perfused with 150mL heparinized saline followed by 400–500mL ice-cold 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). The brains were removed and post-fixed in the same fixative for 4–5h, and cryoprotected in 20% sucrose with 10% glycerin over 2 days at 4°C. Coronal sections of the brain and olfactory bulb were taken at 50μm and sections were mounted on slides at every 200μm for subsequent examination under a fluorescent microscope. Brain sections were examined and photographed using an Olympus BX51 microscope equipped with a digital camera (Spot Insight, Diagnostic Instruments Inc, Sterling Heights, MI, USA) and the images were transferred to Adobe Photoshop CS4 to optimize light and contrast levels.

2.1.4. Statistics

OriginPro 9.0 was used to analyze the datasets. One-way ANOVAs plus post-hoc Bonferroni tests were used to compare different groups in Figures 4-5,7-8,10-11. A two-sample t-test (2-tail) was used in Figure 12. Data are presented as mean ± SEM in Results and Figures.

2.2. Second order conditioning

2.2.1. Animals

Sprague Dawley rats (3–6 months old, weight 400–900 g, in good health) of both sexes (n = 68 total) were assigned randomly to groups. Rats were housed in polycarbonate cages on a 12-hr light/dark cycle, given ad libitum access to food and water, with all behavioural manipulation completed during the light phase of the light cycle. Animals were housed alone for the duration of the experiments. All procedures were approved by the Memorial University Institutional Animal Care Committee and carried out in compliance with the guidelines of the Canadian Council on Animal Care.

2.2.2. Apparatus

In all cases the shock chamber was thoroughly cleaned with 70% ethanol and clean paper towels between exposures. There was a 15 min interval between the chamber cleaning and next exposure, where residual smell and ethanol were removed.

2.2.2.1. Context A

Context A consisted of a custom-made olfactometer for air and odorant delivery attached to the shock chamber: a plexiglass chamber that sits on top of an electrified grid, connected to a shock generator/scrambler (Muromachi Kikai Model SGS-003DX). The sides of the plexiglass chamber were covered with white paper so the animal could not see outside of the box. Polyvinyl carbonate bottles were used for each odor and connected to

the olfactometer by C-flex tubing pinched shut when not in use. Evacuation tubing with a fan was attached to the top lid of the shock chamber to promote odor removal. For Experiments 5 and 6 which required auditory stimulation, computer speakers connected to a laptop were placed on opposite walls outside of the conditioning chamber so the animals could not see them. The volume was set such that the tones were measured at 70dB from inside the conditioning chamber via a decibel meter.

2.2.2.2. Context B

Context B was placed in a separate room from Context A and consisted of a plexiglass chamber covered in a checkerboard pattern such that the animals could not see outside of the chamber while inside. Odor was delivered by soaking filter paper in terpinene diluted in mineral oil and placing small pieces inside of fenestrated 15mL capped tubes, adhered to the four corners of the plexiglass box via tape. A video camera recorded the session and freezing was measured in real time using a stopwatch.

2.2.3. Odorants

Odorants were diluted with mineral oil to specific concentrations. Odorants used were terpinene (6.63%) and octanol (2.67%). These odorants were chosen as they are neither innately appetitive nor aversive to adult rats, and the concentrations were chosen so that the odors would emit a vapor-phase partial pressure of 1 Pascal[330].

2.2.4. Brain mapping of odor used as CS1, CS2 with context CS1, and CS2 with tone CS1

Each experiment differed in the habituation, conditioning, and testing procedures, but remained the same for tissue processing, image analysis, and statistics. Because the nomenclature necessary to identify specific groups within experiments can become cumbersome and lengthy, the groups for each experiment will be defined in each section but subsequently are referred to as "control," "experimental," and "unpaired" with the intention of providing clarity for the reader.

2.2.4.1. Experiment 3: Odor as CS1

2.2.4.1.1. Groups

Two groups were used in this experiment, the experimental group (O^+/S^+) and the control group (O^+/S^-). Experimental animals were given odor and shock on day 3 while control animals were simply given odor. In this way, experimental animals received FOC whereas control animals did not.

2.2.4.1.2. Habituation

All rats were habituated to Context A for one 30 min session each on two consecutive days (days 1 and 2) with clean air pumped through the shock chamber. All behavioural monitoring took place in Context A and rats were handled for 5 minutes each before entrance to and upon exit of the shock box.

2.2.4.1.3. First order conditioning

On the third day, rats were trained individually with four separate exposures to either odor (control) or odor and shock (experimental) at 5, 15, 20, and 30 min during a 30 min training session such that an association was formed between the odor and shock, giving odor the value of conditioned stimulus 1 (CS1) in experimental animals. Odorant (terpinene) was delivered for 1 min at each time point. Shock was delivered at the last second of the odor delivery (0.5 mA for 1 sec). Animals were returned to their home cages immediately following the final odor exposure.

2.2.4.1.4. Testing and final CS1 exposure

On day 4 all rats were individually placed in the shock chamber and exposed to terpinene (CS1) for five minutes. The testing session was recorded and freezing behaviour was measured in real time with a stopwatch. Animals were returned to their home cages immediately following terpinene exposure, their home cages were wrapped in a white paper sheet so they could not see outside, and they were left undisturbed for approximately 80 minutes on a cart outside of the behavioural rooms. Each final CS1 exposure was followed by perfusion 90 minutes later for cFos immunohistochemistry.

2.2.4.2. Experiment 4: Context as CS1 and odor as CS2

2.2.4.2.1. Groups

Two groups were used in this experiment, control (Ct+S/Ct) and experimental (Ct+S/O+Ct). Both groups were shocked in the context on day 1. Experimental animals were given odor and shock on day 2 while control animals were simply placed back in the

context without odor exposure. In this way experimental rats received first and SOC, whereas control rats received first- but not second-order conditioning.

2.2.4.2.2. First order conditioning

On day 1 all rats were first order conditioned individually in Context A with four separate shocks (0.5 mA for 1 sec) at 5, 15, 20, and 30 min during a 30 min training session such that an association was formed between the context and shock, giving Context A the value of conditioned stimulus 1 (CS1). Animals were returned to their home cages immediately following the final shock in Context A.

2.2.4.2.3. Second order conditioning

On day 2 experimental rats were second order conditioned individually by exposing them to terpinene for 5 minutes continuously in Context A such that an association was formed between the odor and the context, giving terpinene the value of conditioned stimulus 2 (CS2). Control rats were simply placed in Context A for five minutes. This training session was recorded and freezing behaviour was measured in real time with a stopwatch to evaluate the extent of FOC from the previous day. Animals were returned to their home cages immediately following odor and Context A exposure.

2.2.4.2.4. Testing and final CS2 exposure

On day 3 all rats were individually placed in novel Context B and exposed to terpinene (CS2) for five minutes. The testing session was recorded and freezing behaviour was measured in real time with a stopwatch. Animals were returned to their home cages immediately following terpinene exposure in context B, their home cages were wrapped in a white paper sheet so they could not see outside, and they were left undisturbed for

approximately 80 minutes on a cart outside of the behavioural rooms. Each final CS2 exposure was followed by perfusion 90 minutes later for cFos immunohistochemistry.

2.2.4.3. Experiment 5: Tone as CS1 and odor as CS2

2.2.4.3.1. Groups

Three groups were used in this experiment, control (T+S/T), experimental (T+S/O+T), and unpaired (T+S/O/T). All groups received tone and shock pairings on day 3, following two days of habituation to the shock chamber. Experimental animals were given tone and odor on day 4 while control animals were simply exposed to the tone without odor. An unpaired group was added to this experiment, and on day 4 they were first given odor presentation, followed by tone presentation 30 minutes later. In this way experimental rats received first and SOC, whereas control rats received first but not SOC, and unpaired animals received FOC, not SOC, but they were exposed to the exact same stimuli for the exact same amount of time compared to the experimental animals across the entire experimental paradigm. I felt this was an important comparison to include, as the control animals experience one less odor exposure compared to the experimental group, and importantly this makes the odor novel for these animals, which is not the case in the unpaired group.

2.2.4.3.2. Habituation

All rats were habituated to Context A for one 30-min session each on two consecutive days (days 1 and 2) with clean air pumped through the shock chamber. All behaviour took place in Context A and rats were handled for 5 minutes each before entrance to and upon exit of the shock box.

2.2.4.3.3. First order conditioning

On the third day all rats were trained individually with four separate exposures to tone and shock at 5, 15, 20, and 30 min during a 30 min training session such that an association was formed between the tone and shock, giving tone the value of conditioned stimulus 1 (CS1). Tone (2 kHz) was delivered for 30 sec at each time point. Shock was delivered at the last second of the tone delivery (0.5 mA for 1 sec). Animals were returned to their home cages immediately following the last shock.

2.2.4.3.4. Second order conditioning

On day 4 experimental rats were second order conditioned individually by exposing them to terpinene and the tone simultaneously for 5 minutes such that an association was formed between the odor and the tone, giving terpinene the value of conditioned stimulus 2 (CS2). Control rats were exposed to the CS1 (tone) for five minutes in clean air. Unpaired rats were exposed to 5-minute presentations of the odor followed by 30 minutes of clean air, followed by a 5-min presentation of the CS1 (tone). This training session was recorded and freezing behaviour was measured in real time with a stopwatch to evaluate the extent of FOC from the previous day. Animals were returned to their home cages immediately following tone or tone/odor exposure.

2.2.4.3.5. Testing and final CS2 exposure

On day 5 all rats were individually exposed to terpinene (CS2) for five minutes. The testing session was recorded and freezing behaviour was measured in real time with a stopwatch. Animals were returned to their home cages immediately following terpinene exposure, their home cages were wrapped in a white paper sheet so they could not see

outside, and they were left undisturbed for approximately 80 minutes on a cart outside of the behavioural rooms. Each final CS2 exposure was followed by perfusion 90 minutes later for cFos immunohistochemistry.

2.2.4.4. Experiment 6: Inhibiting NMDARs in BLA immediately following SOC

2.2.4.4.1. Groups

All animals in this experiment were treated the same behaviourally, except half received intra-BLA infusions of vehicle immediately following SOC while the other half received D-APV. All animals went through the habituation, first-order, and second-order conditioning sessions in the same manner as described in Experiment 5.

2.2.4.4.2. Habituation

All rats were habituated to Context A for one 30 min session each on two consecutive days (days 1 and 2) with clean air pumped through the shock chamber. All behaviour took place in Context A and rats were handled for 5 minutes each before entrance to and upon exit of the shock box.

2.2.4.4.3. First order conditioning

On the third day all rats were trained individually with four separate exposures to tone and shock at 5, 15, 20, and 30 min during a 30 min training session in Context A such that an association was formed between the tone and shock, giving tone the value of conditioned stimulus 1 (CS1). Tone (2 kHz) was delivered for 30 sec at each time point. Shock was delivered at the last second of the tone delivery (0.5 mA for 1 sec). Animals were returned to their home cages immediately following the last shock.

2.2.4.4.4. Second order conditioning

On day 4 rats were second order conditioned individually in Context A by exposing them to terpinene and the tone simultaneously for 5 minutes such that an association was formed between the odor and the tone, giving terpinene the value of conditioned stimulus 2 (CS2). This training session was recorded and freezing behaviour was measured in real time with a stopwatch to evaluate the extent of FOC from the previous day

2.2.4.4.5. Drug infusion

To study the role of BLA NMDA receptors in SOC, a separate cohort underwent cannular implantations. Cannular surgeries were performed 1 week before the behavioral experiments. During surgeries, rats were anesthetized with isoflurane gas and secured in a stereotaxic apparatus. Two holes were drilled 2.5 mm posterior, and 4.9 mm bilateral relative to bregma for the BLA. Guide cannulas were inserted 7.8 mm ventral to the skull surface. Guide cannulas were secured by dental cement to two skull screws. The skin was sutured and the rats were returned to their cages for recovery. Infusion tubing and cannular attachment were performed during habituation for animals to become acclimated to the attachment of the infusion tubing. Immediately following SOC rats received a bilateral infusion of D-APV (5 mM; 1 µl/hemisphere infused over 3 minutes) or vehicle (saline) directly to the BLA in a separate room apart from the behavioural setup. Animals were handled for 5 minutes and returned to their home cages immediately.

2.2.4.4.6. Testing and final CS2 exposure

On day 5 all rats were individually exposed to terpinene (CS2) for five minutes. The testing session was recorded and freezing behaviour was measured in real time with a

stopwatch. Animals were returned to their home cages immediately following terpinene exposure, their home cages were wrapped in a white paper sheet so they could not see outside, and they were left undisturbed for approximately 80 minutes on a cart outside of the behavioural rooms. Each final CS2 exposure was followed by perfusion 90 minutes later for cFos immunohistochemistry.

2.2.5. Perfusion and tissue processing

As cFos protein levels peak at 90 minutes following a stimulus, animals were transported from animal care to the lab at 80 minutes following the final CS2 exposure, given an i.p. injection of pentobarbital (200mg/kg), and when they no longer responded to a toe-pinch, transcardially perfused for 3 minutes with 0.9% ice cold saline and 5-7 minutes with 4% ice cold paraformaldehyde (PFA). Brains were carefully removed and placed in 4% PFA in glass vials for up to 1 week then transferred to PBS until sectioned.

Brains from Experiments 3 and 4 were sectioned on a vibratome (Leica VT1000) while brains from Experiment 5 were sectioned on a compresstome (PrecisionaryVF-210-0Z). Brains were blocked such that the olfactory bulbs and approximately 2/3 of the cerebellum was removed, but a flat surface remained for mounting the brain on the slicing plate or cylindrical specimen holder in the case of the vibratome and compresstome, respectively. The posterior base of the brain was then mounted onto the specimen holder with superglue. It was necessary to add stability to the brain for vibratome slicing, and superglue was added to adhere the cortical regions to the cerebellum and ensure the brain was not pushed while being sliced which would result in uneven sections. When the glue

was dried, the plate was placed onto a vibratome with thickness set at 50 μm. Once the specimen was adhered to the compresstome specimen holder, warm (~45ºC; 2% in water) agarose was poured into the metal sleeve and frozen with a chilled (-20ºC) metal block. Once frozen the apparatus was inserted into the compresstome and bathed in chilled (4ºC, 0.01M) PBS. The brain was serially sectioned from anterior to posterior, collecting 24 sections from each broad region of interest into 24 well plates filled with polyvinylpyrrolidone (PVP) solution. For the anterior piriform cortex collection started at approximately +2.20 mm bregma, 2 sections were collected, 2 sections discarded, and so forth until a 24 well plate was filled with sections. For the posterior piriform, DH, and amygdala collection started at approximately -2.12 mm bregma, 2 sections were collected, 1 discarded, and so forth until a 24 well plate was filled with sections. For the VH and auditory cortex collection started at approximately -4.80 mm bregma and all sections were collected, up to two 24 well plates, as ventral hippocampal slices are difficult to keep intact through the process of IHC and some are unfortunately destroyed. Plates were labelled, wrapped in parafilm, and stored at 4ºC until IHC was performed.

2.2.6. Immunohistochemistry

Prior to initiation of experiments, optimal staining concentrations and parameters were obtained by using an antibody titration for the primary antibody, cFos. Slices from animals exposed to terpinene for 90 minutes and perfused 90 minutes later went through the IHC protocol with cFos concentrations of 1:1000, 1:4000, 1:7000, and 1:10,000. The sections with the highest signal to noise ratio were those that had been exposed to the lowest

concentration of primary antibody, so the experiments used a 1:10,000 dilution for cFos. All IHC procedures were performed on free floating sections. Each new solution was placed in a clean well of a 24 well plate and sections were moved from well to well by a soft paintbrush. Each well always contained 1mL of solution. All washes and incubations took place on a shaker plate at low speed. Four different variations of tris buffer are used in this protocol. Briefly, sections were rinsed in Tris buffer (0.1M, pH 7.6) to remove residual PFA then immersed in 1% hydrogen peroxide for 30 minutes to quench endogenous peroxidase. Sections were rinsed in Tris buffer then washed in Tris A (0.1% Triton-x, 0.1M, pH 7.6) then Tris B (0.1% Triton X, 0.005% BSA, 0.1M, pH 7.6). Sections were then blocked in 10% normal goat serum in Tris B for 1 hour, rinsed with Tris A, Tris B, then incubated in 1:10,000 cFos antibody (Cell Signaling Technology, catalogue #2250S) in Tris B for at least 2 nights but up to a week at 4^{o}C on a rotator in the cold room. Sections were washed in Tris A, Tris B, then incubated for 45 min at room temperature in 1:1000 biotinylated Goat Anti-Rabbit IgG secondary antibody in Tris B. Sections were again washed in Tris A, Tris D (0.1% Triton X, 0.005% BSA, 0.5M, pH 7.6) then the signal was amplified by an avidin-biotin peroxidase kit (ABC kit; Vector labs) for 2 hours by manufacturers instructions. Following another series of washes in Tris buffer, the signal was developed by use of a Vector SG HRP substrate (SG grey; Vector labs) as per manufacturer's directions. Sections were checked periodically under a light microscope for extent of staining and removed from SG grey when desired darkness was achieved, the reaction was quenched in H_2O, and sections were washed for a final time in Tris buffer. Sections were then mounted on labeled glass microscope slides (Leica) and left to air dry in a fume hood overnight. The following day slides were placed in a vertical slide dipper

and submerged in 200 mL of two changes of 50% ethanol, 2 changes of 75% ethanol, 2 changes of 90% ethanol, two changes of 100% ethanol, and 2 changes of xylene. Slides were wiped on the back surface to remove excess xylene, laid flat on the fumehood surface, and cover-slipped with Permount (Fisher Scientific) by dropping a line of Permount at the base of the slide, gently placing a coverslip on top, and gradually letting the coverslip fall onto the slide while the Permount travelled evenly up the slide, minimizing air bubbles between the coverslip and the slide. Slides were left to dry for at least two hours then the excess Permount was removed using a swab soaked in xylene.

2.2.7. Image acquisition and analysis

Images were taken with an EVOS M5000 imaging system (Thermo Fisher Scientific) with consistent brightness, exposure, and gain settings. Images were taken at 10X magnification of the following areas from anterior to posterior: aPC, pPC, BLA, LA, DH, VH, and Au1 (Figure 2C-E). Cells were counted automatically using ImageJ software. Images were transformed to 80-bit, converted to black and white and thresholded manually by an experimenter who was unaware of conditions to maximize the signal to noise ratio. The background became white and positively stained cells were black. The region of interest was selected, and "despeckle" and "watershed" functions were applied to further reduce background and separate any cells that became joined accidentally. The cells were counted using the "analyze particles" function with size set to 15-infinity μm^2 and

circularity defined as 0.50-1.00 and the total number of positive cells was divided by the total area using the scale of 1.6pixels/μm.

2.2.8. Statistics

GraphPad Prism 9.0 was used to analyze the datasets. Two sample unpaired t-tests (2-tail) were used in Figure 14A, Figure 15A, and Figures 16B,C-24B,C. One way ANOVA was used for Figures 16D-24D. Two-way mixed ANOVA was used for Figure 14B2,C2,D2 and Figure 15B,C,D. Bonferroni post hoc tests were employed. Data are presented as mean ± SEM in Results and Figures.

Chapter 3 – Results

3.1. Pheromones can serve as unconditioned stimuli to support higher order odor fear learning

3.1.1. Conditioned fear can be transmitted to conspecifics in the absence of an aversive stimulus

To investigate pheromonally-driven olfactory fear conditioning, a previous honour's student in the lab[333] tested whether the companion rats (O^+/Comp) of the O^+/S^+ conditioned rats were able to form cue-specific fear memory when subsequently exposed to the conditioned odor terpinene. My PhD project probed further into this newly discovered behavioural paradigm (Figure 4A; adapted from Carew[273]). We wanted to know does the stress transferred from the O^+/S^+ to the O^+/Comp rats serve as the US in the conditioning of the O^+/Comp rats? To test this, a subset of the companions were injected with an anxiolytic β-adrenoceptor antagonist propranolol (O^+/Comp + Prop) before the training. Additionally, we tested where the CS-US association occurs by including a group of companion rats that were not subsequently exposed to the conditioned odor (O^-/Comp). The absence of learning in the O^-/Comp group would suggest that any residual terpinene smell on the O^+/S^+ rat is not sufficient to induce associative learning in the O^+/Comp rat during the social interaction, although it does not exclude any priming effects of social interaction on subsequent odor conditioning.

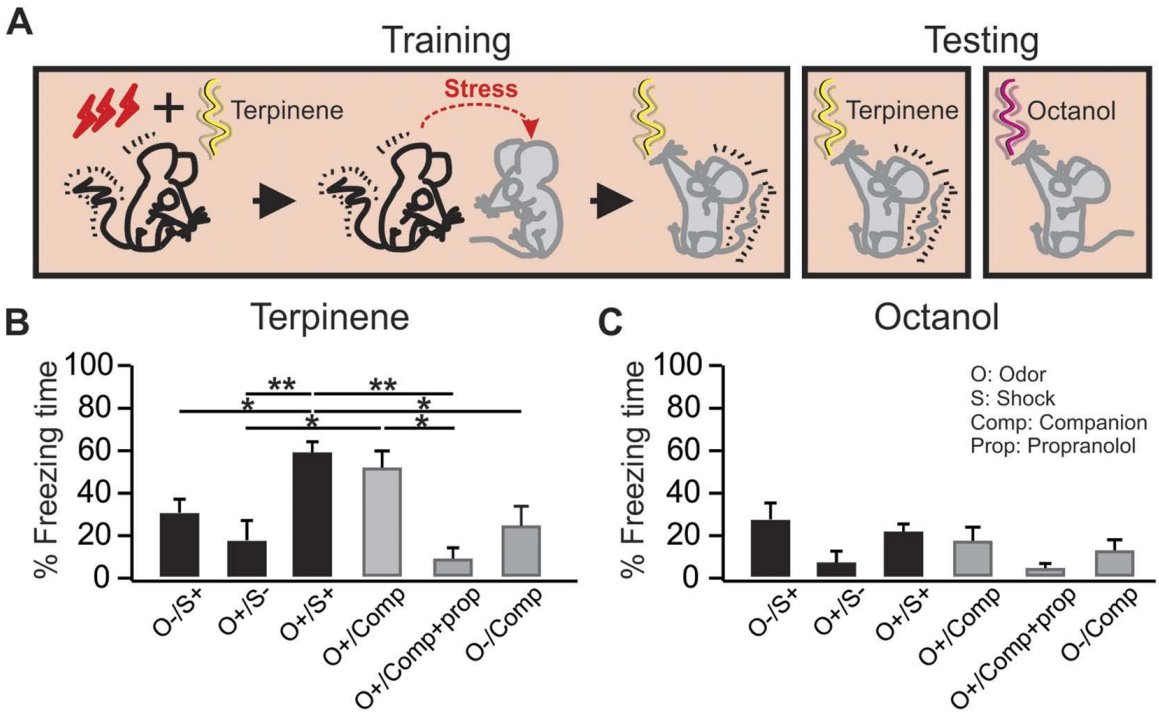


Figure 4. ***Conditioned fear can be transmitted to conspecifics in the absence of an external aversive stimulus.*** **A)** Schematics of the odor conditioning and testing paradigm. **B)** Percentage freezing time during the testing to the conditioned odor terpinene. **C)** Percentage freezing time during the testing to the novel control odor octanol. O−/S+, shock only rats; O+/S−, odor only rats that were caged alone; O+/S+, odor/shock conditioned rats; O+/Comp, odor only rats that were caged with odor/shock conditioned rats; O+/Comp+Prop: O+/S− comp rats that were injected propranolol before training; O−/Comp: companion rats without subsequent odor exposure. *p<0.05, **p<0.01. Error bars, mean±SEM.

Like the O^+/S^+ rats, the O^+/Comp rats developed significant freezing to terpinene. There was a significant difference in the percentage of freezing among different groups in the presence of terpinene ($F_{5,46} = 8.16$, $p = 1.41E\text{-}5$, ANOVA; Figure 4B; adapted from Carew[273]). Post-hoc Bonferroni tests showed significant differences between the O^+/S^+ ($58.91 \pm 4.55\%$, $n = 19$) and O^+/S^- ($17.69 \pm 8.92\%$; $n = 6$, $t = 4.34$, $p = 0.001$), and between the O^+/S^- and O^+/Comp ($54.94 \pm 9.08\%$; $n = 10$, $t = 3.27$, $p = 0.031$). Pre-training infusion of propranolol (O^+/Comp + Prop) prevented the formation of odor-specific memory ($9.5 \pm 3.84\%$; $n = 4$, $t = 3.53$, $p = 0.014$, compared to the O^+/Comp rats). The companion rats without terpinene exposure (O^-/Comp) spent significantly less time freezing in terpinene (25.39 ± 7.46, $n = 7$) than the O^+/S^+ rats ($t = 3.53$, $p = 0.015$). No significant difference was observed in animals exposed to the control odor octanol ($F_{5,44} = 1.67$, $p = 0.16$; Figure 4C; adapted from Carew[273]), or in their baseline freezing level before the odor exposure during testing (Figure 5; adapted from Carew[273]). Further analysis separating sex groups revealed no differences in female and male performance in either the O^+/S^+ or the O^+/Comp groups (Figure 6; adapted from Carew[273]). These results suggest the learning in the companion rat is dependent on NE release, likely induced by the transfer of the stress from the O^+/S^+ rats. Exposure to the conditioned odor following the interaction with a stressed rat was necessary for specific fear odor memory formation in the companion rat.

Additionally, I show that the odor-specific learning is not contingent on the training context, as conditioned rats tested in a different context also showed significant

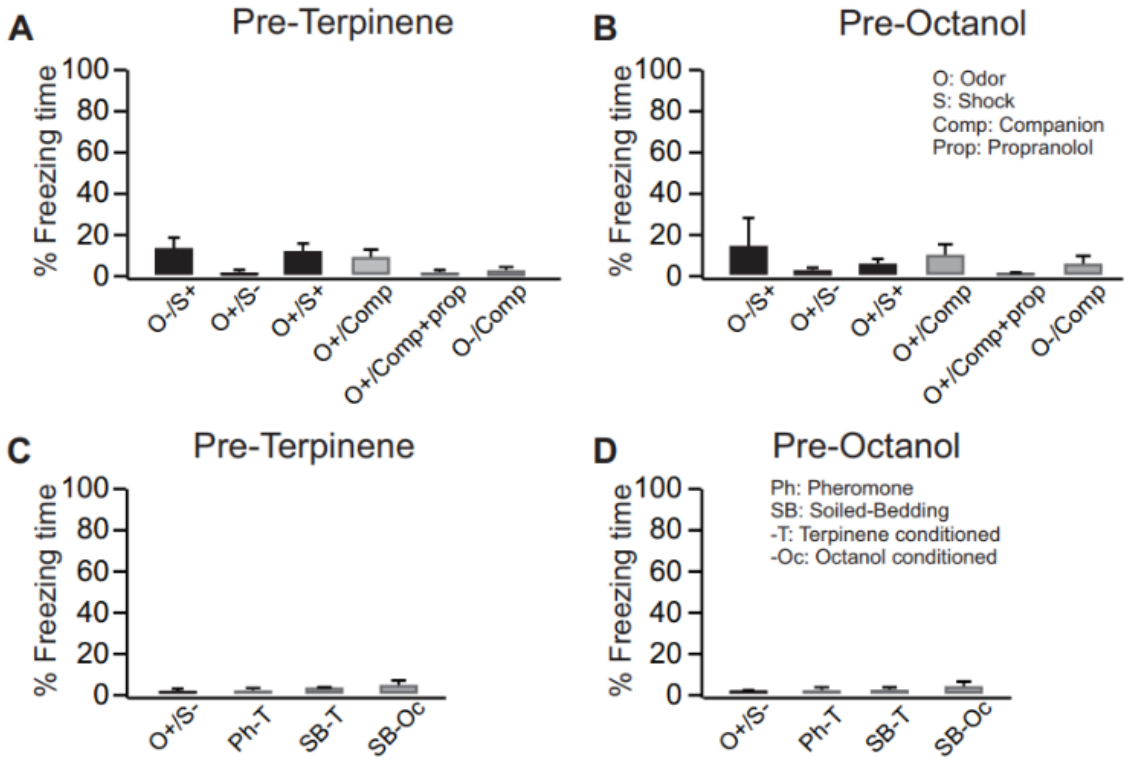


Figure 5. ***Pre-odor baseline freezing.*** **A-B)** Percentage freezing before the odor exposure in Figure 4B,C. **C-D)** Percentage freezing before the odor exposure in Figure 11B,C.

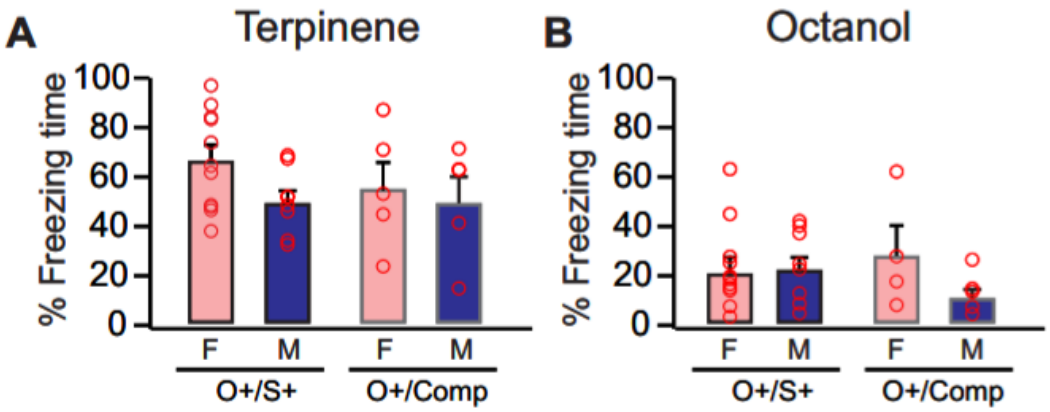


Figure 6. ***No sex differences in classical and pheromone associative learning.*** **A)** Percentage freezing to terpinene in female and male rats in the O + /S+ and O + /Comp groups. **B)** Percentage freezing to Octanol in female and male rats in the O + /S+ and O + /Comp groups. F, female; M, Male.

freezing to the conditioned odor (Figure 7; adapted from Carew[273]) and no general anxiety in an elevated plus maze test (Figure 8; adapted from Carew[273]).

3.1.2. Stressed cage mate-induced odor conditioning activates a classical amygdala fear pathway

I next measured activation profiles of several brain regions critically involved in odor or pheromone processing and fear memory formation using catFISH[104,334]. This technique utilizes the immediate-early genes *H1a* and *Arc* to visualize cells that are active to two temporally distinct events. *H1a* is expressed in the nucleus approximately thirty minutes following a stimulus, while *Arc* appears in the nucleus approximately five minutes after stimulus presentation[115]. Four groups were used for this experiment: O^-/S^+, O^+/S^-, O^+/S^+ and O^+/Comp. Twenty-four hours following odor conditioning, rats were exposed to octanol for 5 min, clean air for 20 min, terpinene for 5 min, and then immediately sacrificed (Figure 9A). Cells expressing *H1a* were those activated by the control odor octanol, while cells expressing *Arc* were those activated by the conditioned odor terpinene (Figure 9B). I systematically measured the *H1a* and *Arc* expression in the dorsolateral and ventromedial mitral cell layer of the MOB, the mitral cell layer of the AOB, the dense cell layer of the OT, layer II of both anterior and posterior PC, and several nuclei of the amygdala (Figure 10). We measured ratios of *Arc*/*H1a* (the ratio of the number of terpinene-activated cells over that activated by octanol) as a way of normalizing the activation profiles in each region. This within-tissue control protocol reduces variation from intrinsic variability in individual animal response levels to odor input and from variability related to differences

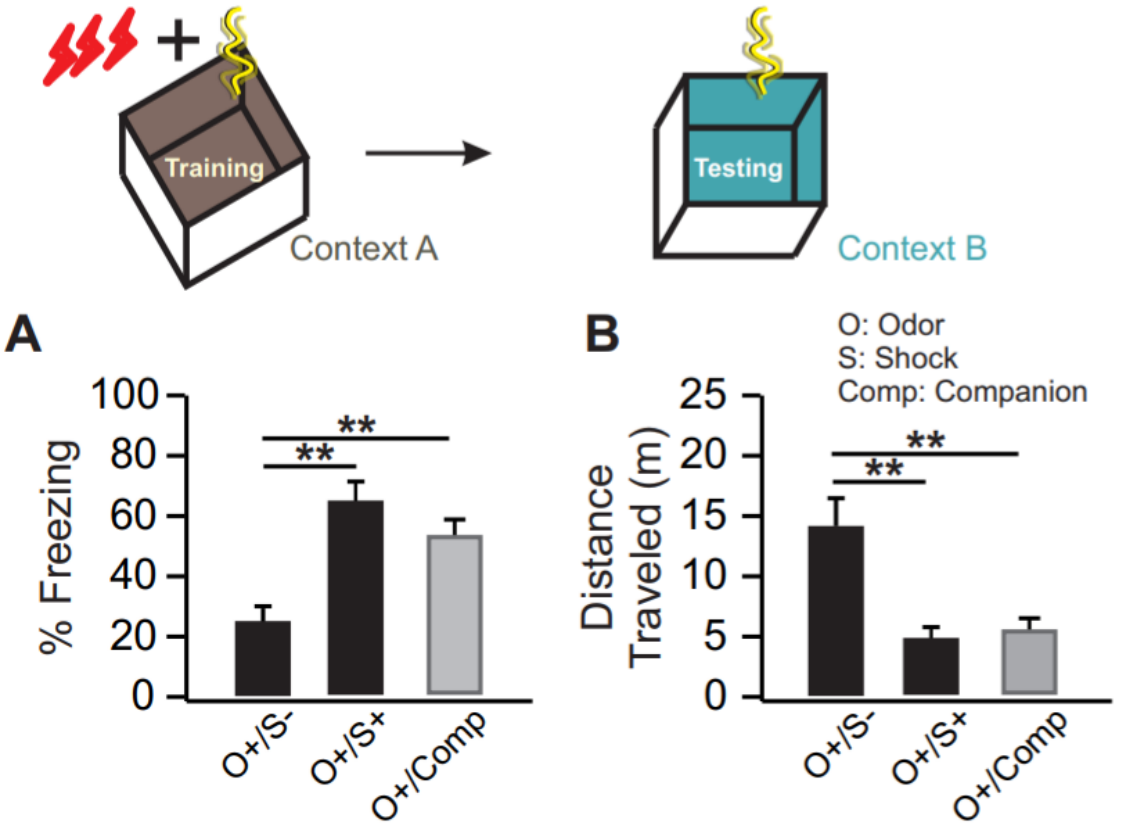


Figure 7. ***Odor-conditioned rats show fear response to the conditioned odor regardless of context.*** Rats were trained in a shock chamber (context A) as in Figure 1 and tested in a different context. **A)** Percentage freezing to the conditioned odor terpinene. **B)** Distance traveled in the context B.

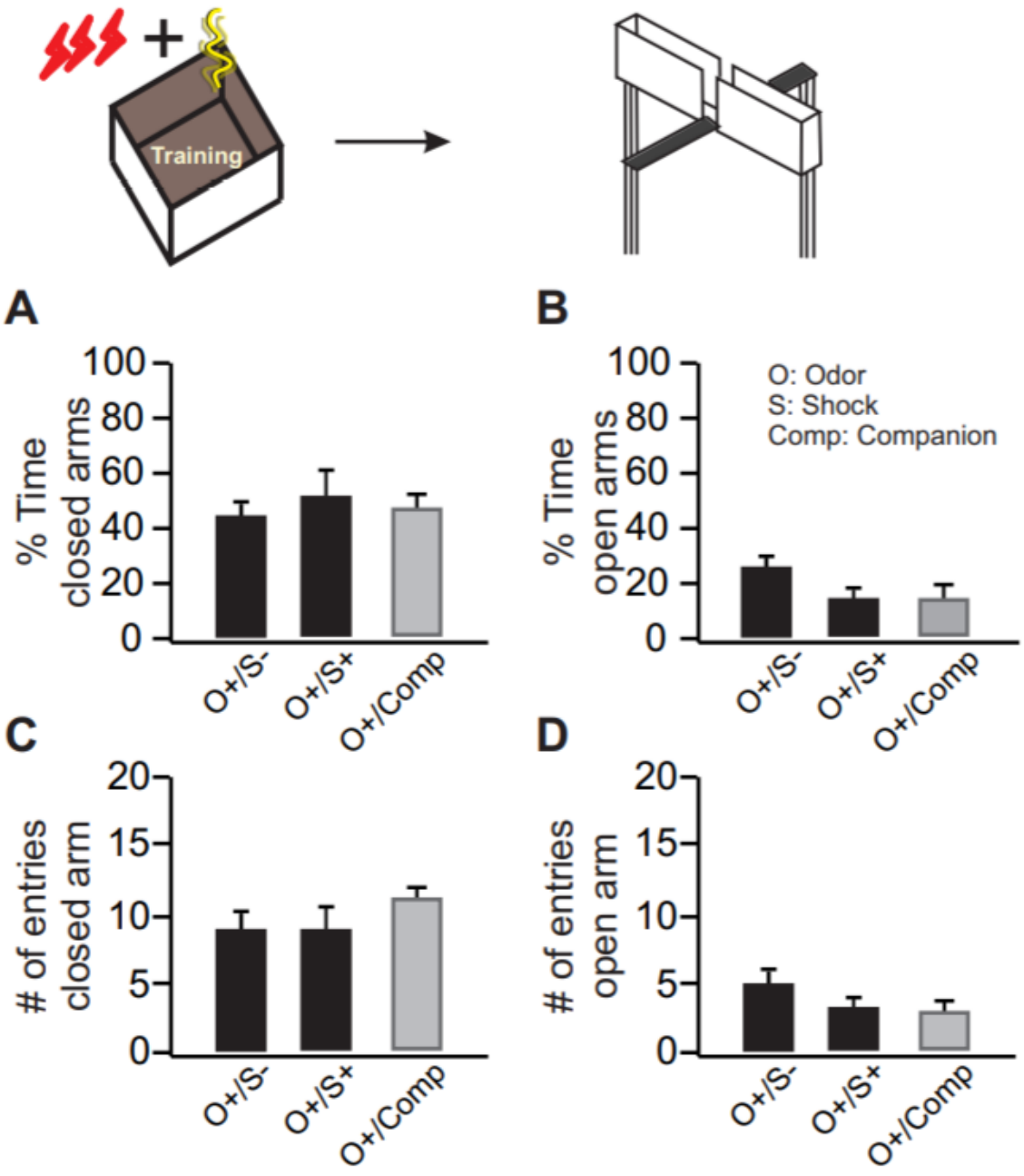


Figure 8. ***No elevated general anxiety following classical and pheromone associative learning.*** **A)** Percentage time spent in the closed arms. **B)** Percentage time spent in the open arms. **C)** # of entries in the closed arms. **D)** # of entries in the open arms.

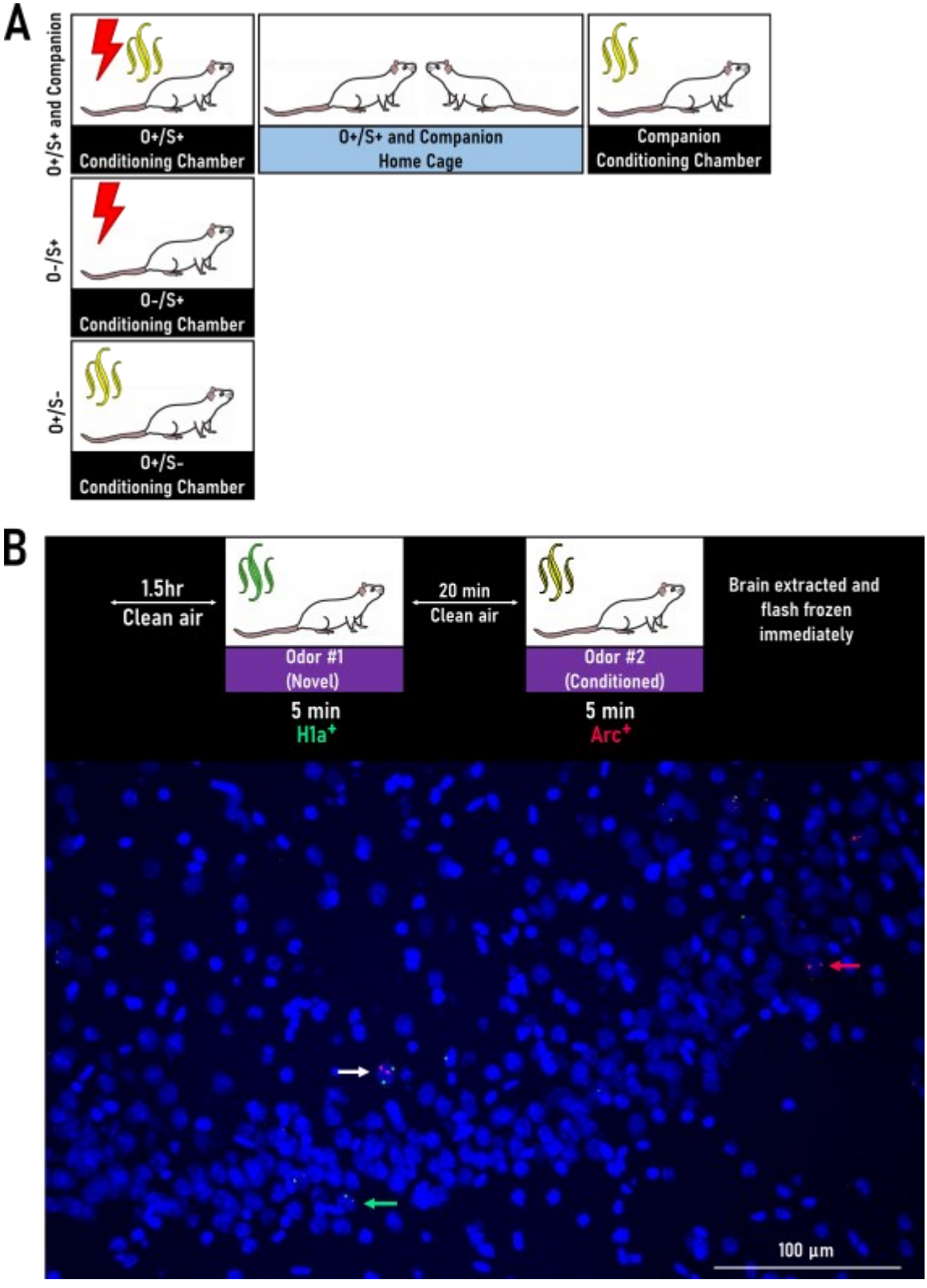
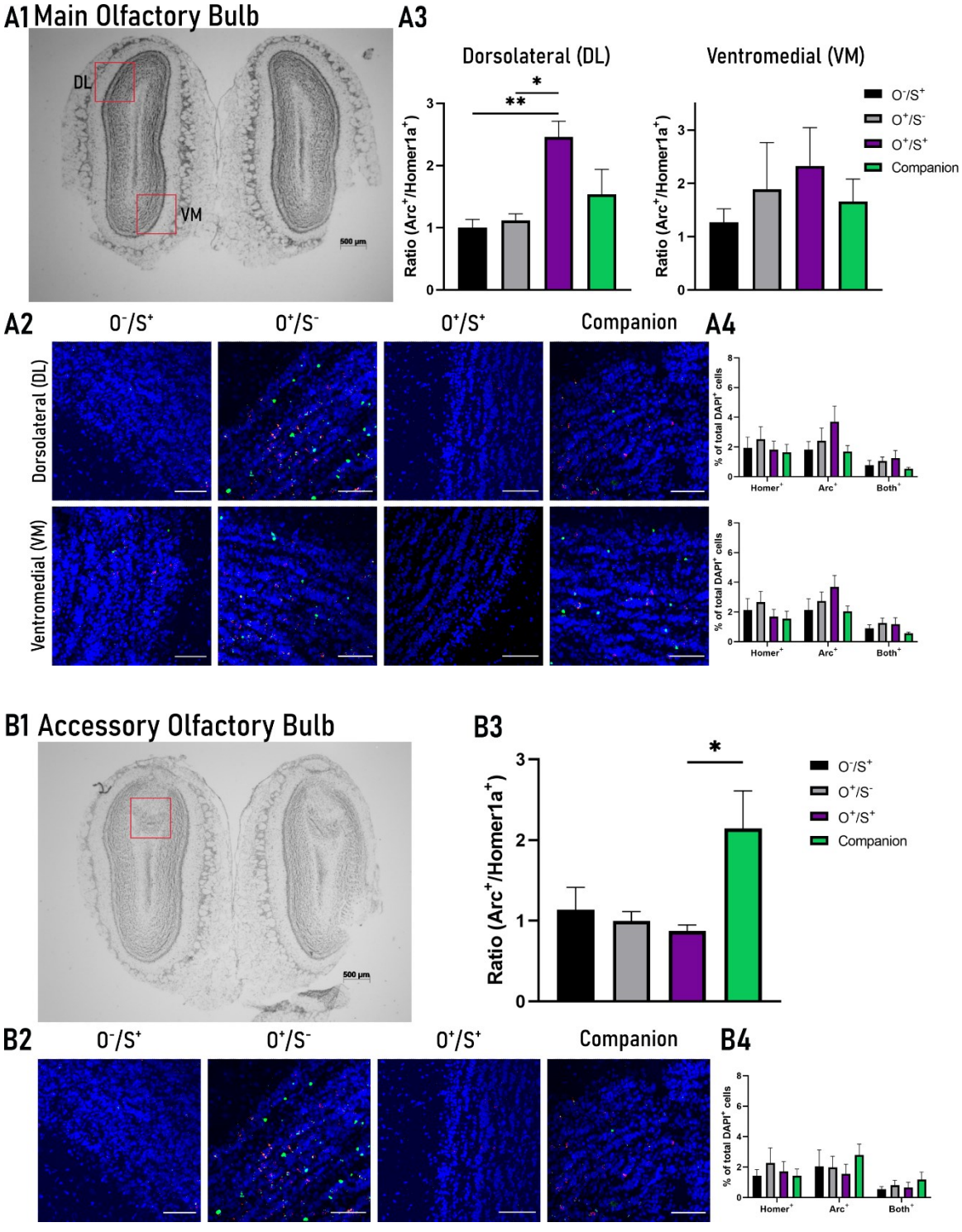
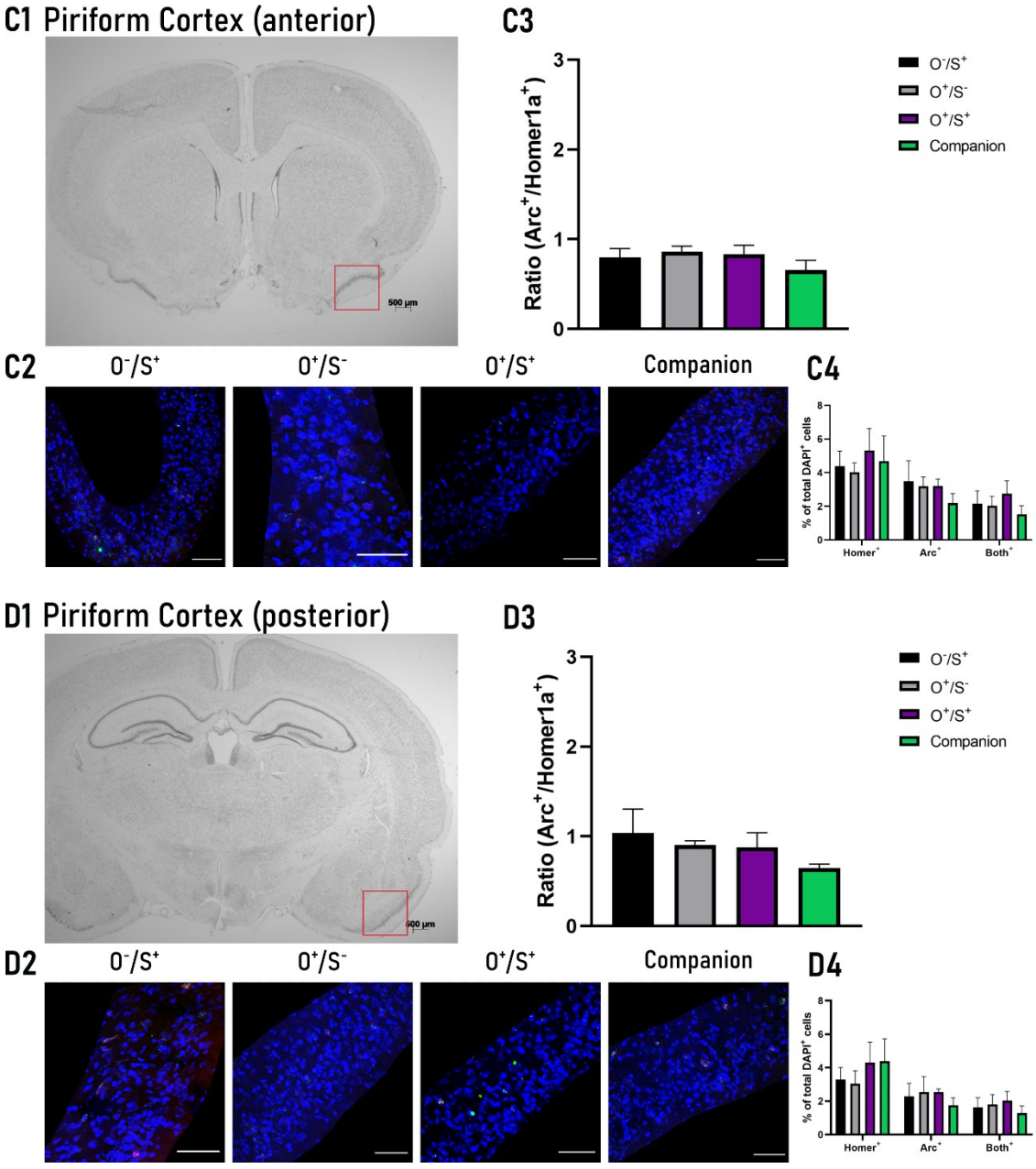
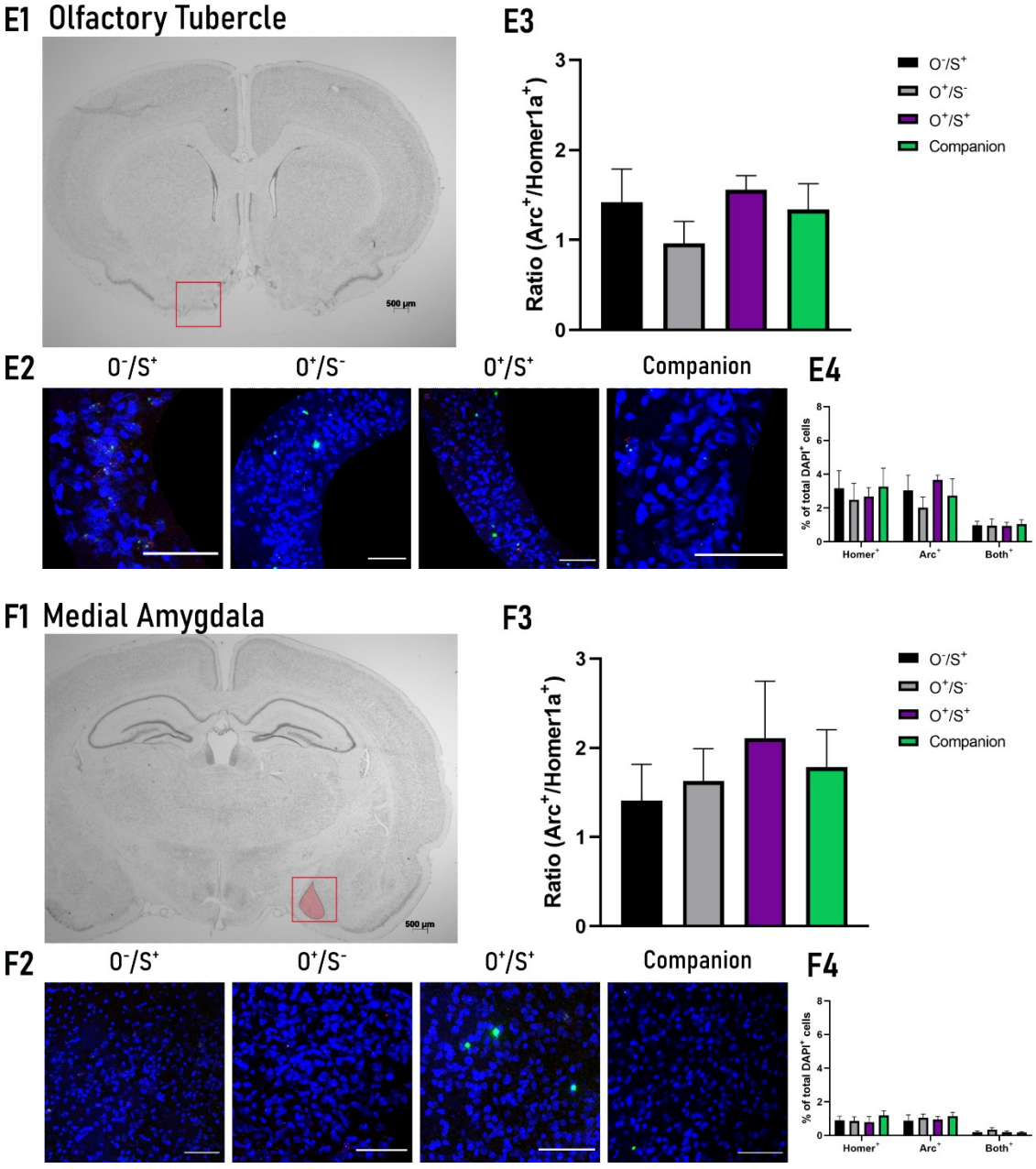
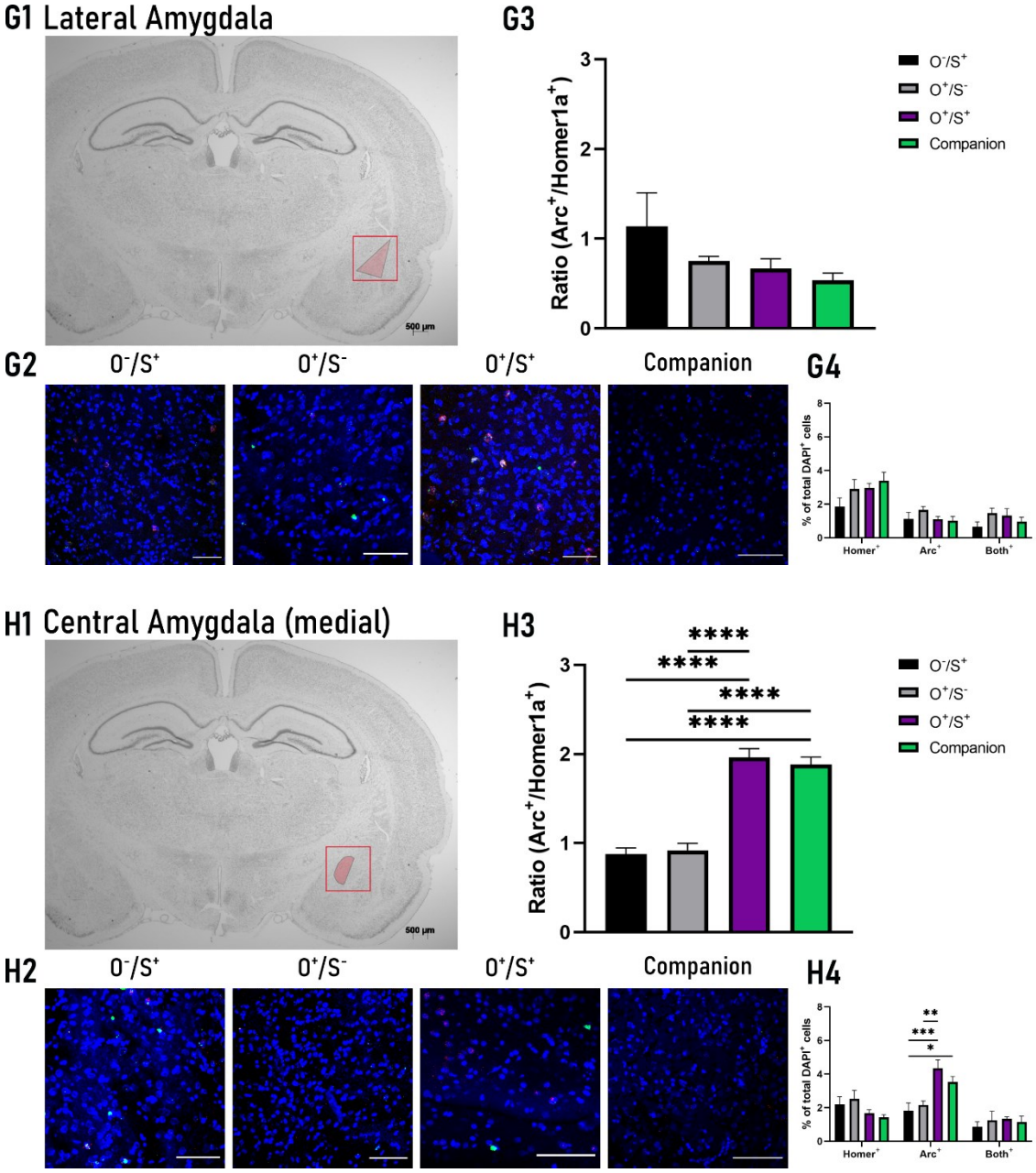

A
O+/S+ and Companion
O+/S+
Conditioning Chamber
O+/S+ and Companion
Home Cage
Companion
Conditioning Chamber
O-/S+
O-/S+
Conditioning Chamber
O+/S-
O+/S-
Conditioning Chamber
B
1.5hr
Clean air
Odor #1
(Novel)
5 min
H1a+
20 min
Clean air
Odor #2
(Conditioned)
5 min
Arc+
Brain extracted and
flash frozen
immediately
100 µm

Figure 9. ***Sample image and pre-sacrifice behavioural paradigm*****. A)** Two days of habituation preceded the experimental paradigms. O+/S+ animals were shocked four times in a 30-minute session preceded by 1 minute of odor delivery at 4, 14, 19, and 29 minute marks in the conditioning chamber. They were then returned to the home cage with the Companion rats for 30 minutes of interaction, after which the Companion rats were placed in the conditioning chamber and given odor only at the same time points as above. O-/S+ and O+/S- rats were given shock only or odor only respectively at the same time points as the O+/S+ rats. **B)** Following the conditioning procedure, on Day 4 animals were placed in a plexiglass chamber and given charcoal filtered air for 1.5 hours prior to any odor delivery. Rats then received a 5-minute exposure to a control odor, octanol, followed by 20 minutes of clean air. Finally, animals were exposed to the conditioned odor (terpinene) for 5 minutes and then immediately anaesthetized by isoflurane gas and decapitated. Brains were removed within 2 minutes of sacrificed and flash frozen in a dry ice/ethanol slurry and placed at -80ºC until they were sliced for catFISH analysis. *H1a* is an IEG expressed in the nucleus within 30 minutes following exposure to a stimulus, while *Arc* is expressed in the nucleus within 5 minutes following a stimulus, so any cells that stained positively for *H1a* (green arrow) were assumed to be activated by the novel odor, while those showing *Arc*+ staining (red arrow) were activated by the conditioned odor. Nuclei showing double labeling were active following both odor exposures (white arrow).


A1 Main Olfactory Bulb
DL
VM
500 µm
A3
Dorsolateral (DL)
Ventromedial (VM)
Ratio (Arc+/Homer1a+)
O-/S+
O+/S-
O+/S+
Companion
A2
O-/S+
O+/S-
O+/S+
Companion
Dorsolateral (DL)
Ventromedial (VM)
A4
% of total DAPI+ cells
Homer+
Arc+
Both+
B1 Accessory Olfactory Bulb
500 µm
B3
Ratio (Arc+/Homer1a+)
O-/S+
O+/S-
O+/S+
Companion
B2
O-/S+
O+/S-
O+/S+
Companion
B4
% of total DAPI+ cells
Homer+
Arc+
Both+


C1 Piriform Cortex (anterior)
500 µm
C3
Ratio (Arc+/Homer1a+)
O-/S+
O+/S-
O+/S+
Companion
C2
O-/S+
O+/S-
O+/S+
Companion
C4
% of total DAPI+ cells
Homer+
Arc+
Both+
D1 Piriform Cortex (posterior)
500 µm
D3
Ratio (Arc+/Homer1a+)
O-/S+
O+/S-
O+/S+
Companion
D2
O-/S+
O+/S-
O+/S+
Companion
D4
% of total DAPI+ cells
Homer+
Arc+
Both+


E1 Olfactory Tubercle
500 µm
E3
Ratio (Arc+/Homer1a+)
O-/S+
O+/S-
O+/S+
Companion
E2
O-/S+
O+/S-
O+/S+
Companion
E4
% of total DAPI+ cells
Homer+
Arc+
Both+
F1 Medial Amygdala
500 µm
F3
Ratio (Arc+/Homer1a+)
O-/S+
O+/S-
O+/S+
Companion
F2
O-/S+
O+/S-
O+/S+
Companion
F4
% of total DAPI+ cells
Homer+
Arc+
Both+


G1 Lateral Amygdala
500 µm
G3
Ratio (Arc+/Homer1a+)
O-/S+
O+/S-
O+/S+
Companion
G2
O-/S+
O+/S-
O+/S+
Companion
G4
% of total DAPI+ cells
Homer+
Arc+
Both+
H1 Central Amygdala (medial)
500 µm
H3
****
****
****
****
Ratio (Arc+/Homer1a+)
O-/S+
O+/S-
O+/S+
Companion
H2
O-/S+
O+/S-
O+/S+
Companion
H4
**
***
*
% of total DAPI+ cells
Homer+
Arc+
Both+

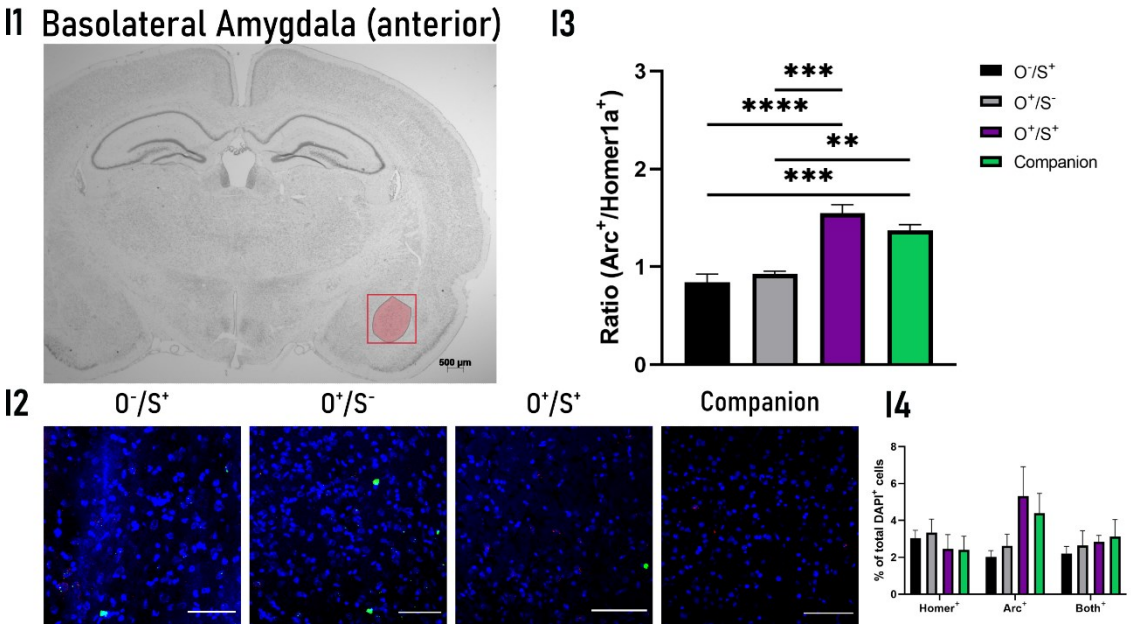

I1 Basolateral Amygdala (anterior)
I3
I2
I4
J1 Basolateral Amygdala (posterior)
J3
J2
J4
O⁻/S⁺
O⁺/S⁻
O⁺/S⁺
Companion
Ratio (Arc⁺/Homer1a⁺)
% of total DAPI⁺ cells
Homer⁺
Arc⁺
Both⁺
500 µm

Figure 10. ***Compared to a control odor, an aversively conditioned odor activates the same classic fear pathway in the amygdala, but different olfactory bulb structures depending on the unconditioned stimulus.*** **Panel 1)** Nissl-stained coronal slices illustrating the area analyzed. **Panel 2)** Representative images of each area from each condition with green arrowheads depicting *H1a*+ signal, red arrowheads depicting *Arc*+ signal, and white arrowheads depicting nuclei expressing both *Arc* and *H1a*. **Panel 3)** The y-axes depict the ratio of *Arc*+ cells (those responding to the conditioned odor) to *H1a*+ cells (which reflect cellular activation to the control odor) within the same animal. *Arc*+ cells are the cells activated by the conditioned odor terpinene. *H1a*+ cells are the cells activated by the control odor octanol. O-/S+, shock only rats; O+/S-, odor only rats that were caged alone; O+/S+, odor/shock conditioned rats; Companion, odor only rats that were caged with odor/shock conditioned rats. **A)** Classically conditioned animals exhibit a higher proportion of neurons responding to the conditioned versus control odor in the dorsolateral region of the MOB but not the ventromedial region. **B)** AOB neurons in companion animals show preferential response to conditioned versus control odor. **C-E)** No differences in response to conditioned vs control odor for any group in the piriform cortex or olfactory tubercle. **F-G)** No differences in response to conditioned vs control odor for any group in the medial or LA. **H)** Both classically conditioned and companion animals showed preferential activity to the conditioned vs control animal in the medial division of the central amygdala. **I)** Both classically conditioned and companion rats exhibit a higher proportion of neurons responding to conditioned over control odor in the aBLA, **J)** while no differences were found in the posterior BLA. **Panel 4)** % of DAPI cells containing *H1a*, *Arc*, or both **A-G)** No significant difference between *Arc*+, *H1a*+, or double labelled cells in the olfactory bulbs, piriform cortex, nor olfactory tubercle. **H)** Both trained groups showed a higher activation of *Arc*+ neurons in the central nucleus of the amygdala compared to controls. **I-J)** No significant difference between *Arc*+, *H1a*+, or double labelled cells in the medial, lateral, or BLA. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$, ****$p<0.0001$. Error bars, mean ± SEM.

in tissue processing. This approach enhanced the signal to noise ratio and resulted in the use of fewer animals for statistical comparisons (compare panels 3 to 4 in Figure 10). Significant differences in the *Arc*/*H1a* ratio between groups were observed in the dorsal lateral MOB ($F_{3,12}$ = 4.89, p = 0.02; Figure 10A3), AOB ($F_{3,12}$ = 4.30, p = 0.03; Figure 10C3), aBLA ($F_{3,12}$ = 23.05, p = 2.87E-5; Figure 10 J3) and medial portion of the CeA ($F_{3,12}$ = 49.65, p = 4.87E-7; Figure 10 G3). O^+/S^+ rats showed significantly more activation in the dorsal lateral MOB (2.35 ± 0.37, n = 4) than O^-/S^+ (0.95 ± 0.15; n = 4, t = 3.45, p = 0.022), and O^+/S^- rats (1.07 ± 0.07, n = 4, t = 3.15, p = 0.037; Figure 10 B3). O^+/S^+ rats also showed enhanced activation in the aBLA (1.54 ± 0.09, n = 4) compared to O^-/S^+ (0.84 ± 0.9, n = 4, t = 7.04, p = 8.12E-5), and O^+/S^- rats (0.93 ± 0.03, n = 4, t = 6.15, n = 4, p = 2.99E-4; Figure 10 J3); and in the CeA (1.96 ± 0.10, n = 4) compared to O^-/S^+ (0.88 ± 0.07, n = 4, t = 9.13, p = 5.71E-6), and O^+/S^- rats (0.92 ± 0.08, t = 8.77, p = 8.70E-6; Figure 10 G3). In contrast, O^+/Comp rats showed more activation in the AOB (2.14 ± 0.47, n = 4), significantly different from O^+/S^+ (1.06 ± 0.33, n = 4, t = 3.20, p = 0.045; Figure 10 C3). Interestingly however, the O^+/Comp rats also showed enhanced activation in the aBLA (1.37 ± 0.06, n = 4) compared to O^-/S^+ (t = 5.28, p = 0.001), and O^+/S^- rats (t = 4.38, p = 0.005; Figure 10 J3); and in the CeA (1.88 ± 0.08, n = 4), compared to O^-/S^+ (t = 8.46, p = 1.27E-5), and O^+/S^- rats (t = 8.10, p = 1.99E-5; Figure 10 G3). No significant differences were found in any of the other areas analyzed (Figure 10). These results suggest that MOB and AOB hold the initial classically conditioned odor and socially transferred stress conditioned odor memory traces respectively and then both conditioning pathways converge on the amygdala fear circuitry to generate conditioned freezing behaviour upon re-exposure to the conditioned odor.

3.1.3. Pheromone mediates the conditioned fear in conspecifics

Rats emit a series of ultrasonic calls when confronting distressful stimuli[335]. To determine whether rats transmit a fear state that supports conditioning by ultrasonic or alarm pheromone communication, I performed additional experiments. One group of rats were exposed to the previously identified shock-induced alarm pheromone molecules 4-methylpentanal and hexanal[39] during the terpinene exposure (Ph-T). Another group of rats were exposed to the soiled bedding (SB) from the donor shocked rats but were never physically in contact with the donor rats. A subgroup of the SB rats were conditioned with terpinene (SB-T) while another subgroup was conditioned with octanol (SB-Oc). All rats were tested for freezing in the presence of terpinene and octanol separately (Figure 11).

There was a significant treatment effect to the terpinene ($F_{3,21}$ = 6.37, p = 0.003; Figure 11B) and octanol ($F_{3,21}$ = 9.04, p = 4.8E-4; Figure 11C). Consistent with pheromone-mediation of odor-specific conditioning, when trained with terpinene as the conditioned odor, the Ph-T group (20.58 ± 5.39, n = 4) showed significantly more freezing to terpinene than the control O^+/S^- group (4.58 ± 1.93, n = 9, t = 2.89, p = 0.041). The SB-T group also showed more freezing (23.65 ± 6.67, n = 6) compared to the O^+/S^- group (t = 3.94, p = 0.004; Figure 11B). In contrast, when SB rats were tested with octanol, the SB-Oc group showed significantly more freezing (25.32 ± 3.86; n = 6) than the SB-T group (9.87 ± 4.20; n = 6, t = 3.32, p = 0.020), or the O^+/S^- group (6.45 ± 2.26, n = 9, t = 4.44, p = 0.001; Figure

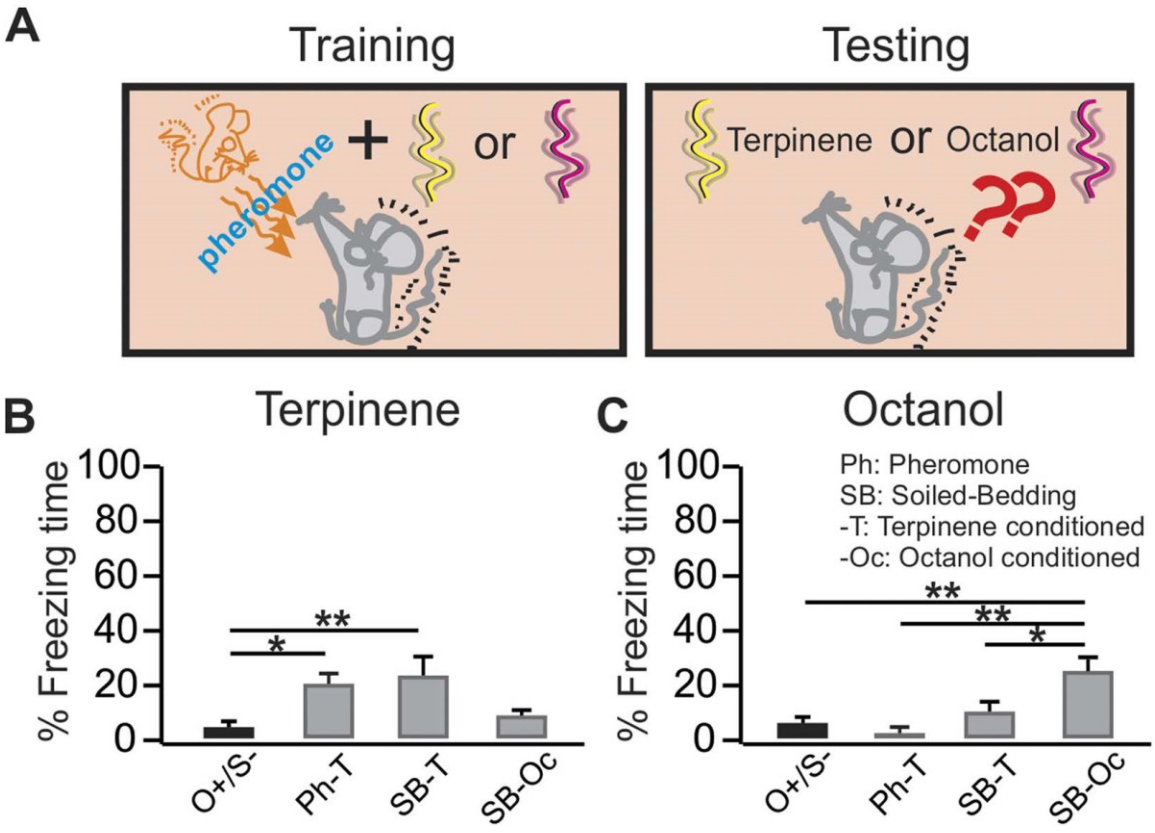


Figure 11. ***Alarm pheromone mediates the fear learning in companion rats.*** **A)** Schematics of the odor conditioning and testing paradigm. **B)** Percentage freezing time during the testing to the odor terpinene. **C)** Percentage freezing time during the testing to the odor octanol. O+/S−, odor only rats that were caged alone; Ph-T, terpinene odor exposed rats that were conditioned with previously identified alarm pheromone molecules; SB-T, terpinene exposed rats that were conditioned with soiled bedding; SB-O, octanol exposed rats that were conditioned with soiled bedding. *p<0.05, **p<0.01. Error bars, mean±SEM

11C). These experiments establish that rats can communicate fear and induce specific odor fear learning via pheromone information.

3.1.4. Basolateral amygdala serves as the common plasticity locus for classical and pheromone conditioning

Infusion of the NMDA receptor antagonist D-APV bilaterally into the BLA during either classical (O^+/S^+) or pheromone conditioned training (using alarm pheromone molecules 4-methylpentanal and hexanal as the US; O^+/Ph) prevented both forms of learning. In O^+/S^+ rats, the D-APV infused group (4.17 ± 1.77, n = 4) showed significantly less freezing than the saline infused control group (72.7 ± 10.20; n = 3, t = 7.79, p = 5.57E-4; Figure 12 A). In O^+/Ph rats, the D-APV infused group (1.75 ± 1.42, n = 4) also spent significantly less time freezing than the saline infused group (29 ± 3.51; n = 3, t = 8.05, p = 4.80E-4; Figure 12 B). This establishes that the BLA is a common plasticity site for both classical odor conditioning and pheromone learning (see Figure 12 C for a proposed pathway).

To further illuminate the routes of information processing from the upstream structures, we injected the retrograde tracer cholera toxin subunit B conjugated to Alexa Fluor-488 unilaterally into the BLA. One week later, we observed robust labeling of neurons in the PC, MeA and CoA, with sparser labeling in the MOB and AOB (Figure 13).

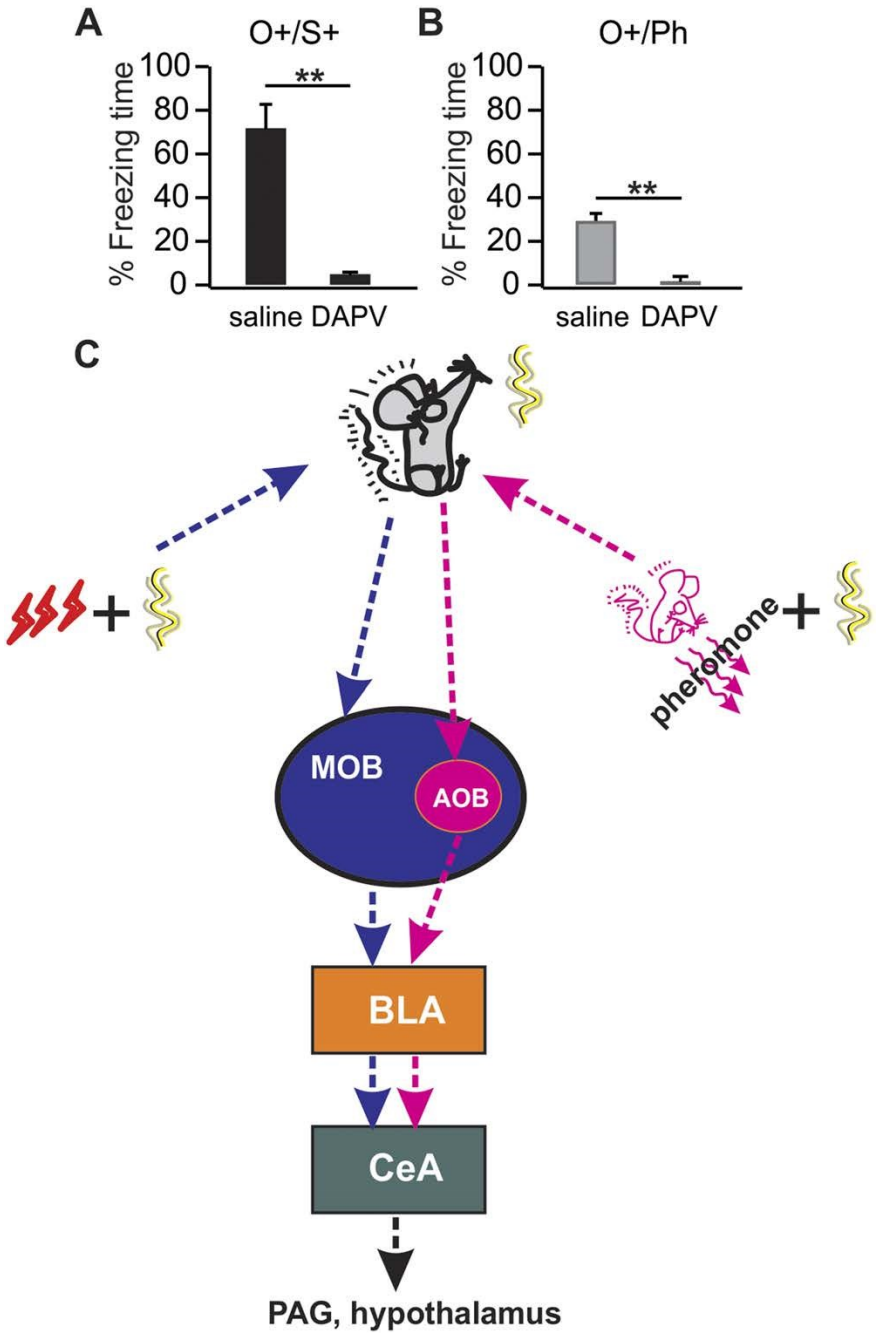


Figure 12. ***D-APV bilateral BLA infusions prevents both pheromone odor conditioning and classical odor conditioning.*** **A)** Percentage freezing time during the testing to the conditioned odor terpinene in odor/shock conditioned (O^+/S^+) rats, infused with either D-APV or saline. **B)** Percentage freezing time during the testing to the conditioned odor terpinene in pheromone molecule conditioned rats (O^+/Ph), infused with either D-APV or saline. **p<0.01. Error bars, mean±SEM. **C)** Converging pathways of classical and pheromone fear conditioning in rats. MOB: main olfactory bulb; AOB: accessory olfactory bulb; BLA: BLA; CeA: central amygdala; PAG: periaqueductal grey.

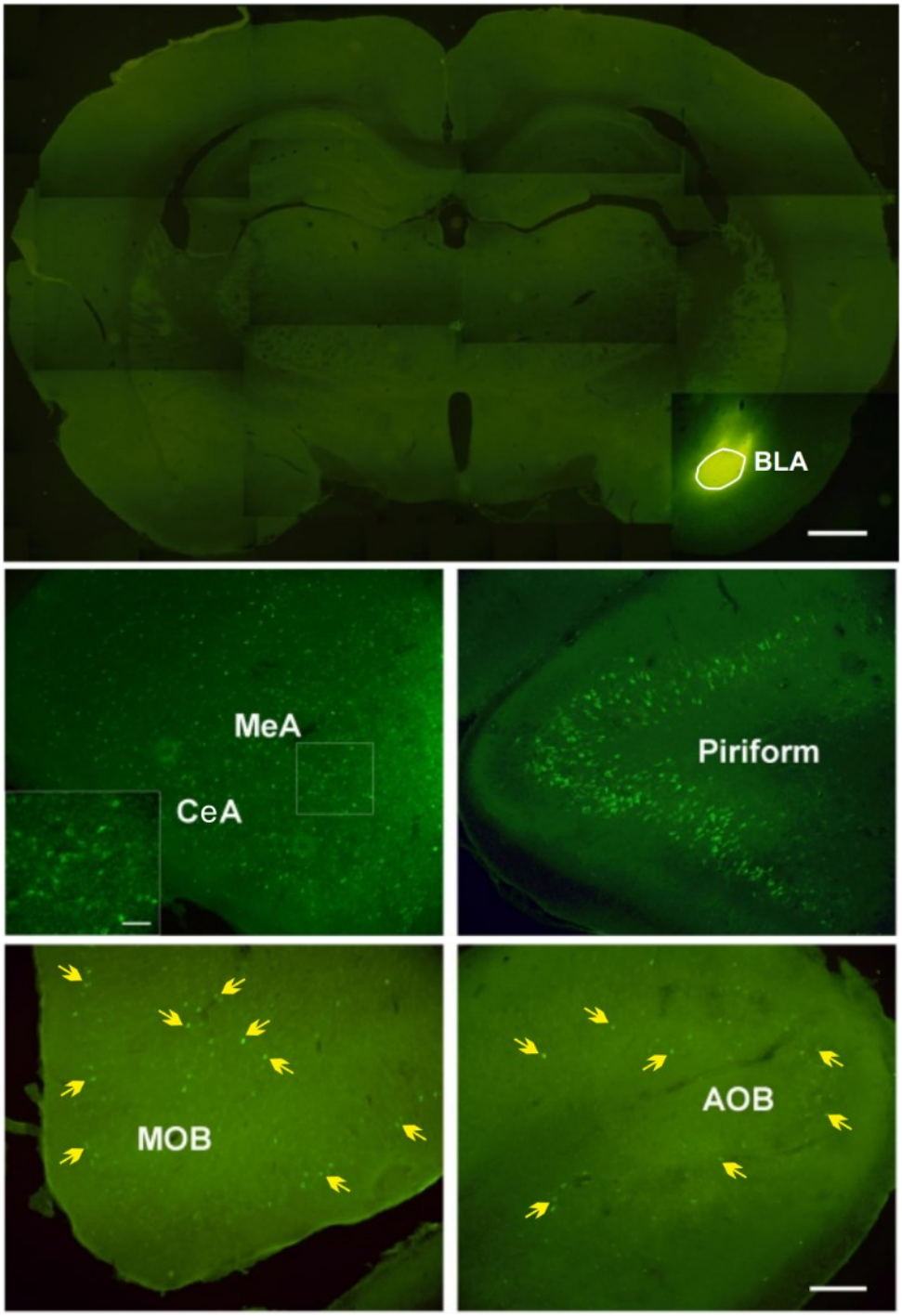


Figure 13. ***BLA receives projections from MOB, AOB, piriform cortex, and other amygdalar subnuclei.*** Retrograde tracer was injected unilaterally into the aBLA. MOB: main olfactory bulb; AOB: accessory olfactory bulb; BLA: BLA; CeA: central amygdala; PAG: periaqueductal grey. Arrows indicate example labelled cells in the MOB and AOB.

Thus, odor and pheromone information could directly transmit to the BLA from the MOB and the AOB, or via the MeA, CoA, or PC[336].

3.2. Second order odor fear learning is supported by contextual and auditory first order fear conditioning

3.2.1. Contextual or auditory first order fear conditioning supports second order odor fear conditioning

When rats are exposed to pairings of a novel odor (terpinene) and a footshock (O^+/S^+), a first-order conditioned fear memory is formed (Figure 14A1) and increased freezing is observed in experimental rats upon subsequent exposure to the odor the following day (58.85 ± 7.29, n=10) compared to control rats who are exposed to the odor in the absence of shock (O^+/S^-; 3.77 ± 2.07, n=11; t=6.961, p<0.0001; Figure 14A2). If a context is used as the first conditioned stimulus (CS1) and is subsequently paired with an odor in the absence of shock (Figure 14 B1), experimental rats that have received both pairings will freeze when re-exposed to the conditioned odor (CS2) in a novel context (41.04 ± 6.68, n=7 p=0.0310; Figure 14 B2, "Odor CS2") significantly longer than control rats who did not receive pairing of the CS1 and CS2 (10.28 ± 3.05, n=7), although both groups freeze for the same amount of time upon re-exposure to the CS1 context (experimental 63.80 ± 10.13, n=7; control 53.72 ± 11.30, n=7; p=0.7215; Figure 14B2,

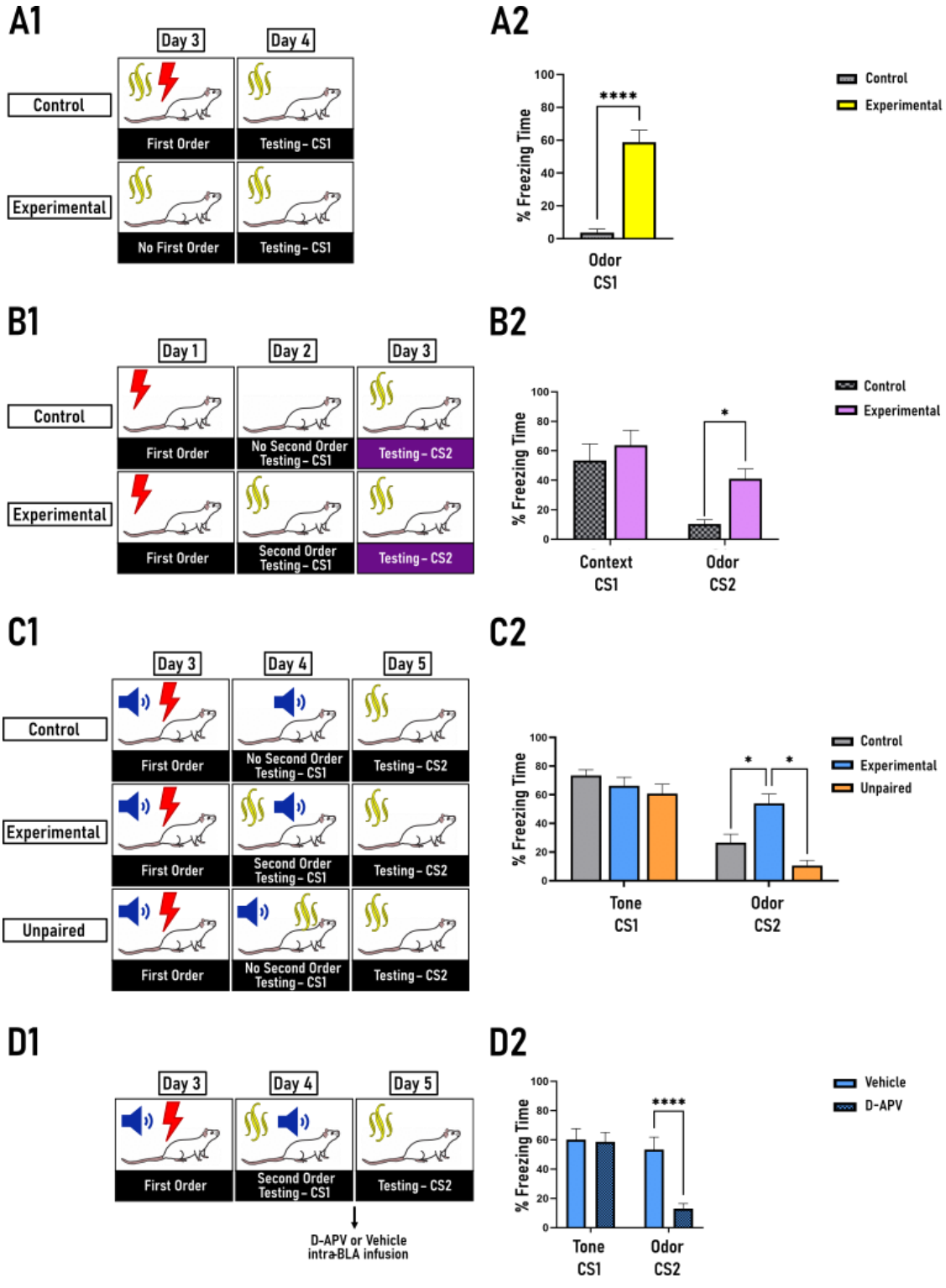

A1
Day 3
Day 4
Control
First Order
Testing – CS1
Experimental
No First Order
Testing – CS1
A2
% Freezing Time
****
Odor
CS1
Control
Experimental
B1
Day 1
Day 2
Day 3
Control
First Order
No Second Order
Testing – CS1
Testing – CS2
Experimental
First Order
Second Order
Testing – CS1
Testing – CS2
B2
% Freezing Time
*
Context
CS1
Odor
CS2
Control
Experimental
C1
Day 3
Day 4
Day 5
Control
First Order
No Second Order
Testing – CS1
Testing – CS2
Experimental
First Order
Second Order
Testing – CS1
Testing – CS2
Unpaired
First Order
No Second Order
Testing – CS1
Testing – CS2
C2
% Freezing Time
*
*
Tone
CS1
Odor
CS2
Control
Experimental
Unpaired
D1
Day 3
Day 4
Day 5
First Order
Second Order
Testing – CS1
Testing – CS2
D-APV or Vehicle
intra-BLA infusion
D2
% Freezing Time
****
Tone
CS1
Odor
CS2
Vehicle
D-APV

Figure 14. ***Establishment of second order conditioning.*** Three different behavioural paradigms were used to compare regional activity in response to odor as a first order conditioned cue (CS1), as a second order conditioned cue (CS2) with context as CS1, and as a CS2 with tone as a CS1. **A) Experiment 3:** Control animals were simply given odor during training, while experimental animals were trained to associate odor with shock. **A2)** Experimental animals spent significantly more time freezing compared to controls. **B) Experiment 4:** Both control and experimental groups were trained to associate the context with shock. The following day, experimental animals received odor (CS2) in the feared context while control animals were simply placed in the context for the same amount of time; freezing behaviour was recorded for the five-minute session. The final day consisted of exposure to the odor in a novel context for both groups while freezing behaviour was recorded. **B2)** Both groups showed a high level of freezing to the context as CS1 but only animals who were exposed to odor in the feared context displayed freezing behaviour in the novel context in response to the CS2. **C) Experiment 5:** Control, experimental, and unpaired groups were trained to associate a tone with a shock following two daily 30 min sessions of habituation to the shock chamber. The following day, control animals were simply exposed to the feared tone, experimental animals were exposed to the feared tone and a novel odor simultaneously, and the unpaired group were exposed to both tone and odor but the exposures were separated by a 30-minute interval; freezing behaviour was recorded. The final day consisted of exposure to the same odor from the previous day while freezing behaviour was recorded. **C2)** All three groups displayed a high degree of freezing behaviour upon exposure to the tone CS1, but only the experimental group which had the CS1 and CS2 paired, showed freezing behaviour when tested with the odor alone on the final day. **D1 Experiment 6:** The same behavioural paradigm used Experiment 5 was employed, with the animals receiving an intra-BLA infusion of either D-APV or vehicle. **D2)** Those given vehicle showed a high degree of freezing to both CS1 and CS2, while animals given D-APV immediately following SOC did not exhibit fearful behaviour following exposure to the trained odor. Statistics in **A** were independent t-tests, and **B-D** were mixed ANOVA. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$, ****$p<0.0001$. Error bars, mean ± SEM.

"Context CS1"), indicating that FOC was successful in both groups. Similarly, if the CS1 is a tone and subsequently paired with a novel odor, the odor becomes a CS2 (Figure 14C1) and experimental rats that have experienced both pairings display increased freezing when re-exposed to the CS2 odor in the absence of the CS1 tone (53.95 ± 6.61, n=7) compared to both control (26.55 ± 5.89, n=6; p=0.0265) and unpaired groups (10.61 ± 3.60, n=6; p=0.0134; Figure 14C2, "Odor CS2"), while freezing to the tone did not differ among the three groups (experimental 66.40 ± 5.78, n=7; control 73.5 ± 3.97, n=6; unpaired 60.83± 6.51, n=6; p>0.9999; Figure 14C2, "Tone CS1").

Prior research has shown that glutamatergic signaling via NMDARs in the BLA is necessary for several types of fear conditioning[337–339]. I next sought to determine whether NMDAR activation in the BLA was required for second-order conditioning. Because context encapsulates cues from multiple sensory modalities, I utilized the odor-tone model from Experiment 5. When D-APV was infused bilaterally into the BLA, freezing to odor CS2 was significantly reduced (12.96 ± 3.53, n=7) compared to when the same animals were trained subsequently with vehicle and a different CS2 odor (53.32 ± 8.55, n=7; p<0.0001; Figure 14D2, "Odor CS2"), while both groups froze similarly to the tone CS1 (D-APV 58.72 ± 6.38, n=7; vehicle 60.19 ± 7.41, n=7; Figure14D2, "Tone CS1"). These results highlight the fact that odor is a very salient stimulus for rodents, show that it can be used to invoke first- or second-order fear conditioning, and is dependent on NMDAR activity in the BLA.

The experiments described in this thesis utilized both male and female rats, and there is some evidence implicating sex differences in the processing and expression of fear memory especially in humans[340,341], as such it is important to assess if any sex differences exist across the current set of experiments. A two-way analysis of variance (ANOVA) with "sex" and "condition" as independent variables revealed a significant effect of condition ($F_{2,20} = 24.433$, $p<0.001$), while sex had no effect on freezing behaviour ($p=0.373$). Mixed analysis of variance revealed no effect of sex ($F_{2,9} = 1.988$, $p=0.193$) but a significant effect of condition ($F_{2,9} = 9.344$, $p=0.006$), and no significant interaction ($F_{2,9} = 0.580$, $p=0.580$, Figure 15 A). Likewise, no effect of sex ($F_{2,29} = 0.358$, $p=0.709$) but a significant effect of condition on freezing behaviour was seen in SOC with tone as CS1 ($F_{4,58} = 9.170$, $p<0.001$; Figure 15 B) with no significant interactions ($F_{4,58} = 0.567$, $p=0.687$; Figure 15C). Paired t-tests for the experiments using D-APV revealed no sex differences across freezing to any of the cues at any point in the experiments ($F_{4,2} = 3.002$, $p=0.265$; Figure 15D).

3.2.2. Similar circuitry utilized by multiple types of fear memories with key differences – the lateral amygdala and auditory cortex are active during recall of auditory-associated odor fear memories

Multisensory memories presumably incorporate neurons from many specialized brain regions into their engrams, but it is unknown whether a SOC fear memory and a FOC fear memory utilize the same, or distinct, neurocircuitry. I utilized cFos immunohistochemistry to label neurons active during recall of each type of odor fear

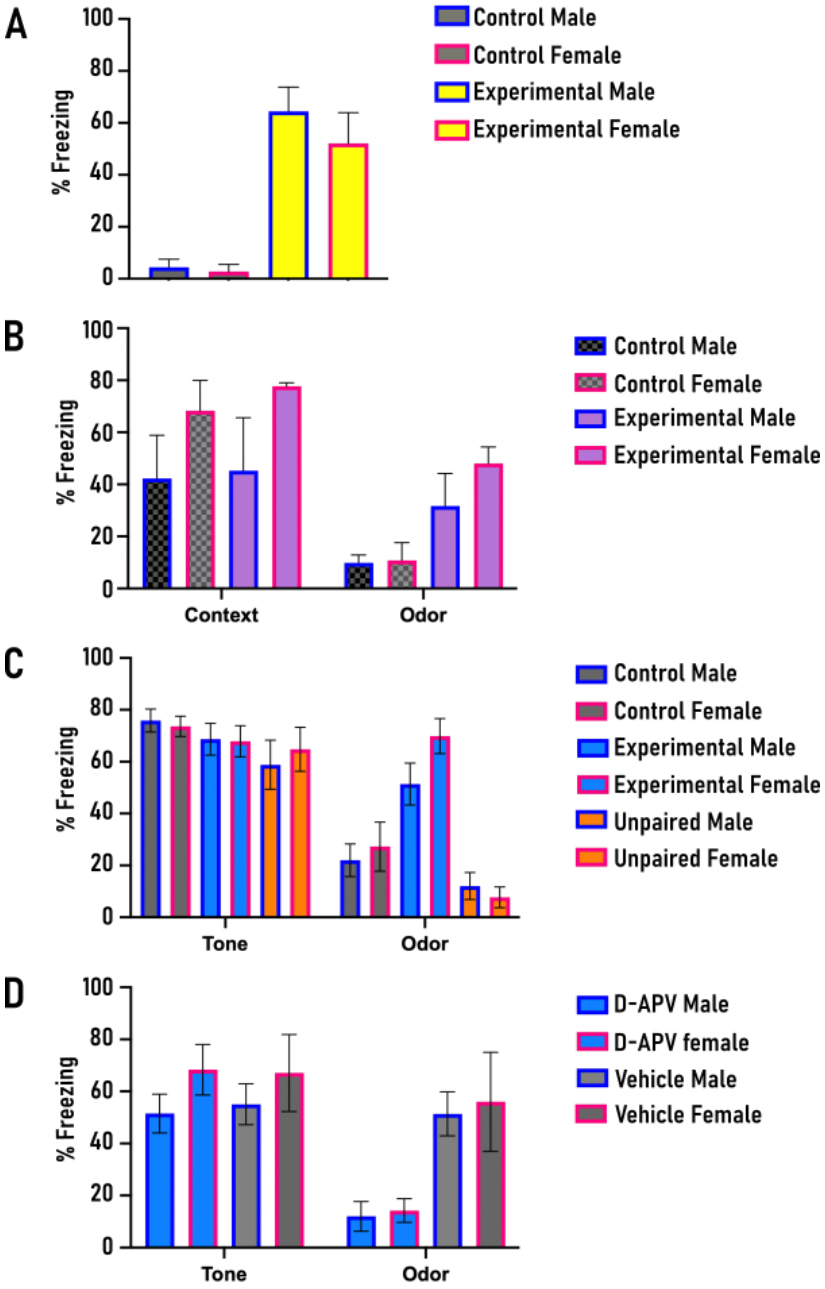


Figure 15. ***No sex differences in first- or second-order conditioned odor fear memory expression.*** **A)** Freezing in control and experimental groups to a first-order conditioned odor fear memory. **B)** Freezing in control and experimental groups to a contextual CS1 and odor CS2. **C)** Freezing in control, experimental, and unpaired groups to an auditory CS1 and odor CS2. **D)** Freezing in D-APV and vehicle to an auditory CS1 and an odor CS2.

memory to elucidate which brain regions were involved and identify one or several area(s) of convergence of information. I then compared areas involved in fear conditioning, relevant sensory cortices, and regions involved in the processing of contextual cues across the three types of odor memory described in Experiments 3-5 (first-order, second-order with context, second-order with tone) to trace the engram complex and determine differences.

I measured brain activation in several key areas of the olfactory fear learning pathway, areas devoted to the processing of auditory stimuli, as well as regions involved in learning, memory, and fear in general, to elucidate the shared and distinct neural pathways involved in first- or second-order odor fear conditioning. I observed no significant differences in the anterior piriform cortex in any experimental paradigm (Figure 16), but significant increases in the percentage of $cFos^{+}$ cells/mm^2 in the posterior piriform of experimental animals (1124.13 ± 101.72, n=11) compared to control (774.06 ± 110.39, n=11; p=0.0303) when odor was used as a first order stimulus or a second order stimulus with context (experimental 1013.90 ± 111.58, n=7; control: 589.11 ± 59.02, n=7; p=0.0056; Figure 17). When odor was used as a second order stimulus with tone as CS1, one way ANOVA reveals a significant between-groups effect (p=0.019) and post hoc tests show that experimental animals (832.44 ± 54.74, n=6) exhibit significantly higher activation in the pPC compared to unpaired (584.27 ± 55.14, n=6; p=0.017), but not control groups (685.24 ± 53.95, n=6; p=0.228; Figure 17). In the auditory cortex, increased cFos expression was

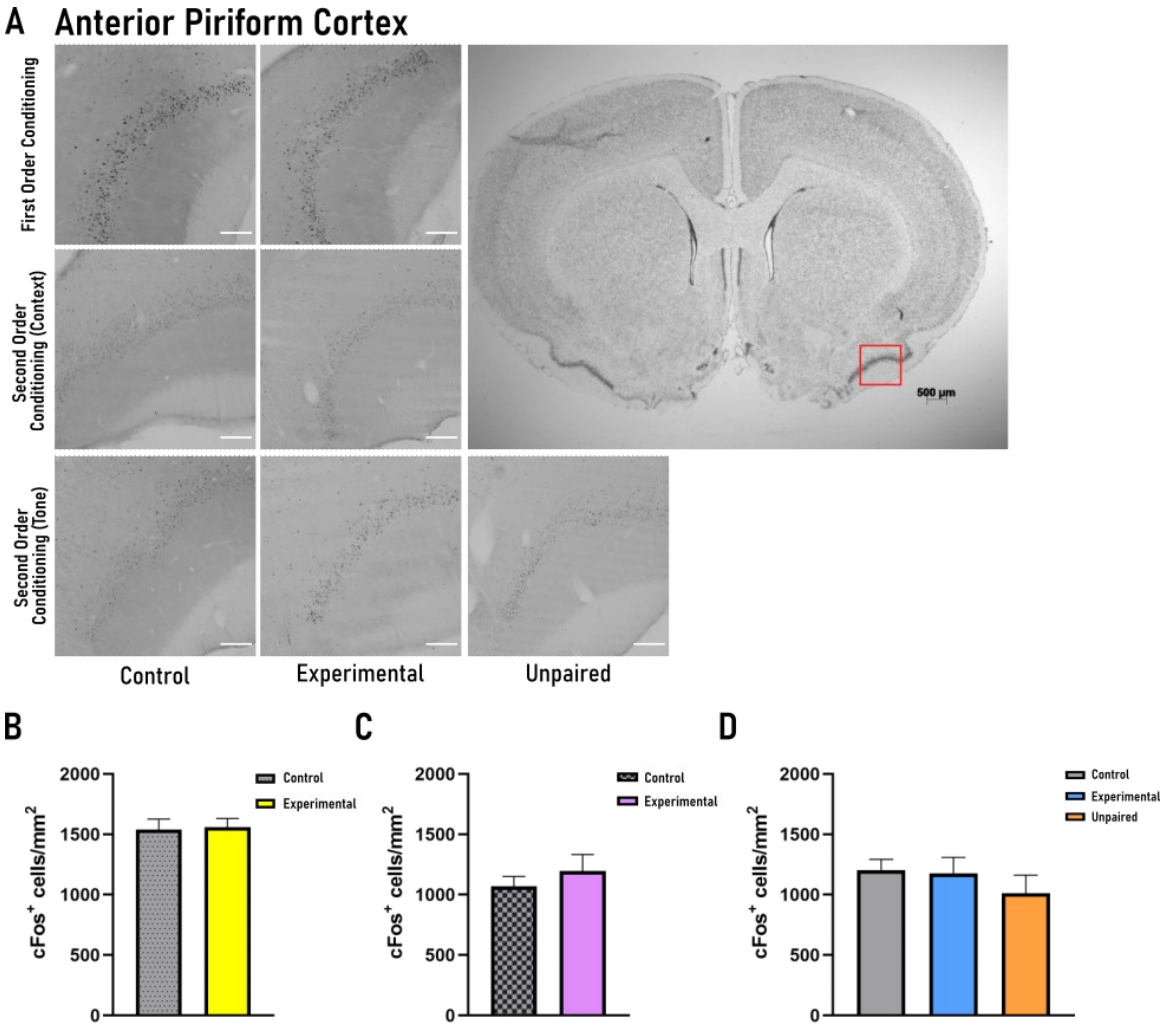


Figure 16. ***Anterior piriform cortex is not activated by recall of odor fear memory conditioned by shock, feared context, or feared tone.*** **A)** Nissl image depicts the location of the anterior piriform cortex, and the red box outlines the region analyzed. Representative cFos images are shown for each experimental condition for FOC (top), SOC with context (middle), and SOC with tone (bottom). Scale bars are 150um. **B)** Control and experimental groups expressed similar levels of cFos following recall of a first order odor fear memory. **C)** Control and experimental animals expressed similar levels of cFos following recall of a second order odor fear memory with context as CS1. **D)** Control, experimental, and unpaired animals expressed similar levels of cFos following recall of a second order odor fear memory with tone as CS1. Statistics for FOC and SOC with context were independent t-tests, and statistics for SOC with tone were one-way ANOVAs. Error bars, mean ± SEM.

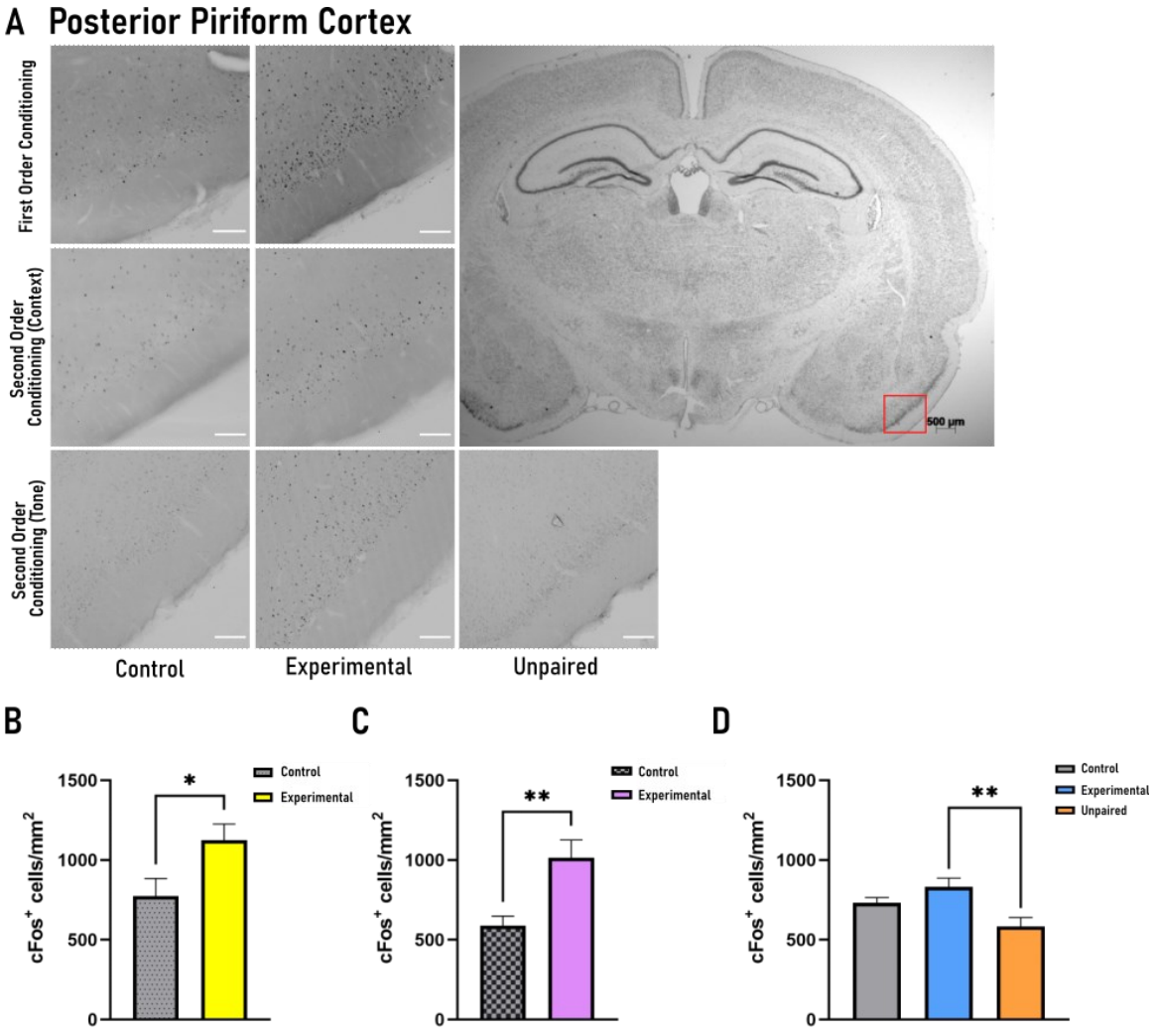


Figure 17. ***Posterior piriform cortex is activated by recall of odor fear memory conditioned by shock, feared context, or feared tone.*** **A)** Nissl image depicts the location of the posterior piriform cortex, and the red box outlines the region analyzed. Representative cFos images are shown for each experimental condition for FOC (top), SOC with context (middle), and SOC with tone (bottom). Scale bars are 150um. **B)** Experimental groups expressed increased levels of cFos following recall of a first order odor fear memory. **C)** Experimental animals expressed elevated levels of cFos following recall of a second order odor fear memory with context as CS1. **D)** Experimental animals expressed higher levels of cFos compared to unpaired animals following recall of a second order odor fear memory with tone as CS1. Statistics for FOC and SOC with context were independent t-tests, and statistics for SOC with tone were one-way ANOVAs. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$, ****$p<0.0001$. Error bars, mean ± SEM.

found in the experimental condition (1033.93 ± 94.88, n=6), compared to unpaired (494.43 ± 80.60, n=6; p=0.001) and control (565.11 ± 60.29, n=6; p=0.003) for the tone-odor paradigm but not the context-odor (experimental 746.45 ± 129.00, n=7; control 844.13 ± 67.44, n=7; p=0.467) or odor CS1 experiments (experimental 790.79 ± 58.56, n=5; control 763.7 ± 66.03, n=5; p=0.773; Figure 18).

In the DH an increase in cFos positive cells in the CA1 of the experimental (748.89 ± 93.28, n=7) compared to the control group (301.41 ± 84.34, n=7; p=0.004; Figure 19) in the context-odor paradigm was observed. Unexpectedly, increased $cFos^+$ cells per mm^2 were found in DH CA1 for the tone-odor (experimental 436.86 ± 32.08, n=6; control 138.71 ± 31.32, n=6, p<0.001; unpaired 100.12 ± 28.27, n=6, p<0.001) and odor as CS1 (experimental 517.77 ± 56.70, n=6; control 249.81 ± 46.64, n=6; p=0.004; Figure 19) experiments. This pattern of expression was mirrored in DH CA3, an increase in cFos positive cells in the CA3 of the experimental group (368.83 ± 52.14, n=7) compared to the control group (175.66 ± 62.36, n=7; p=0.006) was observed in the context-odor paradigm, the tone-odor paradigm (experimental 312.04 ± 40.25, n=6; control 174.27 ± 27.01, n=6, p=0.016; unpaired 153.44 ± 19.01, n=6, p=0.006), and experiments where odor served as CS1 (experimental 286.97 ± 53.87, n=6; paired 148.54 ± 9.63, n=6; p=0.030; Figure 20).

In terms of fear conditioning circuitry, increased cFos was observed in the BLA following re-exposure to the odor, whether it acted as a CS1 (experimental 224.62 ± 29.70,

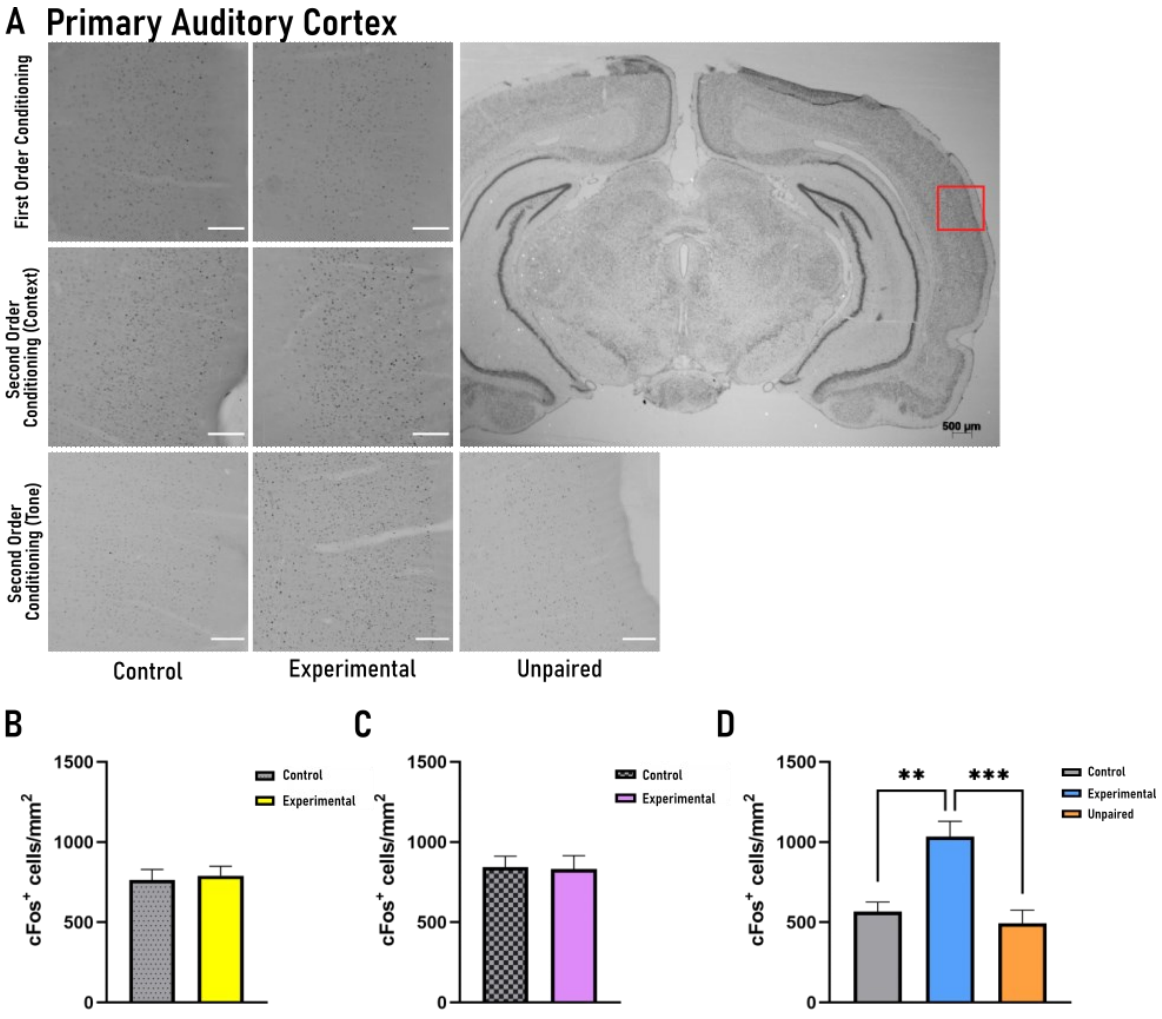


Figure 18. ***Primary auditory cortex is activated by recall of odor fear memory conditioned by feared tone, not by shock or feared context.*** **A)** Nissl image depicts the location of the posterior piriform cortex, and the red box outlines the region analyzed. Representative cFos images are shown for each experimental condition for FOC (top), SOC with context (middle), and SOC with tone (bottom). Scale bars are 150um. **B)** Control and experimental groups expressed similar levels of cFos following recall of a first order odor fear memory. **C)** Control and experimental animals expressed similar levels of cFos following recall of a second order odor fear memory with context as CS1. **D)** Experimental animals expressed higher levels of cFos compared to both unpaired and control animals following recall of a second order odor fear memory with tone as CS1. Statistics for FOC and SOC with context were independent t-tests, and statistics for SOC with tone were one-way ANOVAs. *p < 0.05, **p < 0.01, ***p<0.001, ****p<0.0001. Error bars, mean ± SEM.

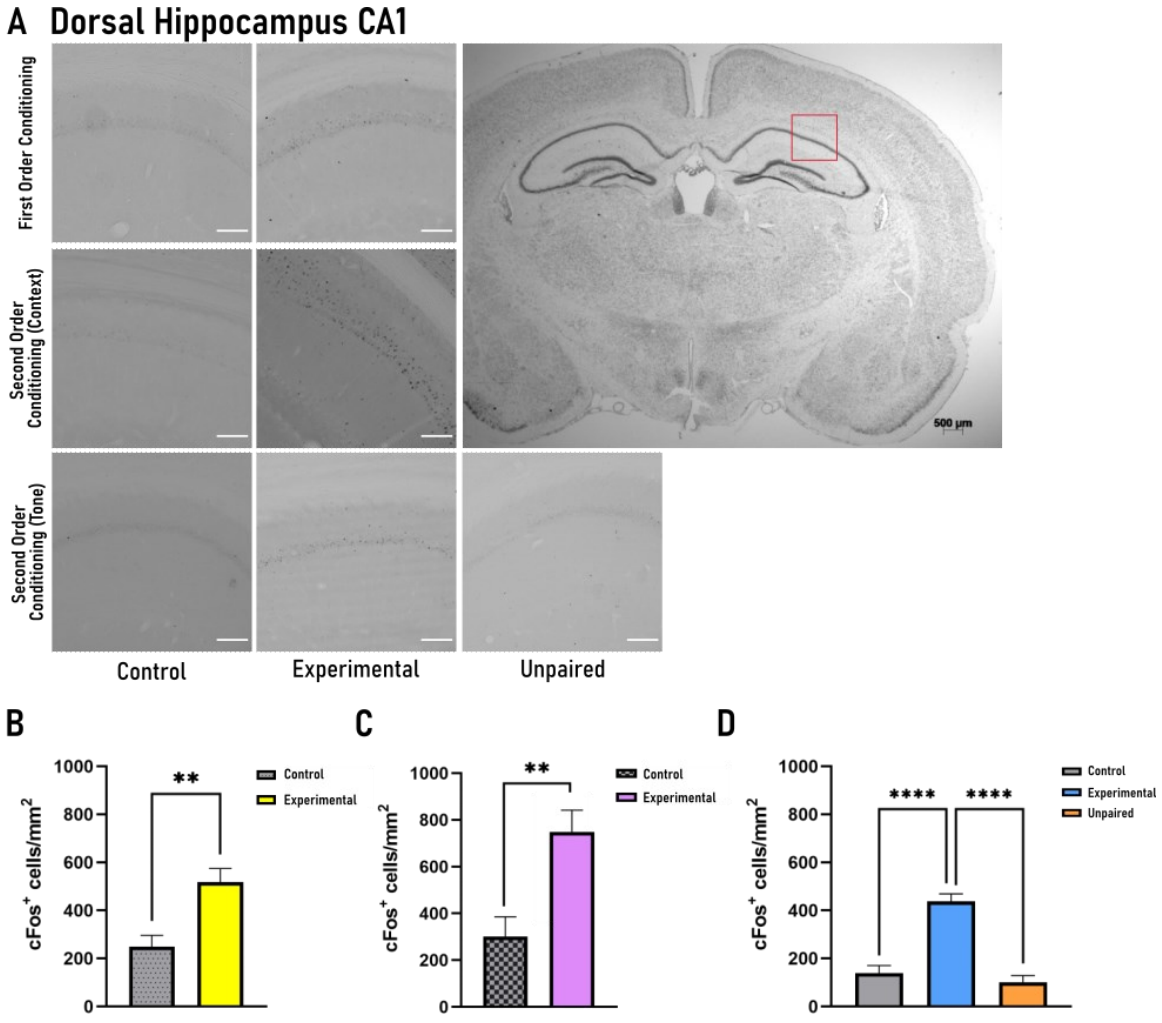


Figure 19. ***Dorsal hippocampus CA1 is activated by recall of odor fear memory conditioned by shock, feared context, or feared tone.*** **A)** Nissl image depicts the location of the posterior piriform cortex, and the red box outlines the region analyzed. Representative cFos images are shown for each experimental condition for FOC (top), SOC with context (middle), and SOC with tone (bottom). Scale bars are 150um. **B)** Experimental groups expressed increased levels of cFos following recall of a first order odor fear memory. **C)** Experimental animals expressed elevated levels of cFos following recall of a second order odor fear memory with context as CS1. **D)** Experimental animals expressed higher levels of cFos compared to unpaired and control animals following recall of a second order odor fear memory with tone as CS1. Statistics for FOC and SOC with context were independent t-tests, and statistics for SOC with tone were one-way ANOVAs. *p < 0.05, **p < 0.01, ***p<0.001, ****p<0.0001. Error bars, mean ± SEM.

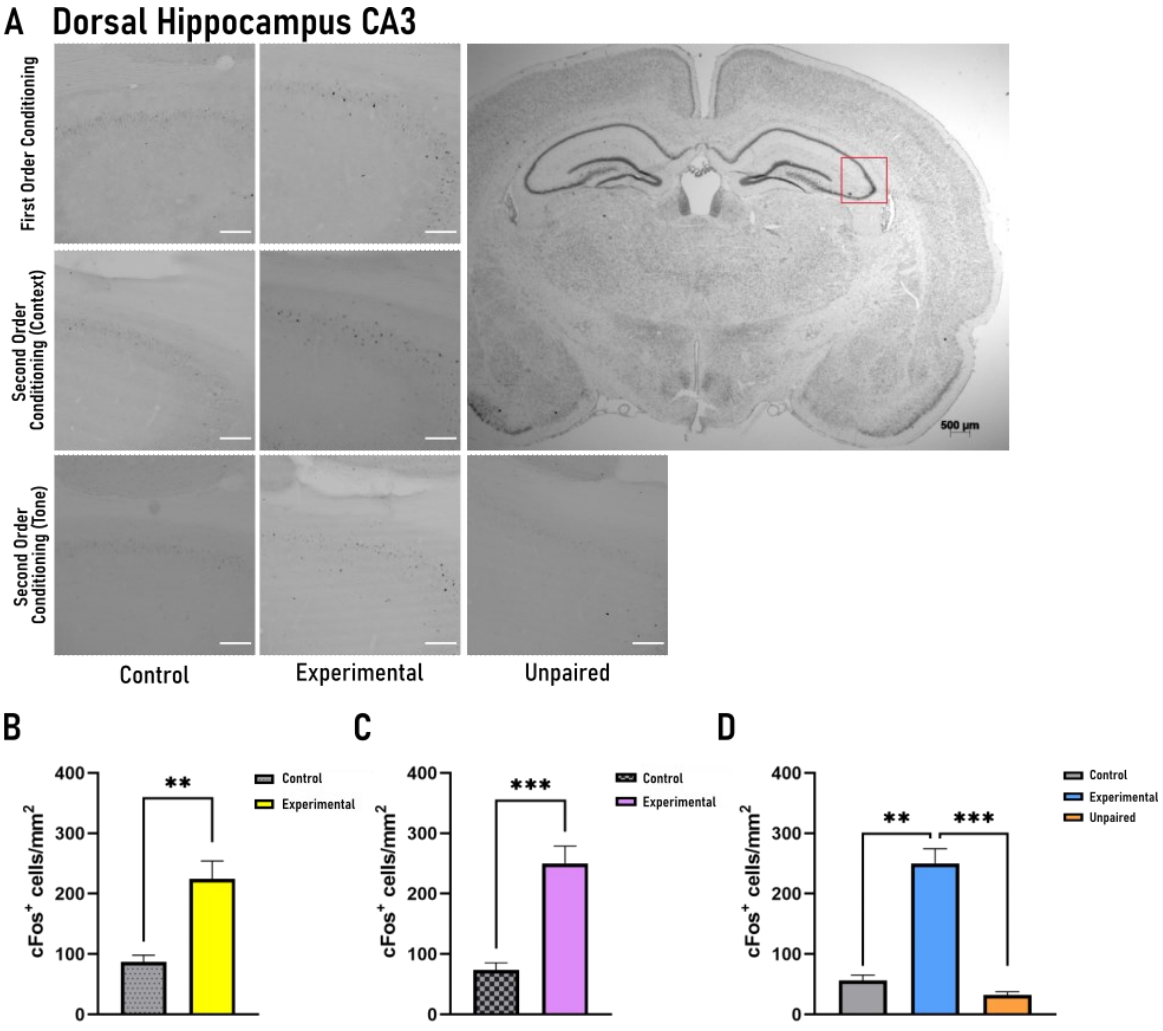


Figure 20. ***Dorsal hippocampus CA3 is activated by recall of odor fear memory conditioned by shock, feared context, or feared tone.*** **A)** Nissl image depicts the location of the posterior piriform cortex, and the red box outlines the region analyzed. Representative cFos images are shown for each experimental condition for FOC (top), SOC with context (middle), and SOC with tone (bottom). Scale bars are 150um. **B)** Experimental groups expressed increased levels of cFos following recall of a first order odor fear memory. **C)** Experimental animals expressed elevated levels of cFos following recall of a second order odor fear memory with context as CS1. **D)** Experimental animals expressed higher levels of cFos compared to unpaired and control animals following recall of a second order odor fear memory with tone as CS1. Statistics for FOC and SOC with context were independent t-tests, and statistics for SOC with tone were one-way ANOVAs. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$, ****$p<0.0001$. Error bars, mean ± SEM.

n=6; control 87.32 ± 10.94, n=6; p=0.001), a CS2 paired with a context (experimental 250.19 ± 28.59, n=7; control 73.60 ±12.02, n=7; p<0.001), or a CS2 paired with a tone (experimental 312.04 ± 40.25, n=6; control 174.27 ± 27.01, n=6, p=0.016; unpaired 153.44 ± 23.60, n=6, p=0.006; Figure 21). Increased cFos expression was observed in the LA in experimental animals (122.57 ± 13.95, n=6) compared to control (56.52 ± 8.99, n=6; p=0.015) and unpaired animals (61.64 ± 18.29, n=6; p=0.026) from the tone-odor behavioural paradigm but not the odor as CS1 (O+S 86.63 ± 10.20, n=6; OO 87.13 ± 11.33, n=6; p=0.974) or context-odor SOC paradigms (experimental 67.48 ± 8.09, n=7; control 66.25 ± 7.85, n=7; p=0.914; Figure 22). In the VH, increased $cFos^+$ cells per mm^2 were observed in the CA1 of experimental animals (365.27 ± 7.37, n=5) compared to control (200.85 ± 13.69, n=5; p<0.001) when odor served as a CS1, a CS2 with context (experimental 369.07 ± 44.11, n=7; control 134.75 ± 17.44, n=7; p<0.001), and a CS2 with tone (experimental 422.01 ± 27.37, n=6; control 197.19 ± 11.28, n=6, p=0.003; unpaired 177.80 ± 34.19, n=6, p=0.001; Figure 23). Like the DH, the patterns in ventral CA3 mirrored those of ventral CA1. When odor was used as a CS1, experimental animals (365.27 ± 7.37, n=5) show a significant increase in $cFos^+$ neurons per mm^2 in ventral CA3 compared to control (200.85 ± 13.69, n=5; p<0.001) and this is also reflected when odor is used as a CS2 with context (experimental 257.20 ± 24.35, n=7; control 92.87 ± 14.49, n=7; p<0.001), or with tone (experimental 291.59 ± 29.60, n=6; control 143.06 ± 14.99, n=6, p=0.002; unpaired 88.07 ± 26.16, n=6, p<0.001; Figure 24).

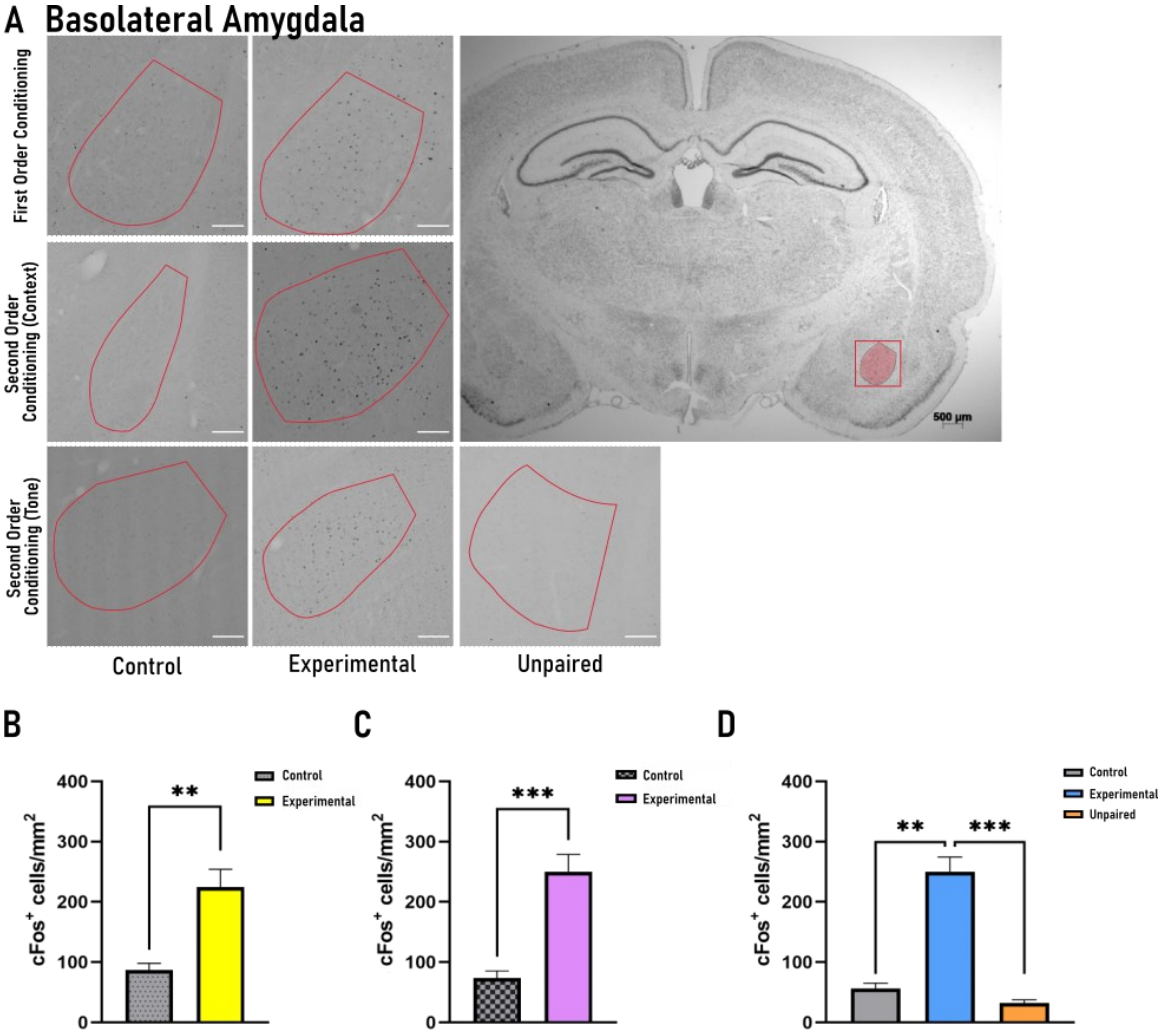


Figure 21. ***Anterior basolateral amygdala is activated by recall of odor fear memory conditioned by shock, feared context, or feared tone.*** **A)** Nissl image depicts the location of the posterior piriform cortex, and the red box outlines the region analyzed. Representative cFos images are shown for each experimental condition for FOC (top), SOC with context (middle), and SOC with tone (bottom). Scale bars are 150um. **B)** Experimental groups expressed increased levels of cFos following recall of a first order odor fear memory. **C)** Experimental animals expressed elevated levels of cFos following recall of a second order odor fear memory with context as CS1. **D)** Experimental animals expressed higher levels of cFos compared to unpaired and control animals following recall of a second order odor fear memory with tone as CS1. Statistics for FOC and SOC with context were independent t-tests, and statistics for SOC with tone were one-way ANOVAs. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$, ****$p<0.0001$. Error bars, mean ± SEM.

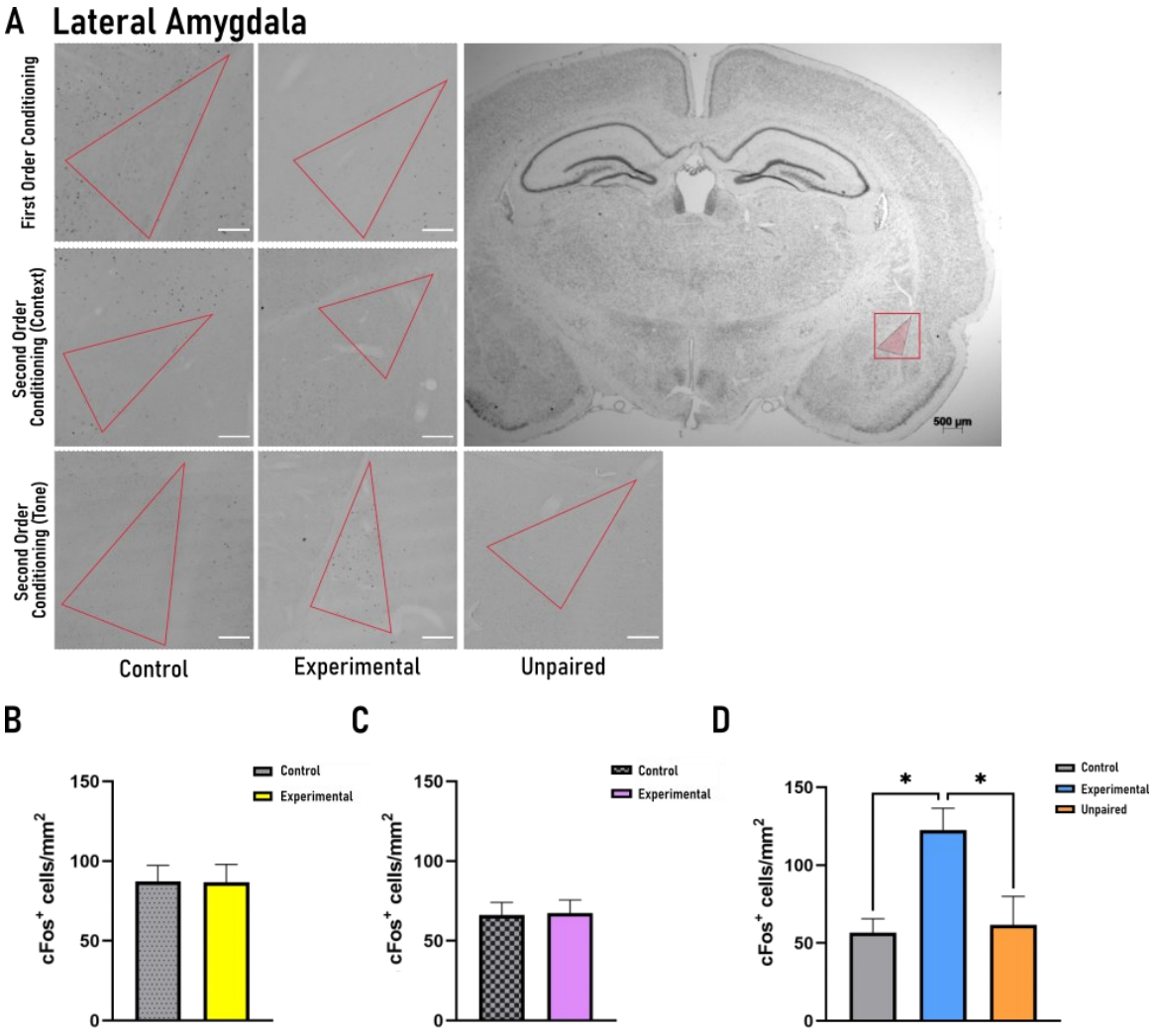


Figure 22. ***Lateral amygdala is activated by recall of odor fear memory conditioned by feared tone, not by shock or feared context.*** **A)** Nissl image depicts the location of the posterior piriform cortex, and the red box outlines the region analyzed. Representative cFos images are shown for each experimental condition for FOC (top), SOC with context (middle), and SOC with tone (bottom). Scale bars are 150um. **B)** Control and experimental groups expressed similar levels of cFos following recall of a first order odor fear memory. **C)** Control and experimental animals expressed similar levels of cFos following recall of a second order odor fear memory with context as CS1. **D)** Experimental animals expressed higher levels of cFos compared to both unpaired and control animals following recall of a second order odor fear memory with tone as CS1. Statistics for FOC and SOC with context were independent t-tests, and statistics for SOC with tone were one-way ANOVAs. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$, ****$p<0.0001$. Error bars, mean ± SEM.

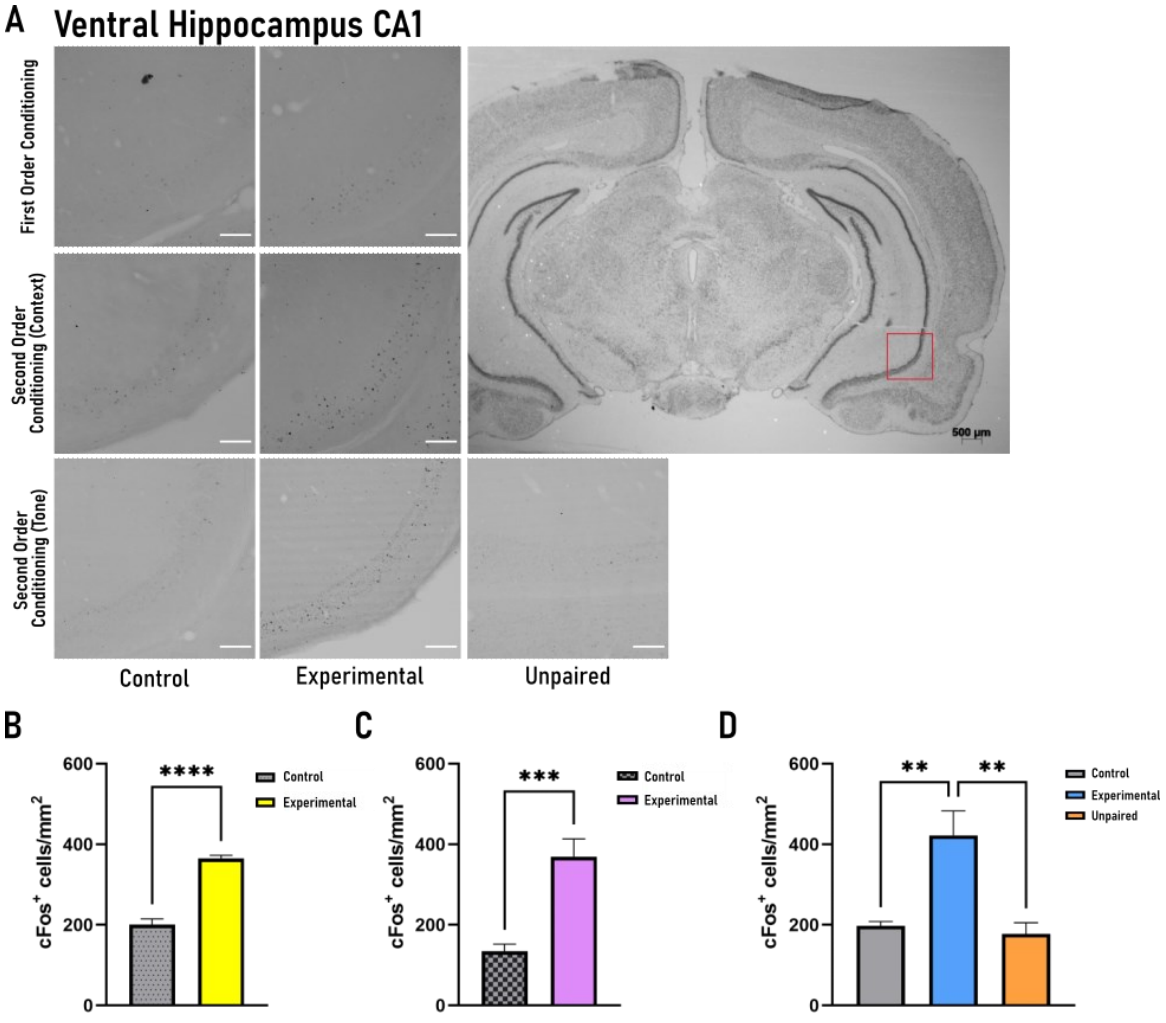


Figure 23. ***Ventral hippocampus CA1 is activated by recall of odor fear memory conditioned by shock, feared context, or feared tone.*** **A)** Nissl image depicts the location of the posterior piriform cortex, and the red box outlines the region analyzed. Representative cFos images are shown for each experimental condition for FOC (top), SOC with context (middle), and SOC with tone (bottom). Scale bars are 150um. **B)** Experimental groups expressed increased levels of cFos following recall of a first order odor fear memory. **C)** Experimental animals expressed elevated levels of cFos following recall of a second order odor fear memory with context as CS1. **D)** Experimental animals expressed higher levels of cFos compared to unpaired and control animals following recall of a second order odor fear memory with tone as CS1. Statistics for FOC and SOC with context were independent t-tests, and statistics for SOC with tone were one-way ANOVAs. *$p < 0.05$, **$p < 0.01$, ***$p<0.001$, ****$p<0.0001$. Error bars, mean ± SEM.

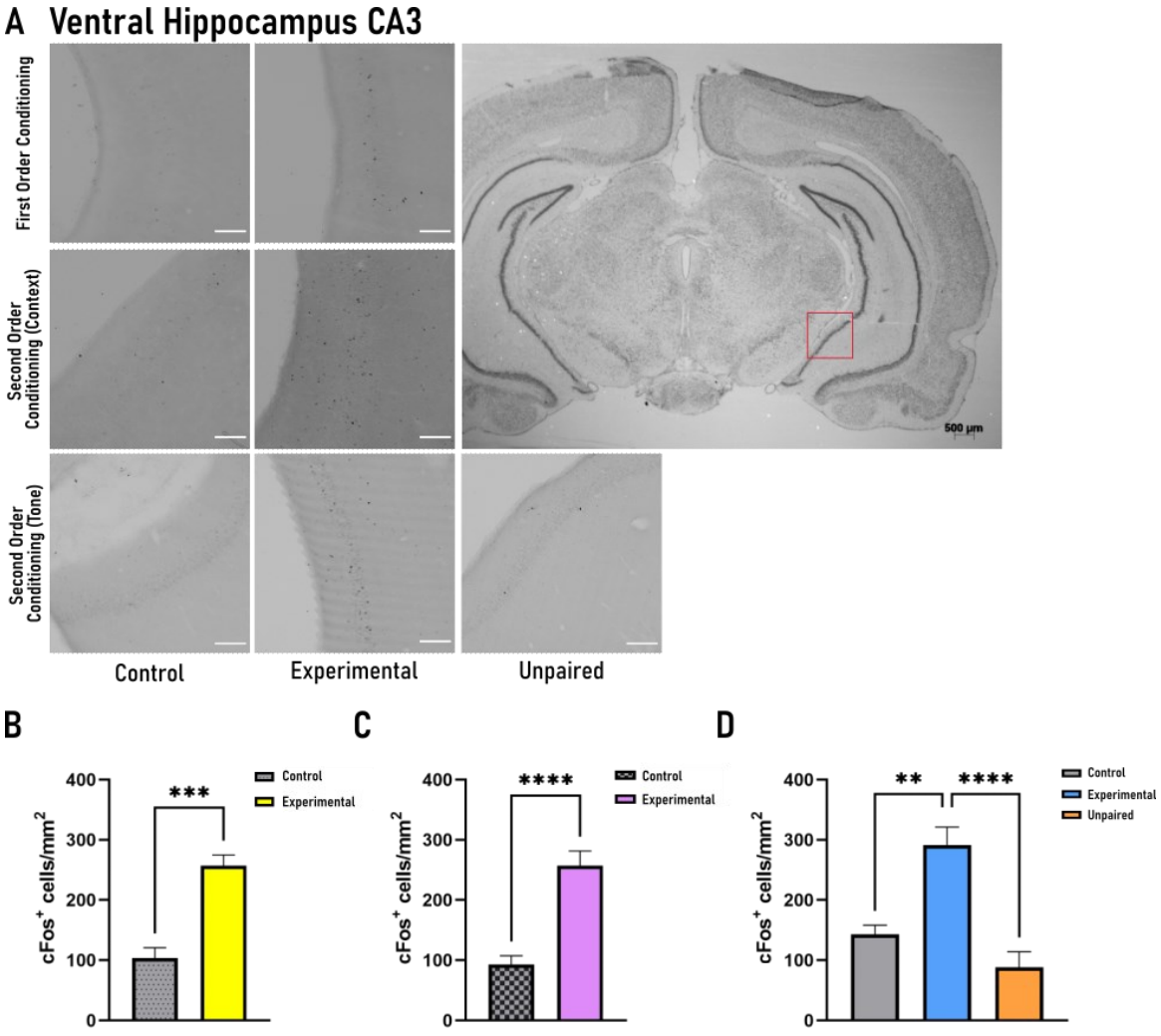


Figure 24: ***Ventral hippocampus CA3 is activated by recall of odor fear memory conditioned by shock, feared context, or feared tone.*** **A)** Nissl image depicts the location of the posterior piriform cortex, and the red box outlines the region analyzed. Representative cFos images are shown for each experimental condition for first order conditioning (top), SOC with context (middle), and SOC with tone (bottom). Scale bars are 150um. **B)** Experimental groups expressed increased levels of cFos following recall of a first order odor fear memory. **C)** Experimental animals expressed elevated levels of cFos following recall of a second order odor fear memory with context as CS1. **D)** Experimental animals expressed higher levels of cFos compared to unpaired and control animals following recall of a second order odor fear memory with tone as CS1. Statistics for FOC and SOC with context were independent t-tests, and statistics for SOC with tone were one-way ANOVAs. *p < 0.05, **p < 0.01, ***p<0.001, ****p<0.0001. Error bars, mean ± SEM.

Chapter 4 – Discussion

Rats can learn that an odor predicts danger in many ways, which is likely an adaptive mechanism with roots in survival. An odor can provoke a conditioned freezing response by its direct association with shock (Figure 4, Figure 14A), its association with alarm pheromones from a conspecific (Figure 4), and by its association with a learned fearful context (Figure 14B) or tone (Figure 14C). Second-order, first-order, and pheromone-mediated conditioning all rely on NMDAR signaling in the BLA (Figure 12, Figure 14D). There are commonalities and differences in the engram complexes recruited for odor fear memories formed with different conditioning stimuli. For example, the aBLA and mCeA are activated upon recall of an odor fear memory if it is associated with either shock or conspecific alarm pheromone (Figure 10A), but pheromone fear memories recruit the AOB while shock-conditioned fear memories elicit activity in the MOB upon recall (Figure 10B). These results along with many others described in this thesis support the notion that the amygdala is crucial for fear memory regardless of how it is formed.

Second- and higher-order conditioning allows for the investigation of specific memory ensembles and their properties in terms of stimulus modality, hierarchy of associations, and future learning based on prior learning. Recall of the exact same CS (terpinene) led to activation in CA1 and CA3 of both dorsal (Figure 19, 20) and VH (Figure 23, 24), aBLA (Figure 21), and posterior piriform cortex (Figure 17), whether it was paired with shock, a context which had previously been paired with shock, or a tone which had

previously been paired with shock. The VH and BLA contain dense reciprocal connections[260,261], and are both involved with numerous aspects of fear learning and memory[8,245,342], so these results were unsurprising and highlight the commonalities observed across the field in areas involved with fear memory reactivation. Since the engram complex was probed in each case by an olfactory cue as the final substrate, in addition to its frequently reported role in aversive odor conditioning, it was also expected to see activation of the pPC across the three types of memory. The DH was surprising, as we intended to habituate the animals to the context in Experiments 3 and 5 to remove contextual fear memory, but potential reasons for this unexpected result are described in detail below. Perhaps the most intriguing finding is that when terpinene is second-order conditioned with an auditory CS1 as in Experiment 5, recall of the odor CS2 induces activation in the LA (Figure 22) and the auditory cortex (Figure 18) in addition to the areas listed above. Considering there is no direct projection from the piriform to auditory cortex[213], this suggests that the ensemble responses in these areas are due to changes induced by associative conditioning. This implies that a recall of a specific conditioned stimulus in a chain of higher-order conditioned cues can reactivate the entire engram, including areas involved in processing other CSs that are absent at recall. The data described in this thesis provides important information on how rats learn danger-associated cues through pain, social communication, and prior experience, shedding light on basic mechanisms of associative memory formation. This could have implications for the way in which traumatic memories are recalled during treatment for PTSD, potentially lessening the burden and re-traumatization involved with the most effective treatments currently available.

4.1. Learning through alarm pheromones

When stressed, rodents release alarm pheromones which provoke anxiety or fearful responses in conspecifics[35,36,39] or even in themselves[36] and this can lead to conspecific avoidance of the immediate danger through increased defensive and risk assessment behaviour[44]. This mode of communication can promote survival of the species at the price of only a few animals experiencing the real danger. However, if the effect of alarm pheromone is short-lived, conspecifics that are warned through an alarm pheromone communication could be compromised when confronting the same danger in the future. To be evolutionarily advantageous, animals should be able to learn to associate relevant cues with the alarm pheromone and obtain the advantage of avoiding the danger in the future by recognizing those cues. Can a memory trace be formed through alarm pheromone and cue association? My research suggests this is the case. Either being caged with stressed rats, being placed over soiled bedding from stressed rats, or being exposed to previously identified alarm pheromone molecules[39] increased rats' freezing to a conditioned odor when tested 24hr later. Fear memory is specific to the conditioned odor cue and can exist despite the presence of a different context. Pheromone conditioning induces an increase in the number of active cells of the AOB upon recall of the odor, while first-order conditioning induces a similar change in the MOB.

Mouse alarm pheromones are detected by a completely different organ also present in rats, called the Grueneberg Ganglion[343]. The primary component of mouse alarm pheromones was identified as 2-*sec*-butyl-4,5-dihyrothiazole (SBT), in contrast to the 4-

methylpentanal and hexanal identified in rat alarm pheromone. Importantly SBT caused an elevation in plasma corticosterone in a receiver mouse, which the authors refer to as a systemic stress response. It is important to note that the alarm pheromone that they utilized was an odor emitted by mice subjected to a lethal CO_2 stressor, a confinement stress, and a cold stressor[40,343]. It is possible that these stressors represent more severe risk and cause release of death-imminent alarm signals rather than a merely painful stressor like a shock. It is tempting to conclude the pheromones in the current thesis are detected by the VNO. However, we cannot rule out the involvement of the Grueneberg Ganglion. Rat pups acquire an odor aversion when a novel odor is paired with their mother's fear[7]. Thus, the ability of pheromones to serve as a US appears early in the life of the rat and continues to function as observed here. Interestingly in neonates, pheromone learning is mediated by the MOB receiving input from the Grueneberg ganglion system, while here the AOB mediates the critical input. Whether adult fear learning with pheromone signals is as enduring (over weeks) as that observed in pups[7] remains to be examined.

4.2. Learning through second-order conditioning

Rats learn second-order associations readily, at least when they utilize contextual, auditory, and odor cues (Figure 14). This thesis has described how a rat can associate an odor with an inherently aversive cue or a learned aversive cue, whether that learned cue is contextual or auditory in nature. Behavioural data from a previous honour's student

showed that the reverse is true in each of these cases; an animal can use an odor as a learned aversive cue to support SOC to a tone or a context[50].

Accumulated evidence from studies that employed extinction (the gradual process by which memories can be overwritten, generally involving repeated exposure to a CS without any further presentation of a US) on CS1 stimuli has revealed that there are multiple accounts of associative linkage underlying SOC[48,344]. The associative chain theory posits that CS2 elicits the CR through an associative chain, such that either CS2 → CS1 → US → CR or CS2 → CS1 → CR[345,346]. The direct association theory instead suggests that the associative linkage forms between CS2 → US, in other words the activation of the US representation occurs during CS1 pairing with CS2, leading to the CS2 being able to directly activate the representation of the US by itself[347]. These two accounts differ by the presence or absence of a role for the representation of CS1 while CS2 is being tested. Studies that used extinction to remove the CS1 as a cue which predicts the US have shown support for both hypotheses[48,348]. When the CS2 and CS1 are presented sequentially during the pairing phase extinguishing CS1 has no effect on the CR in response to the CS2, which would be explained by a direct association account of SOC[48]. However, when the CS2 and CS1 are presented simultaneously during the pairing phase, extinction of CS1 leads to diminished CR to CS2 presentation, fitting the associative chain account of SOC[348].

Since the experiments in the SOC project utilized simultaneous pairing of the CS1 and CS2, it follows that the type of SOC present in this thesis should align with the associative chain explanation. While not tested directly with extinction to any of the CSs, comparison of the engram complexes recruited for each experiment provides some clues. The same basic engram complex was activated by each of the SOC paradigms, but when an auditory CS1 was used in Experiment 5, the LA (Figure 22D) and auditory cortex (Figure 18D) were reactivated following exposure to an odor CS2 despite the absence of the tone during testing. This suggests that information about the CS1 is relevant for the recall of the memory trace for CS2, which does not support the idea that each CS just reactivates the representation of the US, as the engram activated by re-exposure to the CS2 changes depending on how it was conditioned. The US is the same in every experiment: shock (Figure 14). If the engram complexes for context-odor and tone-odor memory were identical, this would provide support for the direct associative chain event, because in both experiments the CS2 and the US were the same. If the sensory properties of CS1 do nothing to influence the ability of CS2 to elicit the CR then the engram complexes should be the same no matter which kind of CS1 is used, as the theory itself posits that each CS is independently capable of reactivating the representation of the US[349]. A causal approach was not taken in the current set of experiments so I cannot say for sure whether the data is explained by the direct associative or associative chain theory, but it appears that activation of the US representation occurs during CS1 and CS2 pairing, leading to CS2 being able to activate the representation of the US by itself and is therefore consistent with the associative chain theory.

4.3. Engram complex differences within pheromone project

It is intriguing that pheromone-conditioned learning in companion rats leads to enhanced AOB activation to the conditioned odor upon memory recall. Although initially regarded as functionally independent systems[300,350], with the MOB system being responsible for volatile odorant detection and the AOB system detecting pheromones, recent evidence has revealed that the two systems have considerable overlap in terms of chemo-signal detection and the behavioural effects they mediate[351,352]. A subset of vomeronasal neurons express odorant receptors and project to the AOB[353]. The AOB system can thus detect both odorants and pheromones[351,352,354]. We suggest potentiation of AOB neurons is linked to pheromone stimulation acting as a US in the associative learning of the CS odor with both signals converging on and potentiating common neurons in the AOB. Another possibility is that amygdala cortifugal input shaped the potentiated responses in the AOB despite an absence of greater activation in the back-projecting cortical amygdala nucleus[336].

Freezing here in the classically conditioned rats (O^+/S^+) is associated with enhanced activation of the MOB and BLA during memory recall. The MOB has been shown to be a critical site for odor associations in multiple learning models[355–359]. The BLA is essential for shock-mediated conditioning including contextual fear conditioning[11,72,360] and odor conditioning[7]. Hebbian plasticity requires coincident inputs of both CS and US onto common postsynaptic cells[64]. While the BLA receives olfactory inputs and somatosensory

inputs such as those induced by electrical shock[361,362], whether MOB neurons receive direct somatosensory inputs is unknown. Alternatively, LC neurons release NE following shock[363,364]. The MOB[365] and BLA[366,367] receive extensive LC projections. Odor and NE inputs could converge on both MOB and BLA neurons to initiate plasticity mechanisms and this could be reflected by reduced freezing behaviour in the companion group when propranolol was used (Figure 4). Both Hebbian plasticity and neuromodulation by NE are required for tone-shock fear conditioning in the amygdala[368]. MOB plasticity could reinforce BLA potentiation either through its direct projections to the BLA, or via the PC or the MeA projections[369–371], as suggested by our retrograde tracing data. In fact, both the PC and BLA exhibit potentiated odor responses following fear learning in another study[372].

4.4. Engram complex differences between pheromone and SOC projects

Classical conditioning by three types of stimuli have been described and illustrated in the pages above; innate (pheromone), inherent (first-order), and learned (second-order) stimuli can serve as cues for future learning about a particular odor, terpinene. Although the same odor CS is used in all experiments, it is important to recognize the major difference in memory recall between the two projects in this thesis. Experiment 1 utilized *Arc*/*H1a* catFISH, a method that allows visualization of two separate behavioural experiences; in this case a control odor and a conditioned odor. The readout chosen to analyze this data was a ratio of cells active to the conditioned odor versus cells active to the control odor, creating an index for responsivity to a CS for each animal and thereby

allowing for specific intra-animal control. IEG mRNA is released at baseline conditions and in response to neutral stimuli[373] but not all is translated into protein, so having an intra-animal control in the *FISH* study should reduce the noise detected in the animals' response. Importantly, the results obtained using the ratio were not mirrored by the raw *Arc*+ and *H1a*+ data and the variability of the raw data is objectively larger than the variability in the ratios (compare panels 3 and 4 of Figure 10), illustrating the inherent variability of neural activity in our animals. In contrast, the second-order conditioning project utilized cFos IHC to map regional activation following recall of the conditioned odor. cFos protein peaks at 90 minutes following stimulation, and therefore its expression corresponds to an experience which occurred 1.5 hours prior, providing an opportunity to overlap exposure groups and include more animals in each experiment. Experiments 3-5, having 3 different behavioural paradigms containing 7 groups, would have been difficult to complete using catFISH and the number of engram complex candidates would have been limited. Even though these three types of memory are recalled with the same cue for the same amount of time and produce the same behavioural response, there are both similarities and differences in the brain regions they recruit for their engram complexes. Importantly, cFos is a protein while *Arc* and *H1a* are mRNA transcripts. While cFos is routinely used to measure neural activity following a behavioural test, factors other than neuronal activity may affect the fidelity with which cFos is translated into protein.

The areas chosen for engram inclusion were not the same across both projects. The pheromone project aim was to compare the effects of an inherent versus innate US on a first-order conditioned odor memory trace. The areas chosen were mostly olfactory related structures and several subnuclei of the amygdala. Results from Experiment 1 of the pheromone project emphasized the importance of the aBLA, how it was active for both types of memory and if NMDARs are blocked so too are both types of memory. As such, we included the aBLA in the second study. The aim of the SOC project was to compare the engram complex formed by an inherent versus learned "US," on a first- or second-order conditioned odor memory trace and to compare engram complexes for an odor CS2 trained from a tone CS1 to an odor CS2 trained from a context CS1. Therefore, auditory and contextual processing areas such as the auditory cortex and DH were included. Although the LA was not identified as part of the memory trace in the pheromone project it is well known to participate in auditory fear learning so it was included for the SOC project. DH and VH were included because of their role in contextual processing and aversive memory, respectively, and the increased appreciation of the differences between the two[374]. The posterior piriform cortex was the only area included in both studies that showed discrepant results.

The posterior piriform has been shown numerous times to participate in odor valence, especially aversive odor memories[184,372]. Interestingly, the pPC was identified as a component of the engram complex in the SOC project (Experiments 3-5) but it was not in the pheromone project (Experiment 1). The pPC of animals who had formed an odor fear

memory, whether through association with shock or pheromone, did not preferentially respond to conditioned odor cues over neutral ones. This is a strange result because the groups in the first-order conditioning experiment of Experiment 3 were treated practically identically as the O^+/S^- and O^+/S^+ groups in Experiment 1. There are a few possible reasons for this discrepancy across the two projects. First, the neutral odor that was delivered prior to the conditioned odor during catFISH sacrifice (Figure 9) was a novel odor to all groups of rats. Odor activity maps are formed in the PC by population coding[172,375], so it is possible that comparing a conditioned odor to a novel odor in the pPC is not a wise choice. The pPC is important for many aspects of odor processing, and it is possible that introduction of a novel odor prior to recall of the conditioned odor occluded some of the *Arc* expression that would normally happen upon reactivation of the memory.

Another potential explanation for this discrepancy could be due to sampling, all brains for Experiment 1 were cut in a block to reduce variability in the FISH procedure, a much more stringent protocol than IHC. Because the IEGs being measured were expressed within 5- and 30-minutes following stimulation, perfusion of these animals was not an option and their brains had to be flash frozen. In order to complete FISH, the brains had to be sliced at 20μm thickness. While many measures were taken to cut the brains evenly with all groups represented in the same block (Figure 3) choosing slides which had sections across groups from the exact same anterior-posterior positions was nearly impossible. The PC is an enormous structure that encompasses almost the entire anterior-posterior axis of the ventral rat cortex. It is possible that the variability in the sections chosen for the pPC is

the cause for the variability in the results. In Experiments 3-5 which used cFos IHC on free floating 50μm perfused sections, great care was taken to ensure slices across groups were taken from the same anterior-posterior position so that each pPC was represented in its entirety, and the pPC was found to be a component of the engram complex in Experiment 3.

The final potential source of these conflicting results could be due to social buffering. A big difference between the two projects is the way the animals were housed. In the pheromone project, both conditioned animals (i.e., shock- and pheromone-conditioned) lived together as cage mates, while in the SOC project all animals were housed singly throughout the experiments. Increased cFos was found in the pPC following social cohabitation experiments[47], so it is possible that the pPC activity of these two animals living together in Experiment 1 cannot be directly compared to the pPC activity of animals who are singly housed in Experiments 3-5.

4.5. Engram complex differences within second-order conditioning project

For all three experiments in the SOC project, consistent activation of the aBLA and dorsal and VH was found during recall of an odor fear memory regardless of how it was formed. When the odor memory is formed by pairing it with a feared tone (Experiment 5), the auditory cortex (Figure 18) and LA (Figure 22) are also activated during recall of the

odor. Post hoc tests showed that there was no significant difference between experimental and control cFos in the pPC, but the unpaired and experimental groups were significantly different from one another.

The activation of the auditory cortex in response to an odor CS2 suggests that unique sensory regions are recruited depending on the sensory features of CSs present in the associative chain. Moreover, this seems to be accompanied by a decreased likelihood of cells in the pPC to respond to the CS2 odor cue, as evidenced by the lack of significant difference between the control and experimental groups in Experiment 5, compared to Experiments 3 and 4 where the difference between control and experimental expression of cFos in the pPC was clear and large. This is, however, further confused by the presence of a significant difference in pPC expression between experimental and unpaired, and the fact that the other experiments did not utilize unpaired conditions.

For the first explanation, an assumption must be made that the unpaired group makes for a better comparison than control in Experiment 5. Experimental and unpaired animals receive the exact same odor and tone cues for the same length of time but they are presented in a way that does or does not lead to SOC fear memory formation, respectively. If, then, the lack of significant difference between control and experimental can be ignored, this implies that the pPC does participate in the engram complex. If this holds true it suggests that a specific link in the associative chain of a fear memory can reactivate the entire engram complex, even areas which encode sensory features of a stimulus in the

absence of that stimulus. This could have implications for the way humans could be treated for traumatic memory disorders, which will be discussed in section 4.9.

An alternative explanation relies on the assumption that the difference between control and experimental in Experiment 5 should be the only comparison made to stay consistent with the design of the other experiments. If, then, there is no memory trace stored in the pPC for this type of memory, it is possible that the auditory cortex preferentially encodes the memory over the pPC. If the piriform cortex preferentially encodes an odor-tone auditory memory over the auditory cortex, this would suggest that the position of the CS in the associative chain determines which brain areas encode the engram. Studies in humans have suggested that the auditory cortex has a poly-sensory role, as audio-tactile[376,377] and audio-visual[378–380] interactions have been found here. Potential audio-olfactory interactions in the auditory cortex have not received due attention, as an anatomical study of the guinea pig showed that the auditory cortex does not project to the piriform cortex, suggesting that the auditory cortex does not process olfactory information[213]. Interestingly, about 30% of neurons in the piriform cortex respond to tones, suggesting that odor sensory input to the PC may be subject to modification by auditory sensory input which could modulate odor memory, perception, or processing[212]. While a direct projection from PC to auditory cortex has not been reported, it has been demonstrated that exposure to pups' body odor reshapes neuronal responses to auditory stimuli in the auditory cortex of a lactating female mouse. The authors of this study suggest that olfactory-auditory integration in the auditory cortex could exist to strengthen bonds between mother and their pups[381]. On the other hand, a study which rewarded presentations

of either an auditory or olfactory cue but did not reward simultaneous presentations of the two cues showed that the learning related changes in beta oscillation which occurred in olfactory cortices did not occur in the auditory cortex[382]. The PC responded to a sound alone, and the multisensory unrewarded cue elicited higher activity in the PC than the odor cue. No such changes were found in the auditory cortex. The contribution of the auditory cortex to an olfactory-auditory memory could depend whether the association between the two stimuli results in a positive outcome for the animal. A readily formed association between pup odor and various auditory stimuli would be evolutionarily advantageous for both pup and lactating mother[381]. Rats completing a difficult task that depends on differentially recognizing odor and sound but not the two together would rely on separating the two stimuli, perhaps by restricting engram complex areas and relying on the polysensory features of the PC[382]. In the current thesis, exposure to odors which were associated with tones that had previously been paired with shock resulted in activation of Au1 and PC. These results are in line with the proposed explanation above; odor predicts tone which predicts pain, so the odor being represented in both PC and auditory cortex could reflect the fact that the two cues being associated is beneficial for the rat's ability to sense incoming danger.

Importantly, the auditory cortex was not the only area that was differentially recruited to the tone-odor memory trace, there were also differences in amygdala involvement (Experiment 5). Surprisingly, both the BLA and the LA were activated during recall of a CS2 odor fear memory conditioned by tone. Contextual and odor conditioning are known to be dependent on BLA signaling while auditory conditioning is known to be

dependent on LA signaling. The fact that both are activated in the tone-odor experiment suggests that both odor-specific and tone-specific brain regions are recruited to the engram, and that both the piriform and auditory cortex are likely to participate in the memory trace, which supports the first explanation given above. Future studies should expand this framework to include the presentation of different types of discrete sensory cues that can be easily separated temporally, like the unpaired group in the tone-odor experiment to support the conclusion that sensory areas are recruited to a memory trace based on their features and that one stimulus in the associative chain can reactivate an entire engram.

4.6. The basolateral amygdala as a site of convergence for innate, inherent, and learned sources of danger

Like classically conditioned fear, stressed cage mate-conditioned fear activates the BLA during memory recall. Abolishing plasticity in the BLA by NMDA receptor blockade prevents both classical and pheromone-conditioned fear memory formation. From the BLA, information flows to the medial portion of the CeA which sends output to the periaqueductal grey and hypothalamus to mediate freezing and autonomic responses associated with fear[11]. Elevated CeA activation was observed in both types of learning in our study. Our results regarding the classical and pheromone conditioning pathways are summarized in Figure 12C.

NMDARs in the BLA were also blocked during odor (CS2) and tone (CS1) pairing, which led to significantly decreased freezing behaviour to presentation of the CS2. This illustrates how NMDAR-dependent activity in the BLA is necessary for second order fear learning with an auditory and an olfactory cue. Assuming that the original fear memory is formed through LA and auditory cortical connections as has been shown[383], it is interesting that both LA and BLA neurons are recruited to the engram instead of one or the other. This supports the notion that these amygdaloid subnuclei differentially support cued learning depending on sensory features of the cue, in other words the LA is involved in auditory fear conditioning while the BLA is crucial for odor and context fear memory, and care should be taken in separating these two structures while dissecting scientific evidence.

4.7. The hippocampus as a hub for nuance and context

Admittedly, when including the DH as a potential engram candidate the intention was to include an area that processes context, akin to the auditory cortex for sounds and the olfactory cortex for odors, because these were the three types of stimuli chosen to be conditioned in the project. It was initially surprising to see increased cFos expression in both CA1 and CA3 in the DH for Experiments 3 and 5 which had included habituation to the context originally intended to minimize encoding contextual fear. Two consecutive daily 30-minute context exploration sessions prior to receiving shock and the CS are sufficient to inhibit the behavioural expression of a fear memory to the context, illustrated by Figure 5, group O^+/S^+ pre-terpinene freezing. Importantly, in O^-/S^+ animals who only

received shock without odor pairing, freezing levels were significantly different between O^-/S^+ and O^+/S^-, illustrating how these animals could be considered as contextually fear conditioned. Animals that were cue trained, i.e., O^+/S^+ animals, did not show freezing specifically to the context (Figure 5). In the SOC project, levels of freezing to the context were not directly measured in the experiments, unless context was a CS as in Experiment 4, but the fact that the context itself did not elicit a freezing reaction when a cue was involved can be observed in Experiment 5. All three groups were first-order conditioned to a tone after being habituated to the context; the difference in the three groups occurred on the fourth day, where control and unpaired did not learn the association between CS2 and CS1, but were still exposed to CS1 and had their freezing behaviour measured. The following day, they were exposed to the CS2 which had no value for them and they did not freeze. These behavioural tests were all conducted inside the context in which they were shocked. If the control and unpaired rats had formed a contextual fear memory, they would have shown higher levels of freezing when exposed to the (value-less) CS2 in the (feared) context. Importantly, all groups were first order cue-conditioned in this context following habituation.

The idea of "context" being comparable to a singular sensory cue was, in retrospect, probably very naïve. While it is not incorrect to say that the DH processes contextual cues, it is an oversimplification of the capacity of this subcortical superhighway. First, the term context encompasses many different things: visual, olfactory, auditory, and tactile cues that are inherent to the physical environment itself, and these are besides the more intricate and

complex concepts of physical space and time. Further complicating the notion of "context;" it has been conceptualized as having internal facets, such as the individual's cognitive, hormonal, and emotional state[253]. The fact that the brain has a single structure in charge of many of these aspects of contextual processing is amazing within itself. Is it even reasonable, then, to expect to effectively inhibit the brain from encoding contextual cues by simply exposing the animal to the context prior to fear conditioning?

Looking again at the Experiment 5, the experimental animals showed increased expression of cFos in CA3 and DH CA1 even though they had been habituated to the context. In fact, hippocampal cFos expression was at practically the same level in experimental animals in both Experiments 4 and 5. Control and unpaired rats who were also first-order fear conditioned in the context did not show increased cFos in CA3 and DH CA1 when exposed to the CS2 in the original context (Figure 19, Figure 20). It seems reasonable to suggest that because the control and unpaired groups from the Ex were exposed to the CS1 in the absence of shock on day 4 in the original context, that perhaps it caused extinction of the context-fear association. However, extinction studies in aversive memory require multiple exposures to the once feared CS, and it is highly unlikely that an extinction memory would overwrite a fear memory because of one exposure following a shock conditioning paradigm (see Maren[384] for a review on fear memory extinction). Importantly, extinction of fear memory has been shown to be transient and context-dependent, relying on the hippocampus[385–388] which further underscores the complex relationship between the hippocampus, contextual cues, and sensory based fear memory.

Extinction experiments have revealed that the hippocampus is important for integrating contextual cues into a CS fear memory, as the normally spontaneous renewal of a cue-based fear memory in a distinct context is inhibited in the absence of a functioning hippocampus. This is intuitive, if a cue no longer predicts danger in a specific context, that will not necessarily translate to all contexts. The mechanisms of extinction are beyond the scope of this thesis, but the general principles it has taught us about the function of the hippocampus in fear memory are important to consider and may be conceptually applied to memory expression.

Even with contextual habituation, individual DH neurons respond to a feared auditory cue only when the animal is freezing in its specific place field[389]. It is possible that the formation of a fear memory induces an excitable state in the hippocampus, which is recapitulated when an additional cue is added to the chain, to enable encoding of contextual information to allow for a richer memory, which may be more valuable in the future to identify impending threats. Evolutionarily speaking, during times of heightened awareness, i.e., having the expectation of an impending painful stimulus by recognizing a cue that has become associated with the pain, it would be advantageous for the animal to have the ability to integrate any contextual cues available into the memory trace in order to better recognize the next dangerous event in the future. For example, if a deer happens upon a bear fishing near a river and narrowly avoids an attack, the deer may associate the sound of rushing water with the visual stimulus of the bear which is associated with a painful attack. Yet deer also require water for survival and need to be able to distinguish between rivers, which

bears frequent, and streams and brooks or other safe sources of water. To think this ability is based on specific auditory cues for each river, stream, or brook, seems irrational. The hippocampus, being a discrete subcortical structure with direct access to inputs and outputs all over the brain (e.g., sensory structures and cortices, amygdaloid nuclei) by way of the EC, is perfectly positioned to integrate multiple aspects of an experience and allow for a more complex, rich, and nuanced memory based on environmental surroundings along with previous experience and internal state of the animal.

Importantly, prior habituation to the context does seem to inhibit both behavioural and neuronal fear memory expression in animals that do not experience a chain of associative events (i.e., unpaired and control animals in the Experiment 5). In cases where the experimental group had been trained to form a first-order or second-order fear memory to an odor CS, the DH was always activated and animals froze significantly more than controls. There are two potential non-mutually exclusive explanations underlying this seemingly contradictory finding. First, it is possible that the DH only participates in an engram complex if the context reliably predicts the presence of a CS. Second, reactivation of a fear memory could induce an excitable, plastic state in the hippocampus so that it is prepared to integrate additional contextual cues if they should arise during presentation of a CS. Clearly, the circuitry in the hippocampus is not as simple as it may seem at first glance, and a complex mechanism likely underlies the integration of contextual cues into fear memories as this is a central feature of survival success. Norepinephrine is an important neuromodulator, and it has been shown to play a role in the pheromone- and shock-

conditioned odor fear memory described in this thesis, as well as several other types of aversive learning[294,366,367,390].

4.8. Norepinephrine and fear learning

The locus coeruleus is the brain's major source of norepinephrine, and has dense reciprocal connections with the BLA, CeA, and DH[391–394]. NE acts on three subtypes of G-protein coupled receptors, α_1, α_2, and β, and they decrease in their affinity for NE in that order[366,395]. α_2 adrenoceptors are inhibitory in nature and G_i-coupled, decreasing cAMP and reducing neuronal excitability[396], while β adrenoceptors are G_s-coupled and produce opposite effects[397,398]. α_1 receptors are generally thought to be excitatory in nature and are G_q-coupled[399].

In the LA, it has been suggested that NE could bi-directionally gate LTP at glutamatergic synapses by suppressing GABAergic inhibition, effects which are dependent on the subtype of the adrenoceptor targeted. Specifically, when NE is applied in the presence of a β-adrenoceptor antagonist, the synaptic response in thalamo-amygdala connections is reduced, but it is enhanced in the presence of the α_2-adrenoceptor antagonist[400]. The observation that aversive conditioning in the BLA is dependent on β-adrenoceptor activity and tonic firing modes from the LC reported earlier this year[294] is in line with this explanation. Interestingly, in the DH NE is released directly by activation of NMDARs[401], and NE can facilitate AMPAR trafficking and LTP induction by its

phosphorylation of the GluR1 subunit[402]. NE application can induce LTP in the LA when paired with electric shock, but not in the absence of any aversive stimuli; in fact Hebbian mechanisms underlying plasticity in the LA need to be coactivated with β-adrenoceptors to engage associative learning[368]. Moreover, NE cannot enhance contextual fear memory in mice lacking the GluR1 NE-dependent phosphorylation sites like it can for WT mice[402]. These observations have led to the hypothesis that increased NE concentration during emotional arousal could lower the threshold for synaptic modifications driven by experience by phosphorylation of GluR1 and facilitate the formation of fear memories[402]. In this way, NE hippocampal release during emotionally heightened experiences could modulate hippocampal-dependent memory by gating the induction of synaptic plasticity in DH circuits. Like the BLA, LC projections to the hippocampus can induce NE-dependent synaptic enhancements[403–405]. The unexpected activation of the DH observed in Experiments 3 and 5 could be explained by CS2 → CS1 → US → CR. The fear reaction from the animal (freezing) could result in NE release to the DH, enabling phosphorylation of GluR1s and facilitating more efficient synaptic plasticity to better integrate further contextual cues and add more predictive value to the memory trace. If this explanation holds true and the SOC experiments were repeated in the presence of a β-adrenoceptor antagonist specifically in the hippocampus, this could recapitulate the results in the tone-odor paradigm, because contextual cues are deemed "unimportant" here and the hippocampus is not required for fear memory acquisition[248–250]. Additionally, it would be interesting to see the capability of integrating a contextual CS into a higher order conditioning paradigm while blocking β-adrenoceptors in the hippocampus.

In the pheromone project, the ability of terpinene exposure following a 10 min exposure to the O^+/S^+ rat to promote equally strong specific cue odor learning in the companion rat is somewhat surprising, given the long-standing evidence that CS must precede or be contiguous with the US in associative learning[5,11,49]. Companion rats were able to associate the pheromone released from a stressed rat with a subsequent odor cue in the absence of the stressed rats, however an association is not formed without the subsequent odor cue (Figure 11 B, C). It is possible that LC NE release during pheromone-induced stress[44] could prime pheromone-activated neurons for later association with odor activation. The odor stimulus would then subsequently become sufficient to drive AOB neurons in the absence of the pheromone stimulus. Our data showing how β-adrenoceptor blockade prevents pheromone learning is consistent with the involvement of NE. Our data do not exclude the possibility that other priming effects may occur during social interaction between the stressed and companion rats such as residual odor smell on the stressed rats or ultrasonic communication. Unfortunately, this project did not include analysis of the hippocampus, so direct implications for hippocampal NE and pheromone learning cannot be inferred.

4.9. Trauma and fear memory

Most human beings experience or witness a traumatic event in their lifetime, but only about 10% will develop PTSD as a result[406,407]. It has been hypothesized that PTSD reflects a "hyper-adrenergic state"[408], as elevated levels of NE were found in urine collected 24 hours following trauma[409]. PTSD patients have been shown to exhibit difficulty

differentiating safety from threat[410,411] while being perfectly capable of forming a cue-based fear memory[412,413], demonstrating that the basic ability of the fear conditioning system to acquire new conditioned responses is not damaged in PTSD. Conflicting results have been found in most avenues of PTSD research and there is not one comprehensive model that recapitulates or explains the collection of symptoms. Abnormalities in fear processing, generalization of fear responses to trauma related stimuli, exaggerated threat detection, diminished extinction, have all been proposed but none offer a complete explanation, missing crucial elements like flashbacks, nightmares, emotional numbing, changes in affective behaviours and cognition, and reckless behaviour possibly leading to more trauma (reviewed by Liberzon[414]).

Recently, a new model has been proposed, based on the idea that people who develop PTSD display a deficiency in contextual processing[414]. As described above, context is *everything*. If we hear an explosion as the clock strikes midnight on December 31st, cheers and laughter erupt, while an explosion at midnight on most other days of the year is cause for alarm. The smell of burning wood at a bonfire is a welcome comfort, while waking up in your home to the same smell signals an emergency. It has been postulated that PTSD could involve a pattern completion bias to threat cues with impaired pattern separation, both hippocampal-dependent processes, making it difficult to separate threat from safety cues[415,416], and contributing to hyperarousal and hypervigilance. This offers a potential explanation for flashbacks as well, partial trauma cues may elicit recurrent

reactivation of trauma memories which could lead to fear responses in a formerly "safe" context[414].

In the past decade, several human studies have provided important support for this contextual deficit model of PTSD. Reduced activity in the hippocampus of PTSD patients during contextual conditioned fear tasks has been observed, and PTSD patients fail to detect danger signals that were contextual in nature[412]. Genetic studies have also implicated hippocampal involvement in PTSD[417–419], and hippocampal size differences[420–422] along with impairments in hippocampal dependent tasks[423,424] could be predictive risk factors for PTSD development. It is currently unknown whether a hypofunctioning hippocampus leads to dysfunctional contextual integration which leads to aberrant fear expression, or if traumatic experiences cause hippocampal hyperexcitability by way of norepinephrine leading to difficulties with contextual integration in susceptible individuals who present with simultaneous hippocampal dysfunction and PTSD symptoms. Reduced hippocampal activity in PTSD patients may reflect damage inflicted by one or more traumatic experiences, reflected by enhanced hippocampal activity seen in the current thesis in the days following a "traumatic experience" (i.e., fear conditioning) upon recall of the memory. If PTSD patients exhibit a bias towards inclusion of cues as described above either throughout their lifetimes because of genetic susceptibility, or following one or more traumatic events, hyperactivity of the hippocampus could lead to excitotoxicity [425–427] which could be observed as reduced hippocampal function in those seeking treatment years later.

A large causal human functional magnetic resonance imaging (fMRI) study engaged 116 people who had been through a traumatic experience in the preceding two weeks. They found that severity of PTSD symptoms at 2 weeks was inversely correlated with hippocampal fMRI response. Importantly, this relationship only held true in patients with high, transient, fear potentiated startle in response to loud auditory cues and negative facial expressions[428]. Having such a large pool of participants who had experienced a traumatic event in the past 2 weeks is extremely rare, and this enabled the authors to investigate activity in specific brain areas in this population in response to aversive cues, potentially shining light on what makes certain individuals prone to developing PTSD, or at least PTSD-symptoms. PTSD can take days, weeks, or sometimes months or years to manifest following a traumatic experience[306], so a longitudinal study following some or all these participants, to see how many actually will go on to receive a PTSD diagnosis would provide valuable information. These authors framed their results in a way that fits with a contextual integration problem at the core of trauma memory intensity. Without a functioning hippocampus, the flexible high-resolution memory of an event is lost, and as a result patients cannot distinguish when it is adaptive to form fear memories, and are biased towards inclusion of more cues leading to more triggers and more fear[428]. It seems like it is possible that a genetically susceptible population of people could display reduced hippocampal function, leading to an inability to appropriately integrate contextual cues into the fear memory surrounding a recently experienced traumatic event, leading to a greater likelihood of developing PTSD. Perhaps the results of enhanced activity in the DH in this thesis reflects the "normal" response to a traumatic event, which is lacking in those predisposed to develop PTSD as a result of a traumatic event.

The most effective, feasible, and clinically validated treatments for PTSD today involve some type of exposure, with or without intentional modification of the original memory. For some people who have developed PTSD, it might be easier to report triggers (i.e., sensory stimuli which cause a fear reaction) and work through those with a therapist rather than trying to remember and describe extremely aversive experiences. The results obtained in this thesis support the notion that cues which have been classically conditioned with an aversive US are represented in the fear memory trace in the brain, and are reactivated when the memory is recalled by a higher order conditioned cue. This suggests that trauma cues which are far removed either temporally or spatially from the original trauma may possess the capacity of activating the entire engram complex, potentially leading to an alterable state that could be exploited by treatment. Future studies should investigate hierarchical relationships between conditioned stimuli in higher-order conditioning to understand the fundamental mechanisms by which aspects of a situation or experience are integrated into a prior fear memory representation.

Chapter 5 – Conclusions, future directions, and caveats

5.1. Conclusions

Alarm pheromone released from a stressed rat can serve as an US and produce associative learning in a receiver rat. Whether additional input from the stressed rat primes or amplifies these effects remains to be clarified, but the pheromone alone experiments demonstrate that this input suffices to produce conditioning. Unlike classical odor conditioning that leaves a memory trace in the MOB, pheromone conditioning potentiates AOB activation and the AOB appears to mediate the specific odor cue association as well. The two forms of learning activate common fear pathways in the amygdala and BLA plasticity is critical for both classical and pheromone conditioned learning. The pheromone project sheds light on how animals communicate with each other in nature and how they may avoid danger through pheromone-mediated associative learning.

Odor can act as a first- or a second-order conditioned stimulus in aversive fear memory, in other words odor can acquire conditioned value based on its direct pairing with shock or by its pairing with a feared tone or context. If odor is first-order conditioned or second-order conditioned with context, the same brain regions are activated upon recall: dorsal and VH, posterior piriform cortex, and BLA. If odor is second-order conditioned with context, the LA and auditory cortex are also activated upon recall of the odor fear memory. These results highlight the importance of the DH in fear memory, which may be

involved with integrating contextual cues while a fear response is occurring, or may reflect an excitable state induced by reactivation of the cells which encode US features. Importantly, the ability of an odor to induce activation in a primary sensory cortex devoted to processing auditory cues implies that members of an associative chain of conditioning from a stressful or traumatic experience can reactivate the entire engram complex.

This thesis demonstrated two forms of higher order conditioning – Pavlovian conditioning based on concepts of social communication and fear derived from learned and not inherently aversive cues. These types of learning could more closely resemble human learning than shock-based classical conditioning, and provide insight into the mechanisms of higher order fear learning that could ultimately lead to informing treatment of trauma related disorders like PTSD.

5.2. Future directions

5.2.1. Does recall of tone in tone-odor fear memory induce activity in the piriform cortex?

The finding which piqued the most personal interest was that an odor CS2 could elicit activation in the auditory cortex if it had been trained with an auditory CS1. This suggests that exposure to a link in the associative chain could cause a reactivation of the entire engram, including in sensory areas for a CS that is absent. It is, however, possible that the position of the CS in the associative chain relative to the US dictates which areas

are activated in response to a particular cue. To determine if the temporal order of CS higher order conditioning is relevant to engram complex activation, future studies could extend the higher order conditioning paradigm, including CSs from the same sensory modality, as well as CSs from varied sensory modalities, and measure activation to each CS in a series of experiments. An experimental technique from the recent study that identified 247 neural ensembles involved in contextual fear conditioning with a modified CLARITY technique[74] would allow for the visualization of connections between ensembles of neurons in the whole brain in response to each CS input in a higher order conditioning paradigm. It is crucial to determine if a single sensory stimulus in a memory chain can reactivate the entire engram complex, as this could explain the bias towards pattern completion and impairment in pattern separation hypothesized to contribute to PTSD symptoms[415,416].

5.2.2. Norepinephrine

The extent to which second-order conditioning in this paradigm depends on norepinephrine is unknown, but given the links to norepinephrine outlined in the pages above, and the fact that both the DH and BLA are persistently activated during recall of an odor fear memory regardless of how it is conditioned, NE dynamics should be explored in similar SOC paradigms. This thesis has demonstrated how NE plays a role in both pheromone and first order classical conditioning at β-adrenoceptors, which ultimately lead to excitation via the G_s pathway to facilitate learning. These receptors have the lowest affinity for NE and are therefore active at very high concentrations of NE, such as during a fear response. It is therefore likely that the second-order conditioning process is affected

by NE as well, although this has not been thoroughly explored. Future studies should infuse propranolol to block β-adrenoceptors at various stages to determine if preventing action of NE could prevent fear conditioning to higher-order conditioned stimuli. Despite dysfunctions in NE systems being one of the earliest genetic factors identified in the pathophysiology of PTSD [414], attempts at using propranolol to prevent consolidation of traumatic memories immediately after a traumatic experience have largely failed [429], but it is likely that timing and a deeper understanding of how NE functions to integrate contextual and cue memories in different brain structures is necessary. Future re-evaluation of propranolol in PTSD treatment in combination with some of the exposure therapies above may be warranted.

5.2.3. Higher order conditioning and generalized anxiety

Humans are shaped by every experience they have. Learning and memory mechanisms are crucial to help us remember what signals danger, pleasure, basic needs, etc. It is the nature of human beings to search for patterns in our surroundings to help us predict what will happen and guide our behaviour towards getting what we want or need. Generalized anxiety disorder (GAD) has increased significantly within the population[306] and affects people differently, hence the term "generalized;" in precisely the same way that plasticity allows us to obtain what we want, it also makes sure we remember the bad things that happen to us in attempts to avoid them. Every individual has a unique set of "triggers," stimuli that reflexively cause an intense emotional reaction, that have developed throughout

their lifetime in response to negative events or traumas. Many times, the triggers are not apparent to the person experiencing them.

At higher tiers of conditioning, for example fourth- or fifth-order conditioning, (which were described by Pavlov but have not since received due attention) perhaps fear reactions start to become generalized. Maybe this depends on the sensory modality of the CSs used throughout the conditioning paradigm. GAD and PTSD could be conceptualized as similar disorders if both involve impairments in contextual cue integration and bias towards pattern completion. It is important to determine if the SOC paradigms described in this thesis reflect mechanisms like those affected in PTSD or GAD (or both) to better aid in treatment efforts. Future studies should focus on carefully measuring responses to neutral stimuli with similar features to the CSs and evaluate the extent to which fear responses are generalized. It would be interesting to repeat this experiment in the presence of β-adrenoceptors to measure their effect on fear generalization as well. Finally, could higher order conditioning itself lead to generalized anxiety if the CSs are unpredictably experienced? Measurements of generalized anxiety (e.g., elevated plus maze, open field test) taken throughout these kinds of experiments can reveal the propensity to develop generalized anxiety in similar or distinct sensory CSs.

5.3. Caveats

For the catFISH experiments, DAPI was used as a counterstain. DAPI stains nuclear DNA, and because *Arc*/*H1a* catFISH involves counting foci it was critical to verify the positive signal was colocalized with a nucleus, as the background stain tended to be speckled. One of the inclusion criteria was colocalization of the nuclear stain and the foci for three consecutive z-stack images. However, because it is a general DNA marker, DAPI stains all cell types. In each region care was taken to avoid glial cells, whether this involved manual or automatic counting. Glial cell nuclei tend to be smaller and rounder than neuronal nuclei and their DNA is more compact. Although *Arc* and *H1a* are expressed solely in neurons, it is possible that some cells mistakenly included in the analysis were glia, but this was consistent across groups as the same experimenter counted all of the images. Further studies could add specific markers to investigate cell types.

cFos protein is expressed in both neurons and glial cells, and no attempt was made to differentiate between cell types in this experiment. It is likely that other cell types were included in the analysis for each region, but this was consistent across groups and experiments. Experiments could be repeated with immunofluorescence and specific cell type markers to elucidate which cell types participate reliably in engram activation, or with another IEG that is specific to neurons.

Importantly, all the catFISH experiments were completed in right hemispheres only, while IHC experiments evaluated both hemispheres equally. While hemispheric differences in fear conditioning have been extensively discussed in the literature, for example unilateral human temporal lobectomy (including the amygdala) impairs fear learning[430] and levels of protein kinase C were differentially expressed in the left versus right hemispheres of trained animals[431], such differences were not considered for the current thesis.

Animals of both sexes were used for all experiments, but no consideration was taken as to the estrous phase of female rats which has been shown to play a role in associative learning, specifically in fear conditioning[432,433]. While no sex differences were found statistically in the current thesis, the number of animals in each group split up by sex are small. This leads to uncertainty in the conclusion that sex differences are not responsible in part for results observed here. Future studies should follow each animal's estrous cycle and only complete behavioural training and testing outside of the pro-estrous phase[432].

Control and unpaired groups in Experiments 4 and 5 were first order conditioned. Whether the CS1 was contextual or auditory in nature, each group received CS1 and shock pairings, and the difference between the groups always occurred during the second stage of CS pairing. Shock itself is such a powerful US and as such it was an important variable to control for, which is why all experimental paradigms included FOC. In contrast, the first-order conditioning SOC experiment does not have a shocked control group. The unpaired

group for the tone-odor SOC experiment was introduced late in the experimental design. The control group for the FOC experiment should have been designed as an unpaired control that receives both odor and shock, but separately and not in a way that they become associated.

Automatic counting was employed for the cFos IHC experiments, which enabled faster analysis and therefore facilitated the inclusion of a larger quantity of engram complex candidates. The protocol used for IHC, which minimized the antibody concentration down to a 1:10,000 dilution, also facilitated the broad search, but importantly this was a free-floating protocol. Each section completed the entire IHC procedure in single well trays, moving each of the slices one by one to the next well with a paintbrush. The stain used was SG grey (Vector Labs) as an alternative to DAB staining, which is a known carcinogen. Each slice was therefore developed in series, and the staining was often variable across individual IHC experiments. To minimize some variability, every group was included for each round of IHC completed, i.e., in the tone-odor paradigm, one round of IHC was completed for 2 control, 2 unpaired, and 2 experimental animals simultaneously. This was not enough to completely minimize the variability in stain intensity. Automatic counting was completed by ImageJ, which contains a function that allows conversion of microscope images into binary (black and white) images, in order to set size and circularity parameters for counting. The images need to first be thresholded, so the computer knows what to count as positive and what to subtract as background. Thresholding was done individually by the same experimenter unaware of the conditions of the animals. A single threshold numerical

value could not be applied to the images, as this value depends on the intensity of the original image, which varied for reasons outlined above. Thresholding was turned up all the way (so that everything was positive) and then gradually reduced until no major background stain remained. All sections were thresholded in the exact same manner, although this resulted in varied numerical thresholding scores. Machine learning has recently been applied to neuroscientific data analysis to reduce experimenter bias (reviewed by Goodwin[434]), and could likely be adapted to fit data such as that described in this thesis.

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Appendix – Animal use protocol

From: maw222@mun.ca
To: Yuan, Qi
Cc: maw222@mun.ca
Subject: Your Animal Use Protocol has been renewed
Date: Tuesday, March 1, 2022 3:28:22 PM

Animal Care Committee (ACC)
St. John's, NL, Canada A1C 5S7
Tel: 709 777-6620 acs@mun.ca
https://www.mun.ca/research/about/acs/acc/

Dear: Dr. Qi Yuan, Faculty of Medicine\Division of BioMedical Sciences

Researcher Portal File No.: 20222358
Animal Care File: 22-04-QY
Entitled: (22-04-QY) Brain circuitry underlying 1st and 2nd order fear conditioning
Status: Active
Related Awards:

Awards File No	Title	Status	
20160955	Memory: Modifiable odor representations, adaptive behavior and Alzheimer's disease	Completed	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20181548	L-type calcium channels in memory	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

Ethics Clearance Terminated: March 01, 2025

Your Animal Use Protocol has been renewed for a three-year term. This file replaces previous File ID [20192529] and Animal Care ID [18-04-QY] as the active ethics clearance associated with this project. Please note the new file ID and Animal Care ID when referring to this protocol.

This ethics clearance includes the following Team Members: Dr. Qi Yuan (Principal Investigator)

This ethics clearance includes the following Sponsors: [[AllSponsorAgencyNames]]
This ethics clearance includes the following related awards:

Awards			

File No	Title	Status	
20160955	Memory: Modifiable odor representations, adaptive behavior and Alzheimer's disease	Completed	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses
20181548	L-type calcium channels in memory	Active	1. Research Grant and Contract Services (RGCS) – St. John's and Grenfell Campuses

An Event [Annual Report] will be required following each year of protocol activity.

Should you encounter an unexpected incident that negatively affects animal welfare or the research project relating to animal use, please submit an Event [Incident Report].

Any alterations to the protocol requires prior submission and approval of an Event [Amendment].

NOTE: You can access a copy of this email at any time under the "Shared Communications" section of the Logs tab of your file in the Memorial Researcher Portal.

Please note that approval of the protocol or amendment does not guarantee space for animal housing or procedures. Coordination with Animal Care Services is required prior to ordering animals.

Sincerely,

MARIE WASEF | ACC COORDINATOR
Department of Animal Care Services
Memorial University of Newfoundland
Health Sciences Centre | Room H1848
P: 709-777-6621
E-Mail: maw222@mun.ca

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