BRIDGING BEHAVIOUR AND MECHANISM IN RELIEF LEARNING IN RATS: A COMBINED BEHAVIOURAL, MOLECULAR, AND CHEMOGENETIC APPROACH

A Thesis Submitted to the Committee of Graduate Studies in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Faculty of Arts and Science

TRENT UNIVERSITY

Peterborough, Ontario, Canada

Psychology M.Sc. Graduate Program

January 2026

ABSTRACT

Bridging Behaviour and Mechanism in Relief Learning in Rats: A Combined Behavioural, Molecular, and Chemogenetic Approach

Lexi Thivierge

Maladaptive fear can develop when nonthreatening stimuli are misinterpreted as dangerous. While fear extinction has been extensively studied, organisms can also learn safety through relief learning, in which cues signalling the termination of an aversive event acquire positive value. Although the medial prefrontal cortex (mPFC) is implicated in regulating responses to threat and safety cues, its role in relief learning remains unclear. In Experiment 1, I validated a relief conditioning paradigm in rats and demonstrated that relief-conditioned animals froze significantly less than fear-conditioned animals during retention. Experiment 2 revealed that relief learning selectively activated the prelimbic cortex (PrL). In Experiment 3, chemogenetic inhibition of the PrL reduced freezing across tones, supporting a causal role in relief expression. Experiment 4 demonstrated that extended training produced more stable and pronounced reductions in freezing than a one-day protocol. Together, these findings identify PrL circuits as key contributors to relief learning.

Keywords: Maladaptive fear, relief learning, fear conditioning, medial prefrontal cortex (mPFC), fear extinction, rat model, behavioural freezing, Fos expression, neural circuitry, conditioned stimuli, aversive stimuli, neuroimaging.

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Dr. Neil Fournier, for his guidance, support, and mentorship throughout my Master's research. His expertise and encouragement were instrumental to the completion of this work. I would also like to thank my supervisory committee, Dr. Hugo Lehmann and Dr. Neal Melvin, for their time, insight, and constructive feedback.

I am grateful to the members of the Fournier Lab for creating a supportive and collaborative research environment. Their assistance, thoughtful discussions, and camaraderie throughout this process made the challenges of graduate research far more manageable.

Finally, I would like to thank all of my lovely family and friends for their constant encouragement throughout my graduate studies. Thank you to my Mom and Dad for always cheering me on. Thank you to my partner, Luke, for always believing in me. Their patience, encouragement, and steady presence are much appreciated, and this work would not have been possible without them.

Table of Contents

ABSTRACT ii
Acknowledgements iii
List of Figures vi
List of Tables vii
List of Abbreviations and Symbols vii
Chapter I: Introduction 1
1. Overview and Rationale 1
2. Neurobehavioural Mechanisms of Threat and Fear Learning 3
2.1 Principles of Associative Learning in Threat and Fear Conditioning 3
2.2 Threat-Predictive Cues and the Importance of Stimulus Contingency 4
2.3 Models to Study Threat and Fear: Cued Fear Conditioning and Contextual Fear Conditioning 5
2.4 Key Brain Circuits in Threat and Fear Learning 6
3. Conditioned Inhibition and Safety Learning 12
3.1 Extinction 13
3.2 Safety Learning 14
3.3 Relief Learning 15
4. Clinical Relevance 25
5. Thesis Aims and Knowledge Gap 27
Chapter II: Materials and Methods 31
2.1 Animals 31
2.2 Conditioning Apparatus 31
2.4 Experiment 1: Characterization of Relief Learning 32
2.4.1 Relief and Fear Conditioning 32
2.5. Experiment 2: Fos Expression in the mPFC Following Recall Testing 33
2.5.1 Perfusions and Fos Immunohistochemistry 34
2.5.1 Quantification of Fos Immunoreactivity 35
2.6 Experiment 3: Effect of Chemogenetic Inhibition of the PrL on Relief Learning 35
2.5.1 Viral Targeting 36
2.5.2 Relief Conditioning 36
2.5.3 Immunofluorescence 37
2.7 Experiment 4: Effect of Extended Relief Learning on Conditioned Inhibition 38
2.7.1 Fear and Relief Conditioning 38

2.8 Statistical Analysis 40
Chapter III: Results 41
1. Experiment 1: Characterization of Relief Learning 41
2. Experiment 2: Fos Expression in the mPFC Following Recall Testing 43
3. Experiment 3: Effect of Chemogenetic Manipulation of the PrL on Relief Learning 45
4. Experiment 4: Effect of Extended Relief Learning on Conditioned Inhibition 50
4.1 Recall Test 1. 52
4.2 Recall Test 2. 53
Chapter IV: General Discussion 54
1. Summary 54
1.1 Behavioural Differentiation of Relief Learning 55
1.2 Neural Correlates of Relief Learning and the Role of the Prelimbic Cortex 56
2. Implications and Clinical Relevance 58
3. Limitations and Future Directions 59
4. Conclusion 60
References 61

List of Figures

Figure 1. The opponent process theory

Figure 2. Fear versus relief conditioning

Figure 3. Comparison of the relief versus fear protocols, highlighting the adjustments in protocol from Experiment 1

Figure 4. Summary of results from Experiment 1.

Figure 5. Summary of results from Experiment 2.

Figure 6. Experimental timeline and confocal images from Experiment 3.

Figure 7. Summary of results from Experiment 3.

Figure 8. Summary of results from Experiment 4.

List of Abbreviations and Symbols

AB – accessory basal nuclei of the amygdala

BA – basal nuclei of the amygdala

CeA – central nucleus of the amygdala

Cg – cingulate cortex

CR – conditioned response

CS – conditioned stimulus

GFP – green fluorescent protein

IL – infralimbic cortex

LA – lateral amygdala

mPFC – medial prefrontal cortex

NaC – nucleus accumbens

NS – neutral stimulus

PAG – periaqueductal grey

PrL – prelimbic cortex

PVN – paraventricular nucleus of the hypothalamus

UCR – unconditioned response

UCS – unconditioned stimulus

vmPFC – ventromedial prefrontal cortex

Chapter I: Introduction

1. Overview and Rationale

Survival depends on the ability to quickly detect and respond to danger, while also recognizing when the threat has passed. From invertebrates to humans, the transition from a state of fear or threat to one of relief or safety is critical for adaptive behaviour. Fear refers to the subjective feeling, along with a complex set of behavioural and physiological changes that occur in the presence of a threat (Johansen et al., 2011). Fear enables an organism to react quickly to a threat by triggering the fight-or-flight response, thereby avoiding injury or death. For example, fleeing a dangerous situation or hiding from predators are fear responses. In contrast, relief refers to the evocation of a transient positive affective state that occurs when an aversive event has ended or subsided (Gerber et al., 2014). This enables the organism to quickly shift from defensive behaviours back to a state of safety, which is rewarding and plays a key role in adaptive behaviour (Leknes et al., 2011; Seymour et al., 2005). If the organism were unable to make this switch, it would remain in a state of extended psychological and/or physiological distress. This can lead to deleterious effects such as elevated and prolonged levels of stress hormones and can suppress the immune response, resulting in damaging effects in the long term (Mariotti, 2015).

While the biology of fear has been extensively studied, far less is understood about the processes underlying relief. Relief refers to the positive, short-term state that occurs after an aversive event ends (Gerber et al, 2014). Relief can be studied in several ways. One common method involves the animal learning to associate specific cues with

the offset of an aversive event (Gerber et al., 2014). The offset of a foot shock followed by a concurrent tone shows positive learned valence, as demonstrated by reduced freezing and increased approach behaviours (Andreatta et al., 2012). Similarly, in invertebrates such as Drosophila, an odour paired with the cessation of a shock supports approach behaviour, indicating conserved relief mechanisms across species (Tanimoto et al., 2014). Despite growing evidence supporting the significance of relief learning, its underlying neurobiological mechanisms remain incompletely understood. Emerging evidence suggests that the medial prefrontal cortex (mPFC), a higher-order association area in the neocortex, plays a central role in integrating information about safety and threat (Chen et al., 2017; Gerber et al., 2014; Ng & Sangha, 2023). However, the precise neural mechanisms by which the mPFC contributes to relief learning remain unclear. This knowledge gap is clinically significant, as disrupted relief and safety learning have been implicated in trauma-related disorders, where individuals may struggle to reduce physiological, behavioural, and subjective aspects of fear, even when danger is no longer present, which is a hallmark of anxiety disorders- some of the most prevalent mental health conditions both in Canada and globally (American Psychiatric Association, 2013; Dozois, 2021; McRae et al., 2016). Understanding how relief learning works at a neural level may therefore not only clarify the transition from fear to safety but also inform therapeutic strategies that rely on recalibrating maladaptive threat responses.

My Master's thesis attempts to address this gap in neurobiological and behavioural understanding of relief learning mechanisms by investigating relief learning in a rodent model, with a focus on the behavioural and molecular mechanisms underlying mPFC involvement. The following sections will first outline the neurobehavioural

mechanisms of threat and fear learning, then review processes related to conditioned inhibition and safety learning. Next, I will discuss the current state of knowledge pertaining to relief learning and discuss the clinical relevance of relief learning in the context of neuropsychiatric conditions. I will conclude this section with a discussion of the overall objective and aims of my thesis research.

2. Neurobehavioural Mechanisms of Threat and Fear Learning

2.1 Principles of Associative Learning in Threat and Fear Conditioning

Pavlovian associative learning, or classical conditioning, is one of the most fundamental processes by which organisms adaptively respond to their environment. First demonstrated by Ivan Pavlov in the 1920s (Pavlov & Anrep, 1927), this learning mechanism involves forming an association between an unconditioned stimulus (UCS), which naturally elicits a response, and a previously neutral stimulus (NS). The UCS triggers an unconditioned response (UCR), one that can occur without prior learning. Through repeated pairings of the NS and UCS, the NS becomes a conditioned stimulus (CS) capable of producing a conditioned response (CR) in the absence of the UCS (McSweeney & Murphy, 2014). In Pavlov's classic experiment, food served as the UCS, and the dog's salivation in response to the food was the UCR (Pavlov & Anrep, 1927). Initially, the sound of a metronome was a neutral stimulus, as it did not cause salivation on its own. However, after repeated pairings of the metronome (NS) with food (UCS), the metronome began to elicit salivation independently. At this point, the metronome became a conditioned stimulus (CS), and the dog's salivation in response to the metronome alone became a conditioned response (CR), which is a learned response to a previously neutral stimulus.

While Pavlov's original experiments involved appetitive stimuli (e.g., food), the same principles apply to aversive stimuli. A neutral cue, such as a tone, can become associated with an aversive event (e.g., an electric shock), resulting in the cue itself triggering fear or avoidance responses in a process known as fear conditioning. Eventually, the tone acquires the ability to elicit innate fear responses that typically occur when the organism is in danger, such as defensive behaviours including freezing, autonomic changes such as raised blood pressure and heart rate, and neuroendocrine responses including the release of stress hormones. The evolutionary relevance of this mechanism allows organisms to predict and respond to threats before they occur, which can be lifesaving. Pavlovian fear conditioning, therefore, is a way to study this innate response to threats in the laboratory. This basic associative process is found across species, from invertebrates to humans, and forms the foundation for much of our understanding of threat learning.

2.2 Threat-Predictive Cues and the Importance of Stimulus Contingency

In standard fear conditioning paradigms, a CS (such as a tone) generally precedes and predicts the UCS (e.g., a foot shock). This temporal structure, known as forward conditioning, allows the organism to learn that the CS signals an imminent threat, triggering anticipatory defensive responses (Bruning et al., 2016). These defensive responses include a variety of different reactions, including increased heart rate, fleeing, or fighting. In rodents, the most commonly measured defensive response is freezing, a suppression of all movement except for those required for respiration (Fanselow & Bolles, 1979), which enhances survival by reducing detection from predators. In forward conditioning, temporal contiguity is critical: the closer in time the CS occurs before the

US, the stronger the association that is formed (Rescorla, 1988). If the CS and US are too far apart, or overlap in an uninformative way, conditioning is weakened. In addition to temporal contiguity, contingency is critical for conditioning. Contingency refers to the degree to which the CS provides information about the likelihood of the US. For example, if a tone consistently precedes a shock (high contingency), the animal rapidly learns the association. In contrast, if the tone and shock occur independently or randomly (low contingency), learning is impaired, even if they are temporally close (Rescorla, 1968). Thus, conditioning requires not only that the CS and US occur near each other in time, but also that the CS serves as a reliable predictor of the aversive event.

2.3 Models to Study Threat and Fear: Cued Fear Conditioning and Contextual Fear Conditioning

Cued fear conditioning is a simple, reliable, and widely validated model for studying threat learning in the laboratory (Fendt & Fanselow, 1999). In this paradigm, an initially neutral stimulus (tone or light) is repeatedly paired with a mild foot shock. Over time, the cue alone triggers conditioned fear responses, even without the presentation of a shock. This approach is highly adaptable across species and experimental contexts, allowing researchers to probe both behavioural and neurobiological mechanisms. In rodents, behavioural responses include freezing, startle potentiation, suppression of exploratory behaviour, and changes in autonomic function (increased heart rate, respiration). Overall, the ease of establishing cued fear, the speed at which animals learn this association, the stability of this fear memory, and the ability to study the innate fear response make cued fear conditioning a great model for studying fear mechanisms.

Contextual fear conditioning is another way to study fear and threat responses in laboratory animals. In this method, the environment or context (i.e. experimental chamber) that the animal is placed in can be paired with an aversive UCS. Therefore, the fear response will occur when the animal is returned to that context, without the need for a cue. This form of conditioning is reliant on the hippocampus, as hippocampal lesions abolish contextual fear memories but not cued fear memories (Kim & Fanselow, 1992).

2.4 Key Brain Circuits in Threat and Fear Learning

A great deal of work has been directed at understanding the neural circuitry that underlies threat and fear learning. To date, this work has highlighted that the medial prefrontal cortex (mPFC), amygdala, hippocampus, and nucleus accumbens serve as key nodes involved in the learning and retention of threat and fear. While each region contributes to distinct aspects of threat learning, they collectively work to support the acquisition, modulation, and expression of learned fear responses. I will now discuss the importance of each of these regions to threat and fear learning.

Medial prefrontal cortex

The rodent mPFC supports higher-order executive functions, which include memory, cognitive flexibility, temporal processing, and inhibitory control, which are important for adaptation and response to environmental threats (Kesner & Churchwell, 2011). Through its extensive connections with the amygdala, hippocampus, and nucleus accumbens, the mPFC integrates sensory, emotional, and motivational information to guide adaptive responses to environmental challenges. The rodent mPFC is divided into subregions, which include the anterior cingulate cortex (ACC), the prelimbic cortex (PL), and the infralimbic cortex (IL), organized from dorsal (ACC) to ventral (IL).

The PL is generally associated with the expression of fear behaviours. When the PL is inactivated using the sodium channel blocker tetrodotoxin (TTX) during conditioning, there is a reduction in freezing to both a tone (cued conditioning) and a context (contextual conditioning) that has been previously paired with a foot shock (Corcoran & Quirk, 2007). However, inactivating the PL prior to conditioning did not prevent the acquisition of fear memory, showing that it is crucial for fear expression but not fear acquisition (Corcoran & Quirk, 2007). It is important to note that this effect is only found in learned fear, and was not shown in innate fear (e.g., exposure to a predator) (Cororan & Quirk, 2007). Anatomically, PL neurons project to the basolateral amygdala (BLA) (Corcoran & Quirk, 2007). It is thought that the PL integrates auditory and contextual inputs and modulates the expression of fear through its projections to the BLA (Corcoran & Quirk, 2007). Additionally, within the BLA, fear neurons project to the mPFC; however, it remains unknown whether these pathways differentially target the IL and PL subregions (Herry et al., 2008).

In contrast to the PL, the IL is linked to fear inhibition and extinction (Sotres-Bayon & Quirk, 2010). IL neurons project primarily to the basolateral amygdala (BLA), forming a pathway that suppresses conditioned fear responses during extinction (Sotres-Bayon & Quirk, 2010). Through these projections, the IL can modulate amygdala output to downstream regions, such as the central amygdala (CeA), which orchestrates the expression of fear-related behaviours (Royer et al., 1999). Lesion studies demonstrate that damage to the IL impairs fear extinction, particularly the retrieval of extinction, indicating that this region is necessary for maintaining the inhibitory memory that suppresses conditioned fear (Quirk et al., 2000). Further evidence from Laurent and

Westbrook (2009) found that temporary inactivation of IL using muscimol, a reversible $GABA_A$ agonist, led to an impairment of long-term retention of extinction in rats. Although the animals were able to undergo extinction during the initial session, they failed to retain the memory when tested the following day, suggesting that IL activity is critical for the consolidation and/or retrieval of extinction memories (Laurent & Westbrook, 2009).

Amygdala

The amygdala is a collection of interconnected nuclei within the medial temporal lobe with importance in processing emotional and motivational information. In mammals, the amygdala is essential for learning and expressing aversive (and appetitive) behaviours. It is comprised of several subregions, including the BLA complex (basal, accessory basal, and lateral nuclei), which integrates sensory input and is heavily involved in associative learning, and the central amygdala (CeA), which is a major output hub for initiating physiological and behavioural responses to emotional stimuli. The intercalated cell masses (ITCs), which serve as inhibitory gates between the BLA and CeA, enable fear suppression and safety signalling (Royer et al., 1999). When the anterior basal nuclei, lateral amygdala, and central amygdala are neurotoxically lesioned before conditioning, rats are unable to learn fear associations with both contextual and auditory CSs, showing attenuated freezing to both (Goosens & Maren, 2001). However, this effect is not found with lesions of the posterior basal nuclei (Goosens & Maren, 2001). Additionally, lesions of the basal amygdala after conditioning completely block the expression of learned fear responses (Anglada-Figueroa & Quirk, 2005).

Early work implicated that the CeA is crucial in fear expression (LeDoux et al., 1988). Projections from the CeA to the hypothalamus are responsible for the autonomic

response to fear, and projections from the CeA to the central grey region are involved in the behavioural output of conditioned fear (LeDoux et al., 1988). Additionally, later work showed that the lateral nucleus (LA) is important for fear acquisition (LeDoux et al., 1990). The LA receives auditory inputs from the auditory thalamus and auditory cortex, and fear conditioning using an auditory CS can use either pathway (Romanski & LeDoux, 1992). CS and UCS information converge in the LA (Johansen et al., 2011) and drive synaptic plasticity, forming the basis for fear memory (LeDoux et al., 1990). The LA then communicates with the CeA through both direct and indirect pathways, including projections from the basal (BA) and accessory basal (AB) nuclei, as well as through intercalated cell masses that modulate output (Johansen et al., 2011). The CeA acts as a major output hub for fear responses, sending projections to the hypothalamus and brainstem, which coordinate the behavioural and physiological expression of fear (Johansen et al., 2011). Specifically, the periaqueductal grey (PAG) mediates freezing, the lateral hypothalamus regulates autonomic responses such as heart rate, and the paraventricular nucleus (PVN) initiates the release of stress hormones via the HPA axis (Johansen et al., 2011). Lesions of both the LA and CeA disrupt fear conditioning (LeDoux et al., 1990; Nader et al., 2001). Additionally, functional inactivation research has demonstrated that the CeA is important for fear expression and the learning and consolidation of fear conditioning (Wilensky et al., 2006). When the CeA is inactivated using the $GABA_A$ agonist muscimol, animals are unable to learn fear conditioning, and it leads to an almost complete abolishment of freezing, therefore affecting both fear acquisition and expression (Wilensky et al., 2006). Additionally, when protein synthesis is blocked within the CeA immediately after training, rats tested twenty-four hours later

show impairments in fear memory recall, demonstrated by reduced freezing compared to controls, suggesting that the CeA plays a role in the consolidation of fear memory (Wilensky et al., 2006)

Hippocampus

The hippocampus (HPC) is a medial temporal lobe structure critical for functions including contextual processing and memory formation in rodents. It consists of interconnected subfields, including the CA1, CA2, CA3, and dentate gyrus (DG). The HPC is particularly important for associating environmental cues with specific outcomes, making it essential in contextual fear conditioning (Kim & Fanselow, 1992).

In 1992, Kim and Fanselow showed that hippocampal lesions disrupted contextual, but not cued, fear conditioning. In 1999, Rudy and O'Reilly demonstrated that this is due to contextual fear conditioning relying on a holistic, conjunctive memory of the context as opposed to a separate cue. In other words, rather than associating fear with one isolated feature, the hippocampus allows the animal to bind together multiple contextual cues (e.g., lighting, odours, textures) into a unified memory that can then be associated with the aversive event. When the HPC is lesioned, this process is disrupted because the animal cannot retrieve the whole-context memory, so their fear response is dampened (Kim & Fanselow, 1992; Phillips & Ledoux, 1992). Notably, these impairments are greater when lesions are performed shortly after conditioning, indicating that recent memories depend more strongly on hippocampal integrity (Maren et al., 1997). Lesion studies reveal that both the dorsal and ventral HPC are independently necessary for the long-term expression of learned fear in contextual conditioning (Wang et al., 2013). They contribute in different ways: the dorsal hippocampus is primarily involved in processing spatial and contextual details of the environment, whereas the

ventral hippocampus is more strongly linked to emotional regulation and the strength of fear expression (Kjelstrup et al., 2002; Moser & Moser, 1998; Wang et al., 2013). While both are additionally responsible for forming a contextual representation, the ventral HPC also regulates defensive behaviours (Wang et al., 2013). The hippocampus also sends excitatory projections to the PL and IL of the mPFC (Hoover & Vertes, 2007). Ventral hippocampal projections may also preferentially target interneurons in the mPFC, further regulating mPFC output (Sotres-Bayon et al., 2012). When the ventral HPC is inactivated, PL interneuron activity is decreased, and the animals show increased fear responses, suggesting a modulatory role in top-down fear regulation (Sotres-Bayon et al., 2012). The HPC also supports fear renewal, linking context with relapse after extinction (Orsini et al., 2011; Zelikowsky et al., 2012)

Nucleus accumbens

The nucleus accumbens (NAc), a key region of the ventral striatum, is traditionally associated with reward and motivation, but it also plays a critical role in aversive learning (Bradfield & McNally, 2010; Ray et al., 2020). In rodents, the NAc integrates excitatory inputs from the amygdala, mPFC, and HPC (Bagot et al., 2015). Its involvement in salience processing highlights the NAc as a central hub that translates threat-related information into adaptive actions (Ray et al. 2020). Functionally, the NAc core and shell serve differing roles. The NAc core is essential for adjusting the fear response based on the degree of threat (Ray et al., 2020). Conversely, the NAc shell is responsible for associative learning between neutral stimuli (e.g. tone) and aversive events (Bradfield & McNally, 2010). The NAc shell is also linked to the expression of defensive behaviours such as freezing (Piantadosi et al., 2020).

Together, the mPFC, amygdala, HPC, and NAc form an integrated network for detecting threats, forming aversive associations, and regulating fear expression. The mPFC exerts top-down modulation over amygdala activity, the HPC provides contextual output, the amygdala encodes and drives fear responses, and the NAc integrates these signals to guide adaptive behaviour.

3. Conditioned Inhibition and Safety Learning

A core feature of adaptive defensive behaviour is the ability to not only learn when a stimulus predicts danger but also when it signals the *absence* or *termination* of danger. This latter process is captured by conditioned inhibition, a form of learning where a stimulus signals the absence or omission of an expected aversive event, thereby predicting safety rather than threat (Pavlov, 1927; Rescorla, 1969). Conditioned inhibition provides the foundation for related phenomena such as extinction, safety learning, and relief learning, which all involve learning that danger is no longer imminent. In 1971, Robert Rescorla created two behavioural tests that are used to confirm whether a CS is a conditioned inhibitor. In the summation test, the CS should reduce fear when paired with a threat signal (Rescorla, 1971). If a tone predicts a shock and a light predicts safety, when the light is paired with the tone, the rat should show less fear to the combined cue than to the tone alone. In the retardation test, the CS should be harder to turn into a danger signal later. If a light is trained as a safety signal and then the experimenter turns it into a threat signal, the rat will learn much more slowly that the light predicts danger compared to a neutral stimulus. These behavioural assays remain critical for distinguishing true conditioned inhibitors from simple reductions in responding.

3.1 Extinction

Extinction learning is a fundamental process that allows organisms to update previously learned associations when they are no longer predictive of meaningful outcomes (Pavlov, 1927). In the context of fear conditioning, extinction occurs when a conditioned stimulus (CS), which previously signalled danger, is repeatedly presented without the expected aversive stimulus (US). Over time, this results in a gradual reduction of conditioned fear responses, such as freezing behaviour in rodents (Myers & Davis, 2007). Extinction does not erase the original fear memory; instead, it involves the formation of a new inhibitory memory that suppresses the expression of conditioned fear (Bouton, 2004). This explains why extinguished fear can return under certain conditions, such as spontaneous recovery (the re-emergence of fear over time), reinstatement (return of fear following re-exposure to the US), or renewal (reappearance of fear when the CS is presented outside the extinction context) (Bouton, 2004; Vervliet et al., 2013).

Extinction learning is highly context-dependent, meaning that the suppression of conditioned fear is often tied to the environment in which extinction occurred (Maren et al., 2013). For example, a CS extinguished in one context may elicit robust fear responses when presented in a different context, demonstrating that the original fear memory remains intact but is modulated by contextual cues (Maren et al., 2013). Neural evidence indicates that extinction relies on many of the same circuits involved in threat learning, particularly the medial prefrontal cortex (mPFC), amygdala, and hippocampus (Quirk & Mueller, 2008). The ventromedial prefrontal cortex (vmPFC) plays a critical role in inhibiting fear responses by exerting top-down control over the amygdala, allowing an organism to recognize that a previously threatening cue no longer predicts danger (Milad

& Quirk, 2012). Specifically, extinction relies on IL projections to the BLA (Bloodgood et al., 2018). In contrast, the HPC contributes to context-dependent extinction recall, meaning that fear suppression is often tied to the specific environment in which extinction occurred (Maren et al., 2013). Overall, while extinction learning reduces conditioned fear, it is often fragile and susceptible to spontaneous recovery, renewal, and reinstatement (Vervliet et al., 2013).

3.2 Safety Learning

Safety learning involves explicit learning that a particular cue predicts the absence of an aversive outcome (Gerber et al., 2014). Here, a "safety signal" acquires inhibitory properties by being consistently presented in situations where the UCS is omitted (Rescorla, 1988). Safety learning can occur in two ways: explicit unpairing or differential conditioning. In explicit unpairing, the CS- is never presented in temporal proximity to the US, making it a consistent predictor of safety (Rescorla, 1988). In differential conditioning, one cue (CS+) predicts shock, while another cue (CS-) signals the absence of shock, enabling animals to discriminate safety from threat. The CS- then becomes a conditioned inhibitor (Kong et al., 2014).

Neurobiologically, safety learning engages partially overlapping but distinct circuits from those supporting extinction. While extinction primarily relies on IL projections to the BLA to form a competing inhibitory memory that suppresses conditioned fear (Bloodgood et al., 2018), safety learning additionally recruits inhibitory microcircuits within the amygdala, particularly the intercalated cell masses (ITCs), which actively suppress central amygdala (CeA) output during safety cue presentation (Royer & Paré, 2002).The HPC supports contextual discrimination, allowing safety signals to be

interpreted relative to the broader learning environment (Maren et al., 2013). When animals learn to discriminate between safety and fear conditions, the anterior medial bed nucleus of the stria terminalis (BNST) is also recruited compared to animals who only experience the fear condition, suggesting a role for the BNST in mediating safety-threat distinctions (Foilb et al., 2021). Together, these differences suggest that safety learning not only suppresses fear but also actively signals predictive safety and approach behaviours, distinguishing its circuitry and functional outcomes from those of extinction.

While safety learning and extinction both result in the suppression of fear responses, they differ in mechanism and behavioural expression. Extinction involves repeated non-reinforcement of a threat cue, whereas safety learning depends on explicit discrimination between danger and safety cues. Moreover, safety signals can serve as conditioned inhibitors that actively block fear expression, whereas extinction memories are often fragile and context dependent. These differences have important clinical implications, as safety signals may confer more durable and generalizable reductions in fear compared to extinction (Christianson et al., 2012).

3.3 Relief Learning

Relief learning is a related but unique form of associative learning in which a neutral stimulus becomes a conditioned cue signalling the termination or offset of an aversive event, thereby acquiring positive motivational valence (Leknes et al., 2011). Unlike safety learning, which involves the explicit prediction of the absence of threat, relief learning specifically depends on the temporal relationship where the CS follows the aversive UCS offset, a process termed backward conditioning (Heth & Rescorla, 1973).

This temporal structure is crucial: the CS signals that the aversive event has ended, eliciting a state of relief that can motivate approach behaviours (Leknes et al., 2011). This learning is thought to engage reward-like processes, since the offset of aversive stimulation itself is reinforcing (Gerber et al., 2014). Although relief is often categorized as a type of reward, it is distinct from other forms of reward in meaningful ways (Leknes et al., 2011). Traditional rewards are typically defined by the inherent pleasantness of the stimulus itself, such as food or social interaction. In contrast, the pleasantness of relief depends not on the intrinsic qualities of the stimulus but on the prior experience of aversive states (Leknes et al., 2008, 2011). In other words, relief is derived from the offset of discomfort, rather than the rewarding properties of a stimulus.

3.3.1 Opponent Process Theory

Much of the framework for understanding relief learning has centred on the opponent process theory of emotion proposed by Solomon and Corbit (1974). Their theory posits that emotional responses are regulated by two linked processes: a primary "a-process" (Fear, distress, pain) and a secondary opponent or "b-process" (relief, calm, positive affect), which counteract the primary emotion (see Figure 1). The a-process is fast to activate, stimulus-driven, and decays rapidly after the aversive event ends. The b-process lags in onset, is slower to decay, and functions to restore affective homeostasis.

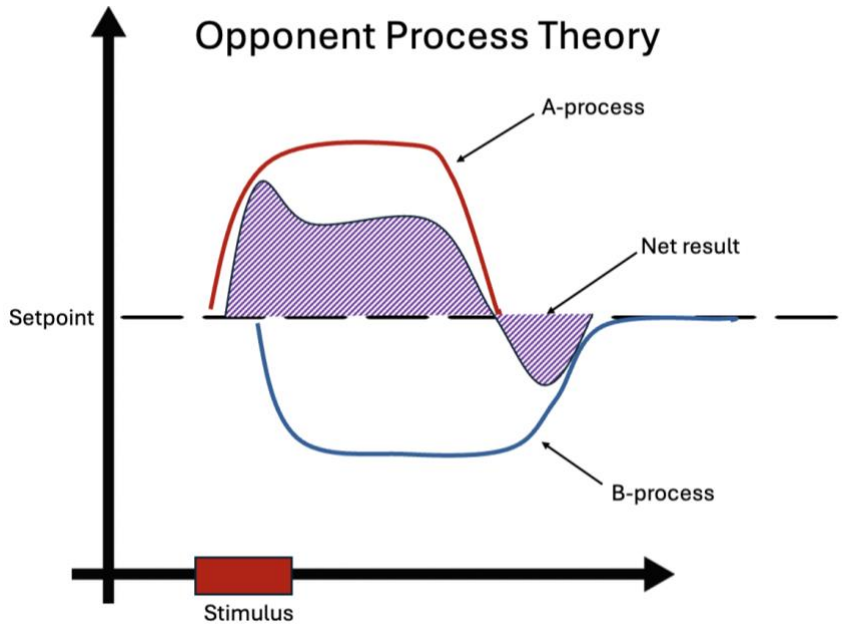


Figure 1. The opponent process theory. The a-process is the initial response to the stimulus. The b-process counteracts it to return to homeostasis.

In the context of Pavlovian fear conditioning, presentation of an aversive UCS (e.g., foot shock) initiates a strong negative a-process. Once the UCS terminates, a relief-related b-process emerges, generating a rebound of positive affect. Relief learning harnesses this temporal rebound, associating external cues with the onset of the b-process. Over repeated pairings, these cues acquire positive motivational value, capable of eliciting relief in the absence of the aversive event. Neurobiologically, the opponent process theory maps onto known circuits of fear and reward. The aversive a-process is encoded by the amygdala, which initiates fear expression, while the b-process is encoded by the NAc, which allows the organism to feel the positive reinforcing effect of relief (Navratilova & Porreca, 2014). At the neurochemical level, dopaminergic signalling in the NAc is critical for the b-process. UCS offset triggers the release of dopamine in the NAc shell, allowing relief cues to be reinforced like natural rewards (Luo et al., 2018).

Importantly, the mPFC orchestrates the shift between fear and relief through top-down control of the amygdala and NAc (Goodpaster et al., 2025). Recently, research has investigated neural substrates that support the opponent process theory of emotion. Two mutually inhibitory intercalated cell clusters in the amygdala, the dorsomedial (ITCdm) and ventromedial (ITCvm) ITCs, selectively respond to aversive and relief/rewarding stimuli, respectively (Hagihara et al., 2021). Most rodent work on the opponent process theory focuses on drug exposure. Supporting the opponent process framework, withdrawal from acute opiate exposure in rats induces a negative affective state via the mesolimbic dopamine system (Radke et al., 2011). Specifically, reduced opiate receptor stimulation in the ventral tegmental area (VTA) potentiates anxiety-like behaviour, which can be alleviated by dopamine receptor agonists, indicating that the emergence of negative affect during withdrawal arises from the same reward circuitry initially engaged by the drug. Consistent with opponent process theory, the nucleus accumbens appears to mediate both the positive and negative motivational aspects of drug use (Koob et al., 1989). While acute opiate exposure produces intense hedonic effects, the adaptive processes in this circuitry generate opposing negative affect during withdrawal, suggesting that the same neural substrate underlies both the rewarding 'high' and the aversive consequences of abstinence.

3.3.2 Neural Circuits and Neurotransmitters Involved in Relief

Relief learning differs from both extinction and safety learning in that the cue does not signal *non-occurrence* of an aversive event but rather its *explicit offset*, which acts as a reinforcer. Relief learning engages a network that overlaps with, but is distinct from, classical fear and safety circuits.

Medial Prefrontal Cortex

The rodent mPFC is divided into the prelimbic (PL) and infralimbic (IL) cortices, which have well-characterized roles in fear expression and extinction, respectively (Sotres-Bayon & Quirk, 2010). By analogy, it is thought that the mPFC may similarly modulate relief learning: the IL could facilitate suppression of fear when a cue signals aversive offset, whereas the PL may influence the behavioural expression before the relief cue (Sotres-Bayon & Quirk, 2010). However, direct experimental evidence linking mPFC subregions to relief learning remains sparse, leaving a critical gap in our understanding of how this region contributes to the formation, consolidation, and expression of relief memories.

Although relief-specific studies are limited, research in related paradigms suggests that the mPFC is capable of direct associative learning. For instance, optogenetic stimulation of mPFC pyramidal neurons can serve as a conditioned stimulus (Wu et al., 2015). This indicates that the mPFC is not solely a regulatory or inhibitory structure but can actively encode predictive relationships, mechanisms that may be used during relief learning. Evidence from fear and extinction studies suggests that the mPFC can coordinate interactions between fear and reward networks. IL projections to the amygdala facilitate suppression of conditioned fear (Quirk & Mueller, 2008), while mPFC-NAc connections may allow cues signalling aversive offset to acquire positive motivational significance (Lai et al., 2024). These interactions suggest a plausible role for the mPFC in integrating relief-predictive cues with downstream reward circuits. Emerging work in pain relief paradigms shows that the mPFC contributes to learning when cues predict the

cessation of ongoing aversive stimuli, highlighting a broader role in encoding positive outcomes following negative events (Zhang et al., 2018). These findings hint at a general function for the mPFC in relief learning, but the specific subregional mechanisms and neural circuit dynamics remain largely unexplored.

In summary, while the mPFC is a prime candidate for supporting relief learning, direct empirical evidence is scarce. Current data suggest that it may modulate the behavioural and motivational expression of relief and coordinate with reward circuits such as the NAc. The limited understanding of mPFC involvement provides a strong rationale for the present study, which aims to clarify how this region contributes to relief learning and its distinction from both fear extinction and safety learning.

Amygdala

Safety learning involves activation of inhibitory amygdala pathways, a feature not found in relief learning (Andreatta et al., 2012). The amygdala is traditionally associated with fear processing and emotional memory, yet its involvement in relief learning, where cues predict the cessation of an aversive event, is not well characterized. This gap in knowledge underscores the importance of investigating the amygdala's contributions to relief learning, as understanding its role could provide deeper insights into the neural mechanisms underlying adaptive behaviour and emotional regulation. Emerging evidence suggests that the amygdala and NAc are functionally connected and may jointly contribute to emotional learning processes (Ambroggi et al., 2008; He et al., 2023). For instance, studies have shown that the BLA projects to the NAc, and this pathway is involved in reward-seeking behaviours and reinforcement learning (Ambroggi et al.,

2008). These findings raise the possibility that the amygdala-NAc circuit could be engaged during relief learning, with the amygdala providing emotional context to the NAc's encoding of reward-related information. However, direct evidence linking amygdala-NAc connectivity to relief learning is currently lacking. Given the amygdala's established roles in emotional processing and memory, and its potential interactions with the NAc, it is plausible that this structure contributes to relief learning. However, the specific mechanisms by which the amygdala may influence relief learning remain unclear. Addressing this gap is crucial for a comprehensive understanding of the neural circuits involved in relief learning.

Hippocampus

The hippocampus (HPC) is a medial temporal lobe structure critically involved in contextual processing, memory formation, and spatial navigation. In classical fear conditioning, the HPC is essential for encoding the context in which aversive events occur, supporting context-dependent fear expression and extinction (Kim & Fanselow, 1992; Rudy & O'Reilly, 1999). However, its role in relief learning is comparatively underexplored, representing a significant gap in the field. While dorsal HPC is primarily associated with precise contextual encoding, the ventral HPC is thought to modulate emotional and motivational aspects of learning (Wang et al., 2013). In the context of relief learning, it is hypothesized that dorsal HPC may contribute to the formation of context-specific relief memories, ensuring that cues predicting aversive offset are tied to the appropriate environmental conditions. Ventral HPC may influence the emotional salience and motivational significance of relief cues, potentially interacting with the NAc and mPFC to regulate behavioural responses (Alemán-Andrade et al., 2025).

Evidence from fear extinction and safety learning suggests that the HPC interacts extensively with the mPFC and amygdala to encode and retrieve context-dependent memories (Orsini et al., 2011; Zelikowsky et al., 2012). By analogy, these same interactions may be critical in relief learning: the HPC could provide contextual information that allows an organism to predict when an aversive stimulus will terminate, enhancing the adaptive value of relief cues. Moreover, hippocampal projections to the NAc may help assign motivational value to relief-predictive cues, bridging contextual encoding and reward-related circuits (Hoover & Vertes, 2007; Bagot et al., 2015). Despite these plausible roles, direct experimental evidence for hippocampal involvement in relief learning is limited. Most existing studies focus on fear, extinction, or safety learning, leaving a critical gap regarding how the HPC contributes specifically to learning from aversive-offset cues. Understanding these mechanisms is essential, as the HPC may support both temporal and contextual specificity in relief memory formation and may interact with subcortical reward circuits to guide adaptive behaviour.

Nucleus Accumbens

The NAc has traditionally been studied in the context of reward, motivation, and reinforcement learning. However, recent work demonstrates its critical role in aversive-offset learning. Importantly, the nucleus accumbens (NAc) and its dopaminergic inputs play a central role, reflecting the reward-like properties of aversive offset (Andreatta et al., 2012). For example, Andreatta et al. (2012) showed that the onset and offset of aversive events engage distinct neural systems: shock onset recruits the amygdala-centred fear network, whereas shock offset engages mesolimbic dopamine pathways, with the NAc serving as a key integrative hub. Importantly, inactivation of the NAc impairs relief

learning while sparing safety conditioning, suggesting that the two processes are neuronally distinct (Mohammadi et al., 2014). This dissociation underscores that relief is not a passive state of "no threat," but rather an active positive signal encoded by reward-related circuitry. Relief cues thus acquire motivational salience through the NAc. Additionally, Mayer et al. (2018) found that dopaminergic projection from the posterior medial ventral tegmental area (pmVTA) to the nucleus accumbens shell (AcbSh) is selectively activated by aversive stimuli and is critical for relief learning. Lesions of the pmVTA or chemogenetic silencing of the pmVTA–AcbSh pathway impaired relief learning without affecting fear or safety learning. These findings indicate that this mesolimbic dopamine pathway specifically encodes relief.

Several lines of research demonstrate that relief learning depends on plasticity-related molecular mechanisms within the NAc. Bruning and colleagues (2016) found that temporarily inhibiting protein synthesis within the NAc using local infusions of anisomycin into the NAc shell or core immediately after relief conditioning disrupted the consolidation of relief memories into long-term storage. However, inhibiting protein synthesis 4 hours post-conditioning did not affect memory consolidation, suggesting a critical time window for protein synthesis in the NAc during the consolidation process. Similarly, Soleimanpour et al. (2021) investigated the molecular mechanisms underlying relief learning, focusing on the phosphorylation of cAMP response element-binding protein (CREB) in the NAc. The authors found that relief conditioning led to increased CREB phosphorylation in the NAc at 6 hours post-conditioning compared to controls. This phosphorylation was primarily mediated by dopamine D1 receptor activation, involving protein kinase A (PKA) and potentially other kinases downstream of NMDA

receptors. These findings suggest that CREB phosphorylation in the NAc is crucial for the acquisition and consolidation of relief learning, highlighting the role of cellular adaptations in the NAc during this process.

Relief learning is also shaped by multiple neurotransmitter systems within the NAc. Dopamine plays a central role: co-activation of dopamine D1 receptors and NMDA receptors within the NAc is necessary to encode relief (Bergado Acosta et al., 2017). This aligns with the broader literature on NAc-mediated reinforcement learning, where dopamine-glutamate interactions are critical for plasticity (Yagishita et al., 2014). Additionally, the endocannabinoid system modulates relief acquisition: blockade of CB1 (cannabinoid receptor 1) receptors in the NAc impairs learning but not retention, indicating a role in early phases of relief memory formation (Bergado Acosta et al., 2017). More recently, metabotropic glutamate receptor 7 (mGluR7) activity, which modulates appetitive processes within the NAc, has been implicated, further highlighting the diversity of neurotransmitter systems involved in shaping relief associations (Kahl & Fendt, 2016).

Together, these findings converge on the view that the NAc serves as the central locus for relief learning, translating aversive-offset signals into positively valenced associations. Relief learning depends on protein synthesis and transcriptional regulation within the NAc, requires coordinated dopamine and glutamate signalling, and is modulated by cannabinoid and metabotropic glutamate systems. Importantly, the NAc's necessity for relief, but not safety, provides strong support for opponent process theory, which posits that the termination of aversive states triggers an active and rewarding

opponent process. Thus, relief learning cannot be reduced to diminished fear but instead represents a qualitatively distinct learning process, mediated by reward circuitry, that may serve as a powerful mechanism for promoting safety in the aftermath of threat.

4. Clinical Relevance

The inability to transition from a fear response to a state of relief is a hallmark of anxiety disorders. These disorders are among the most prevalent mental health conditions both in Canada and globally (Dozois, 2021; McRae et al., 2016). Statistics Canada (2023) reported that the prevalence of anxiety disorders in Canadians aged 15 and older doubled between 2012 and 2022, rising from 2.6% to 5.2%. According to The *Diagnostic and Statistical Manual of Mental Disorders* (5th ed.; DSM–5; American Psychiatric Association, 2013), generalized anxiety disorder (GAD), which is the most common anxiety disorder, is characterized by persistent, excessive anxiety and worry across a range of situations or activities for a period of at least 6 months. Individuals with GAD often struggle to control their worry, resulting in significant emotional distress or functional impairment. Common associated symptoms include irritability, difficulty sleeping or concentrating, and restlessness (American Psychiatric Association, 2013). The impact of anxiety disorders extends beyond the individual, affecting family members, relationships, and broader social systems. Moreover, the economic burden is substantial: recent estimates suggest that anxiety disorders cost the Canadian economy approximately $17 billion annually (Dozois, 2021).

Post-traumatic stress disorder (PTSD) is another condition in which individuals struggle to access or initiate relief-related responses. PTSD develops following exposure to a traumatic event and is characterized by persistent intrusion symptoms, even after the

threat has passed (American Psychiatric Association, 2013). These may include intrusive and distressing memories of the event, flashbacks that stimulate the re-experiencing of the trauma, and heightened psychological or physiological reactivity to cues that resemble aspects of the original event (American Psychiatric Association, 2013). In addition to intrusion symptoms, individuals with PTSD often exhibit negative alterations in cognition and mood. These include persistent negative emotions, distorted beliefs about oneself and the world, dissociative amnesia, emotional numbing, and detachment from others (American Psychiatric Association, 2013). Changes in arousal and reactivity are also common, such as heightened irritability, reckless or self-destructive behaviour, hypervigilance, and exaggerated startle responses (American Psychiatric Association, 2013).

While fear is an essential emotion for survival, persistent fear can have unwanted effects. If relief from the fear is not initiated, it leads to extended psychological and physiological distress. Understanding the full scope of behavioural and psychological responses to painful or traumatic experiences requires attention not only to fear learning but also to relief learning. This dimension is particularly relevant when considering the development and persistence of both adaptive behaviours, like appropriate avoidance, and maladaptive conditions, including excessive safety-seeking, self-harm, risk-taking, panic disorders, and post-traumatic stress disorder (Gerber et al., 2014). Investigating how these behaviours are shaped by both fear- and relief-based learning processes may offer important insights into why certain therapies succeed or fail (Gerber et al., 2014). Therefore, continued research into the neurobiological mechanisms underlying

punishment and relief learning is essential for both theoretical understanding and clinical application.

5. Thesis Aims and Knowledge Gap

The present study investigates the behavioural and neural mechanisms underlying *relief learning*, a form of affective learning in which a previously neutral cue acquires motivational significance by predicting the offset of an aversive event. While much of the existing literature on associative learning has focused on fear acquisition and extinction, relatively little is known about how animals learn from the cessation of threat, an emotionally significant transition that may be highly relevant for understanding resilience and trauma recovery.

The first objective of this research is to gather behavioural evidence of relief learning in rodents using a backward conditioning paradigm. This is important because relief learning is less well characterized than fear conditioning, and I need to be able to demonstrate it behaviourally in the lab as a necessary foundation for examining its neural mechanisms. I hypothesize that rodents exposed to backward conditioning will show reduced freezing to the tone, consistent with relief learning.

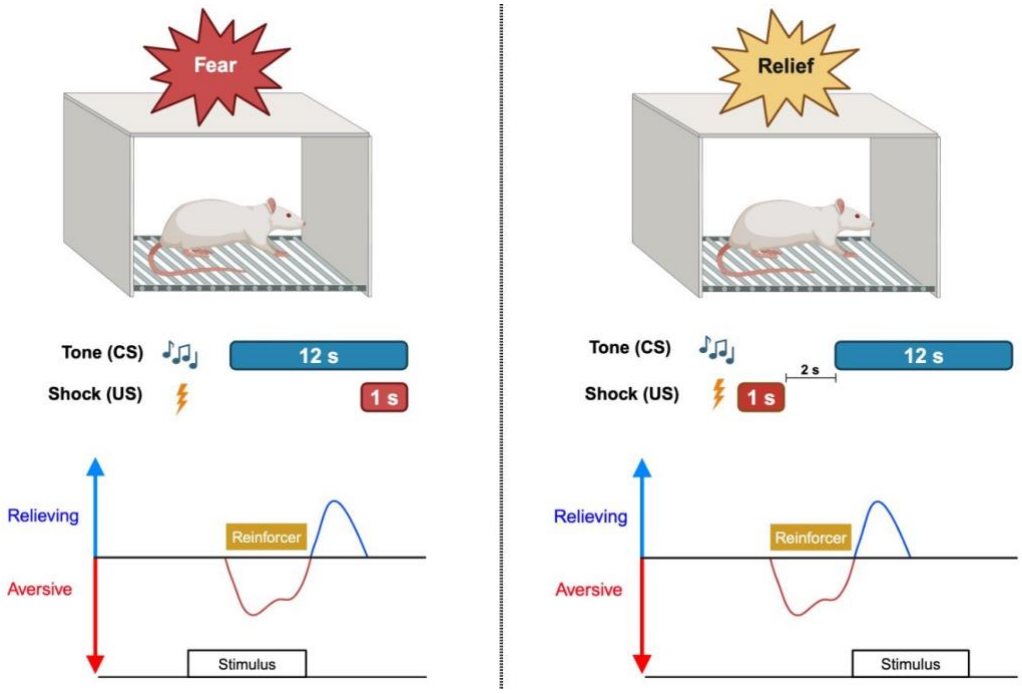


Figure 2. If a stimulus (tone) precedes an aversive reinforcer (US), the stimulus can serve as a signal predicting the upcoming aversive state, eliciting anticipatory fear responses. In contrast, when the stimulus (tone) closely follows the cessation of the aversive reinforcer, it can become a signal for relief from the aversive state. In this backward pairing, the association between the unconditioned stimulus and the conditioned stimulus may lead to appetitive-like responses and induce approach behaviours.

The second objective is to examine immediate early gene (IEG) expression, specifically c-Fos, in the medial prefrontal cortex. The mPFC is strongly implicated in affective learning, including both fear and safety processes, but its role in relief learning has not been well defined. By comparing c-Fos expression in animals exposed to relief learning versus fear conditioning, I aim to identify unique circuit-level correlates of relief. I hypothesize that relief learning will recruit IL activity to a greater extent than fear

learning, reflecting its role in inhibiting fear responses. I also predict that activity in the PrL will be dampened in relief compared to fear learning, due to its role in initiating fear responses. The third objective is to determine the functional contribution of the PrL to relief learning through chemogenetic inhibition. Chemogenetics is a reversible, cell-type-specific method for controlling neuronal activity. It utilizes engineered receptors activated by otherwise inert ligands (Roth, 2016). The chemogenetic tool that I will utilize is Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), which are modified muscarinic G-protein-coupled receptors that do not respond to endogenous neurotransmitters but can be selectively activated by synthetic ligands (Urban & Roth, 2015). They respond specifically to clozapine N-oxide (CNO), but not acetylcholine. Depending on the receptor subtype, DREADD activation can either increase or decrease neuronal excitability. Excitatory DREADDS such as hM3Dq activate signalling pathways that increase intracellular calcium signalling and enhance neuronal firing (Roth, 2016). Conversely, the inhibitory hM4Di receptor suppresses neuronal excitability by reducing intracellular signalling and hyperpolarizing neurons, thereby decreasing the likelihood of an action potential being generated (Roth, 2016). DREADDs are typically delivered via viral vectors, allowing targeted expression within specific brain regions or neuronal populations, and their effects are temporally controlled by systemic ligand administration. This technique offers a powerful means of testing the causal role of defined neural circuits in behaviour while avoiding the permanence of lesion approaches (Urban & Roth, 2015). I will utilize a viral vector delivery of an inhibitory hM4Di DREADD to temporarily inhibit the PrL during relief training. The PrL is typically associated with fear expression, and its inhibition may facilitate the

expression of relief by reducing fear-related outputs. I hypothesize that PrL inhibition during training will enhance behavioural evidence of relief during recall testing, as shown by reduced freezing, supporting the idea that PrL activity prevents the expression of relief.

Although relief learning has received increasing attention in recent years, several important gaps remain. Most affective neuroscience research has focused on threat acquisition, with limited emphasis on how the absence or offset of threat is encoded at both behavioural and neural levels. Additionally, the neural correlates of relief learning, especially c-Fos expression across mPFC subregions (e.g., PrL, IL, ACC), remain largely unexplored. Based on prior work and the theoretical framework of opponent process theory, the following hypotheses are proposed: Animals exposed to a tone that follows shock offset (relief-predictive cue) will develop an appetitive or approach-like response to the cue, as opposed to animals in a standard fear-conditioning group who will exhibit aversive responses (e.g., freezing). Additionally, relief-conditioned animals will show increased c-Fos expression in brain regions associated with reward and safety signalling (e.g., infralimbic cortex), and decreased activation in classical fear-related regions (e.g., prelimbic cortex) compared to fear-conditioned animals. The overall pattern of regional brain activity will distinguish relief learning from fear learning, supporting the notion that relief is not merely the absence of fear, but a distinct learning process with its own circuitry. Finally, chemogenetic inhibition of the PrL will lead to a greater reduction in fear to the relief signal due to the PrL's role in fear expression.

Chapter II: Materials and Methods

This study consisted of four experiments designed to investigate the behavioural, cellular, and circuit-level mechanisms of relief learning. Each experiment used a separate cohort of animals and distinct methods, as detailed below.

2.1 Animals

Fifty-two adult male Long-Evans rats were used across these four experiments. All rats were obtained from Charles River Laboratories (QC, Canada). All animals were initially group-housed (4 rats per cage) upon arrival before being separated and housed in pairs in standard rectangular cages. The animals were housed in a temperature-controlled room (25 ± 2°C) at 60% humidity with *ad libitum* access to food and water. Experiments began after 1 to 2 weeks of handling. Experiments were performed during the light phase of a 12-hour light/dark schedule (lights on at 0700 h). All procedures were approved by the Trent University Animal Care Committee and followed the Canadian Council on Animal Care guidelines.

2.2 Conditioning Apparatus

Ugo Basile (Varese, Italy) conditioning boxes were used across all experiments. The boxes measure 25.4 x 25.4 x 36.5 cm and are made of Plexiglas, with a circular front opening door. The floor consisted of 21 metal rods (3 mm diameter), spaced 1.2 cm apart from center to center. Each conditioning box was housed in a sound-attenuating chamber (54.3 x 46.4 x 55.1 cm). The shocks were delivered through the metal rods on the floor, which were connected to a shock generator and scrambler (Ugo Basile, Varese, Italy). The boxes were cleaned using Oxivir Five 16 concentrate (1:16 dilution) before and after

each rat underwent conditioning or retention testing. All conditioning and retention testing sessions were video recorded using a USB 2.0 Monochrome Industrial Camera (DMK 22AUC03, Imaging Source, Charlotte, NC) placed above the conditioning chamber and connected to a Dell laptop computer.

2.3 Drugs

In experiment 3, Clozapine-N-oxide dihydrochloride (CNO, 1 mg/kg, HB 6149, Hello Bio) was dissolved in 0.9% (w/v) saline and freshly prepared at the time of use. CNO injections (1 mg/kg, i.p.) were given 30 minutes before the training session.

2.4 Experiment 1: Characterization of Relief Learning

This experiment aimed to examine whether a backward conditioning protocol (shock followed by tone) could produce a measurable relief learning response in rats, distinct from the traditional fear response elicited by forward (tone-shock) pairings. By comparing freezing behaviour during a recall test, this experiment sought to determine whether animals trained with relief conditioning would exhibit reduced conditioned fear responses to the tone relative to fear-conditioned animals. This paradigm allowed for a direct behavioural comparison of aversive versus relief learning, helping to establish the validity of backward shock-tone pairings as a model of relief in rodents.

2.4.1 Relief and Fear Conditioning

Rats were acclimated to the conditioning chambers for 15 minutes before being randomly assigned to either the fear (n=6) or relief (n=6) conditioned groups. Twenty-four hours later, the groups were conditioned in the same chambers. During the first 2 minutes, no stimuli were presented. After this period, the fear conditioning group received six CS-UCS pairings consisting of tones (CS, 12 s, 5 kHz, 80 dB) that co-

terminated with a foot shock (US, 1 s, 1.0MA), with an inter-tone interval of 90 s. The relief conditioning group received the same number of pairings with the UCS-CS backward presented with shocks (1.0 mA, 1 s) always preceding the onset of the tone (12 s, 5 kHz, 80 dB) by 2 s. This temporal gap following shock offset has been previously shown to correspond with the strongest relief learning effect in rodents (Andreatta et al., 2012; Mohammadi et al., 2014). The inter-tone interval also matched that of the fear conditioning group. Thus, the relief learning procedure was identical to that of fear conditioning with respect to the number and duration of pairings but only varied in the inter-stimulus interval.

Twenty-four hours after the last training session, a recall test was performed. Rats were returned to the same conditioning chambers and allowed to habituate for 90 seconds. They were then presented with six test tones (12 s) with an interval of 60 s between tones. No shock was delivered during the recall test.

Defensive freezing, defined as the absence of observable movements except those necessary for respiration, was measured using an automated freeze detection system (Any Maze, Stoelting, Wood Dale, IL, USA). “Freezing” was scored when the movement index fell below a threshold of 70 arbitrary units for a minimum duration of 1 s. The amount of time freezing to each tone was expressed as a percentage of freezing to the tone.

2.5. Experiment 2: Fos Expression in the mPFC Following Recall Testing

This experiment aimed to examine whether relief and fear conditioning differentially activated subregions of the medial prefrontal cortex (mPFC), a brain area implicated in emotional regulation and defensive behaviour. By quantifying expression of the

immediate early gene c-fos, a marker of neuronal activation, across the prelimbic (PrL), infralimbic (IL), and cingulate (Cg) cortices, this study sought to identify circuit-level differences associated with relief learning. These data were used to determine whether relief conditioning engages distinct prefrontal pathways compared to traditional fear conditioning.

2.5.1 Perfusions and Fos Immunohistochemistry

Ninety minutes after undergoing recall testing, all rats were anesthetized with a solution of sodium pentobarbital (340 mg/ml, Euthansol, Merck Animal Health Canada) and underwent transcardial perfusion with 0.1 M phosphate-buffered saline (PBS, pH=7.4) followed by ice cold 4% (w/v) formaldehyde fixative (pH=7.4) freshly prepared from depolymerized paraformaldehyde. Brains were removed and postfixed in the same solution overnight at 4°C and then transferred into 30% (w/v) sucrose and 0.01% (w/v) sodium azide dissolved in PBS. A sliding microtome was utilized to cum 50 μm-thick coronal sections. All sections were stored at −20°C in a cryoprotectant solution containing 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol in 0.2 M PBS until processed.

As described elsewhere (Kalinina et al., 2021), immunohistochemistry for c-Fos was conducted on free-floating sections incubated with a polyclonal rabbit anti-c-Fos antibody (1:15,000, EMD Millipore Canada) diluted in 5% (v/v) normal goat serum, 1% bovine serum albumin and 0.3% (v/v) Triton X-100 dissolved in PBS. Sections were then incubated in biotinylated goat anti-rabbit secondary antibody (1:500, Vector Laboratories) solution for 2 hrs at room temperature, followed by incubation in avidin-biotin complex at 4°C for 1 hr (1:500, Vectastain ABC Elicit, Vector Laboratories, Newark, USA). Finally, the reaction was visualized using 2.5% (w/v), 0.02% (w/v) DAB, and 0.000083% (v/v)

$H2O_2$ in 0.175 M sodium acetate to produce a blue and black product. Sections were mounted on slides, dehydrated through a series of alcohols, cleared in xylene, and cover slipped. Sections from all groups were processed at the same time using the same conditions and reagents to minimize variability.

2.5.1 Quantification of Fos Immunoreactivity

Quantification of Fos-positive (Fos^+) cells was performed using the Automated Batch BioImage Analysis (ABBA) (Chiaruttini et al., 2024) pipeline in conjunction with QuPath (Bankhead et al., 2017). Brain sections were imaged using a Nikon TI2-E inverted microscope at 4× magnification for anatomical orientation and 10× magnification for cell quantification (Nikon Instruments, USA). High-resolution images were exported in TIFF format and analyzed in QuPath. Regions of interest (ROIs) were delineated based on the Paxinos and Watson rat brain atlas (2007) and included the medial prefrontal cortex (prelimbic cortex, cingulate cortex, and infralimbic cortex). Two coronal sections were analyzed per animal. Within QuPath, the positive cell detection algorithm was applied to identify Fos^+ nuclei using consistent parameters for optical density thresholding, cell size, and circularity across all sections. Batch quantification and data extraction were managed using the ABBA workflow, allowing for standardized detection of Fos^+ cells across experimental groups and brain regions.

2.6 Experiment 3: Effect of Chemogenetic Inhibition of the PrL on Relief Learning

This experiment aimed to investigate whether activity in the prelimbic cortex (PrL) is necessary for the expression of relief-learned behaviours. Building on prior evidence of increased PrL activation during relief learning (see Experiment 2), a chemogenetic approach was used to selectively inhibit excitatory neurons in this region during

conditioning. By comparing freezing responses between DREADD-inhibited and control animals, this experiment tested whether silencing PrL neurons would impair the expression of relief learning, providing causal evidence for the role of this circuit in modulating defensive behaviours.

2.5.1 Viral Targeting

Rats were anesthetized with isoflurane (5% induction, 2.5% maintenance), head-fixed into a stereotaxic frame, and then treated with an analgesic cocktail (carprofen, 10 mg/kg, i.p.; buprenorphine, 0.05 mg/kg, i.p.) and physiological saline (5 mL) to minimize pain and reduce fluid loss during surgery. Injections of either AAV8-CaMKIIahM4D(Gi)-mCherry (2.4 x 1013 GC/ml) or AAV8-CaMKIIa-EGFP (2.1 x 1013 GC/ml) viral construct were made bilaterally into the prelimbic cortex at the following coordinates: +2.76 mm (AP), +/- 0.6 mm (ML), and -4 mm (DV) (Paxinos & Watson, 2007). Each site was injected with 200 nl of virus at a rate of 50 nl/min. The needle remained in place for an additional 5 minutes after each injection to facilitate diffusion of the virus before being slowly retracted. The incision site was closed with staple clips, cleaned, and the animal was placed under a heat pad until ambulatory. Post-operative care included injections of buprenorphine (0.05mg/kg) for 3 days following surgery and carprofen (5mg/kg) for 5 days following surgery. Staple clips were removed around one week after surgery.

2.5.2 Relief Conditioning

Three weeks after injection of the DREADD construct, rats received an injection of clozapine-N-oxide (CNO, 1 mg/kg, i.p., Hello Bio, n=5) or 0.9% (w/v/) saline (n=5) and underwent relief conditioning. The procedure for relief conditioning and recall

testing was similar to that described above (Experiment 1), with the foot shock intensity increased to 1.5 mA. This modification was introduced to ensure a robust behavioural response in control animals, thereby maximizing the likelihood of detecting an effect of chemogenetic inhibition.

2.5.3 Immunofluorescence

Rats were deeply anesthetized and transcardially perfused with 0.1 M phosphate-buffered saline (PBS), followed by 4% (w/v) paraformaldehyde (PFA) in PBS 90 minutes after recall testing. Brains were extracted and post-fixed overnight in the same fixative at 4 °C. Following fixation, tissues were cryoprotected in an ascending sucrose gradient (15% and then 30% w/v sucrose in PBS) until they sank. The brains were then flash-frozen on dry ice and sectioned at 40 µm in the coronal plane on a freezing stage microtome (Leica SM2010R, Leica Canada) and stored at 4°C in PBS containing 0.001% sodium azide to prevent bacterial growth.

Free-floating sections were rinsed several times under gentle agitation in PBS before being incubated for 30 min in a blocking solution containing 5% normal goat serum (v/v) and 1% bovine serum albumin (BSA) in PBS with 0.3% Triton X-100 (PBSx). Sections were then incubated overnight at 4 °C with rabbit anti-mCherry (1:2000, Abcam #167453) diluted in blocking solution. After three 10-minute PBS washes, sections were incubated for 2 h at room temperature in the dark with Alexa Fluor 568 goat anti-rabbit IgG (1:500, Invitrogen) prepared in PBSx. Sections were washed three times in PBS (10 min each), mounted onto Superfrost Plus microscope slides, and allowed to air-dry in the dark for approximately 30 min. Slides were then cover slipped with antifade mounting

medium containing DAPI (Vector Laboratories, H-1800), sealed with clear nail polish along the edges, and stored at 4°C until imaging.

Fluorescent viral labelling was visualized using a Nikon Ti2-E inverted microscope equipped with a C2+ laser-scanning confocal system and DU3 high-quantum-efficiency photomultiplier detectors. Images were acquired with NIS-Elements Confocal software using 405 nm, 488 nm, and 561 nm, laser lines and Plan Apo λ 20×/0.75 NA or 60× oil 1.4 NA objectives. Laser power, gain, offset, and pinhole size were kept constant across sections and experimental groups to ensure imaging consistency. Optical sections were captured using Plan Apo λ 10×/0.45 NA and 20×/0.75 NA objectives. Imaging parameters, including laser power, detector gain, offset, and pinhole diameter, were held constant across sections and experimental groups to ensure consistency. Representative images were processed uniformly for brightness and contrast adjustments using NIS-Elements AR or Fiji (ImageJ). Any animal that contained unintended viral beyond the prelimbic cortex was removed from the study.

2.7 Experiment 4: Effect of Extended Relief Learning on Conditioned Inhibition

This experiment aimed to investigate the effects of different durations of relief learning, comparing one versus three days of training, on conditioned freezing responses during retention testing. Additionally, it sought to compare behavioural outcomes between relief and fear conditioning protocols using an extended training paradigm designed to enhance learning.

2.7.1 Fear and Relief Conditioning

After one day of habituation (15 min), rats were randomly assigned to one of three groups: relief 1-day (relief 1D, n=10), fear 1-day (fear 1D, n=10), or relief 3-day

(relief 3D, n=10). The relief 1D and fear 1D groups underwent a single day of relief or fear conditioning, respectively, while the relief 3D group received relief conditioning for three consecutive days. This design aimed to investigate whether extending the number of training days, and thereby increasing the number of shock-tone pairings, would enhance the behavioural response observed during retention testing. Relief and fear conditioning was conducted twenty-four hours later, similarly to the methods described in Section 2.4.1, with the following modifications. The fear and relief conditioning protocols were extended to a total duration of 26 minutes and 4 seconds. Additionally, each protocol consisted of 10 pairings, compared to 6 pairings in Experiment 1, with the fear group receiving tone-shock pairings and the relief group receiving shock-tone pairings. In addition, the tone duration was increased from 12 to 20 seconds, and in the relief protocol, the shock was presented 3 seconds before the tone onset instead of 2 seconds. These adjustments were made to optimize learning based on prior literature and pilot testing from Experiment 1.

Retention testing was conducted on the two consecutive days following the last training session for all groups. The retention testing protocol was 13 minutes in duration. During this time, six 30-second tones were played, and freezing percentages were analyzed during each tone.

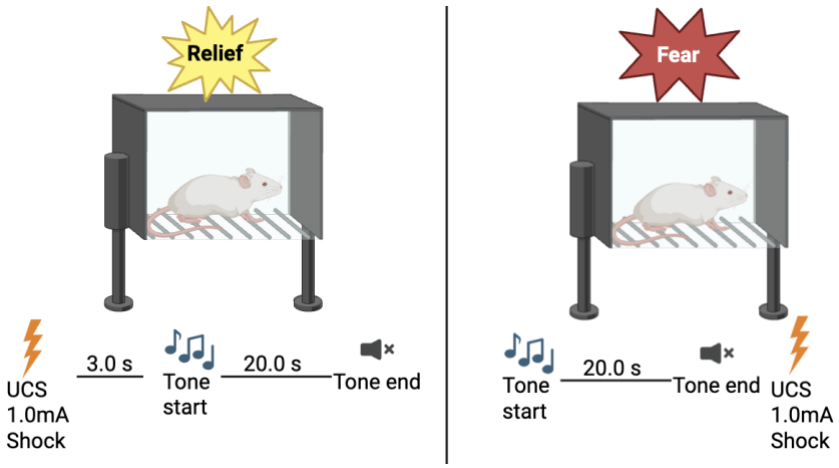


Figure 3. Visual comparison of the relief versus fear protocols in Experiment 4, highlighting the adjustments in protocol from Experiment 1. In this protocol, the tone lasted 20 seconds instead of 12 seconds. Additionally, for the relief protocol, the shock came 3 seconds before the tone started as opposed to 2 seconds.

2.8 Statistical Analysis

All statistical analyses were performed using JASP (version 0.95.3) and GraphPad PRISM (version 10.6.1). A significance threshold of $\alpha = 0.05$ (two-tailed) was used for all tests. For comparisons between groups, independent sample t-tests were used to assess differences in freezing behaviour. Where appropriate, repeated-measured ANOVA was used to evaluate changes across multiple tone trials or recall sessions, with Greenhouse-Geisser correction applied when the assumption of sphericity was violated.

Post hoc comparisons were conducted using Fisher's Least Significant Difference (LSD) test to explore significant main effects and interactions. All data are presented as mean ± standard error of the mean (SEM) in the figures. Effect sizes were reported using Cohen's *d* or η^2 to aid interpretation of the magnitude of the observed effects. No corrections for multiple comparisons or unequal variances were applied, as such

adjustments are overly conservative in small-sample designs and were not deemed necessary based on experimental goals. Visualizations were created in GraphPad PRISM to support data interpretation by visually summarizing group means, variability, and statistical outcomes across conditions.

Chapter III: Results

1. Experiment 1: Characterization of Relief Learning

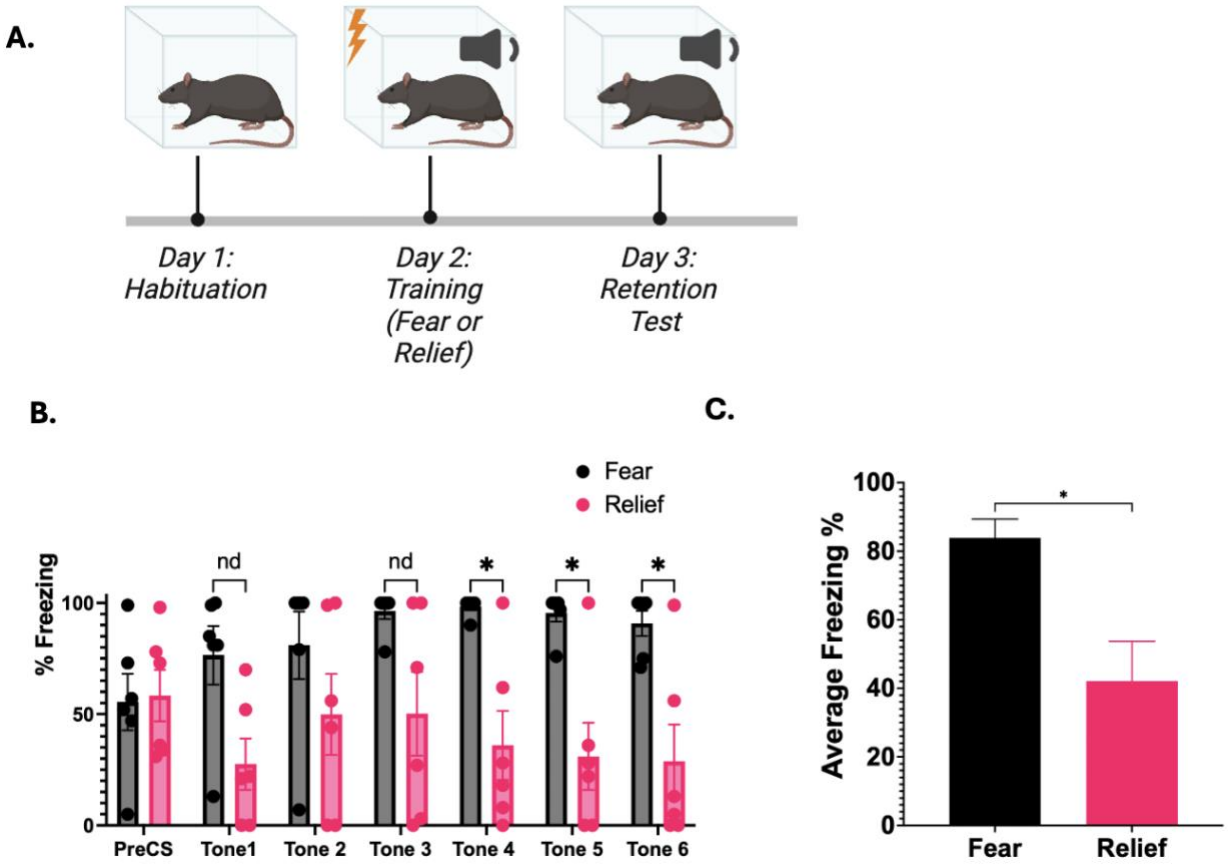


Figure 4. **A.** The experimental timeline for Experiment 1. **B.** Freezing responses during PreCS and Tones 1–6 for Fear and Relief groups. Data are shown as mean ± SEM. The Fear group displayed higher freezing than the Relief group across all tones. Tones 1 and

3–6 showed significant differences ($p < 0.05$) PreCS baseline did not differ between groups. **C.** The average freezing % was significantly lower in the Relief group compared to the Fear group ($t(11.48) = 6.00$, $p < .0001$)

Figure 4.A. outlines the experimental procedure. Rats underwent fear (n=6) or relief (n=6) conditioning. During fear conditioning, tone stimuli (conditioned stimuli; CS) co-terminated with the foot shocks (unconditioned stimuli; US). In contrast, during relief conditioning, foot shocks and tones were explicitly backward paired with each shock terminating 2 s before the onset of the tone. This timing ensured that the tone signalled for period of physical and emotional relief immediately following the cessation of the aversive stimulus. Tone-evoked freezing was assessed twenty-four hours after conditioning to evaluate memory for the fear- or relief-associated cues. Analysis of freezing behaviour before presentation of the first test tone (pre-CS) revealed no significant differences between the fear and relief conditioned animals [fear: 55.5 ± 9.88%, n=6; relief: 58.3 ± 13.56%, n=6; Student's *t*-test, $t(10) = 0.165$, $p = .872$], indicating a comparable level of baseline freezing across groups before tone onset. However, group differences emerged once the tones were presented. A mixed-design ANOVA with tone (Tones 1 through 6) as the within-subjects factor and group (Fear vs. Relief) as the between-subjects factor revealed a significant main effect of Group [$F(1, 70) = 41.98$, $p < .0001$, $\eta^2_p = .375$], indicating that fear-conditioned animals froze more overall than relief-conditioned animals. No significant main effect of Tone [$F(6, 70) = 0.592$, $p = .735$, $\eta^2_p = .048$] or Tone × Group interaction [$F(6, 70) = 1.74$, $p = .125$, $\eta^2_p = .130$] was observed. A follow-up *t*-test comparing average freezing across all tone presentations confirmed that fear-

conditioned animals froze significantly more than relief-conditioned animals ($t(11.48) = 6.00, p < .0001$).

Overall, these findings indicate that relief-conditioned animals exhibited significantly lower levels of freezing during most tone trials compared to fear-conditioned animals, supporting the interpretation that the task successfully differentiated between aversive and relief learning behaviours.

2. Experiment 2: Fos Expression in the mPFC Following Recall Testing

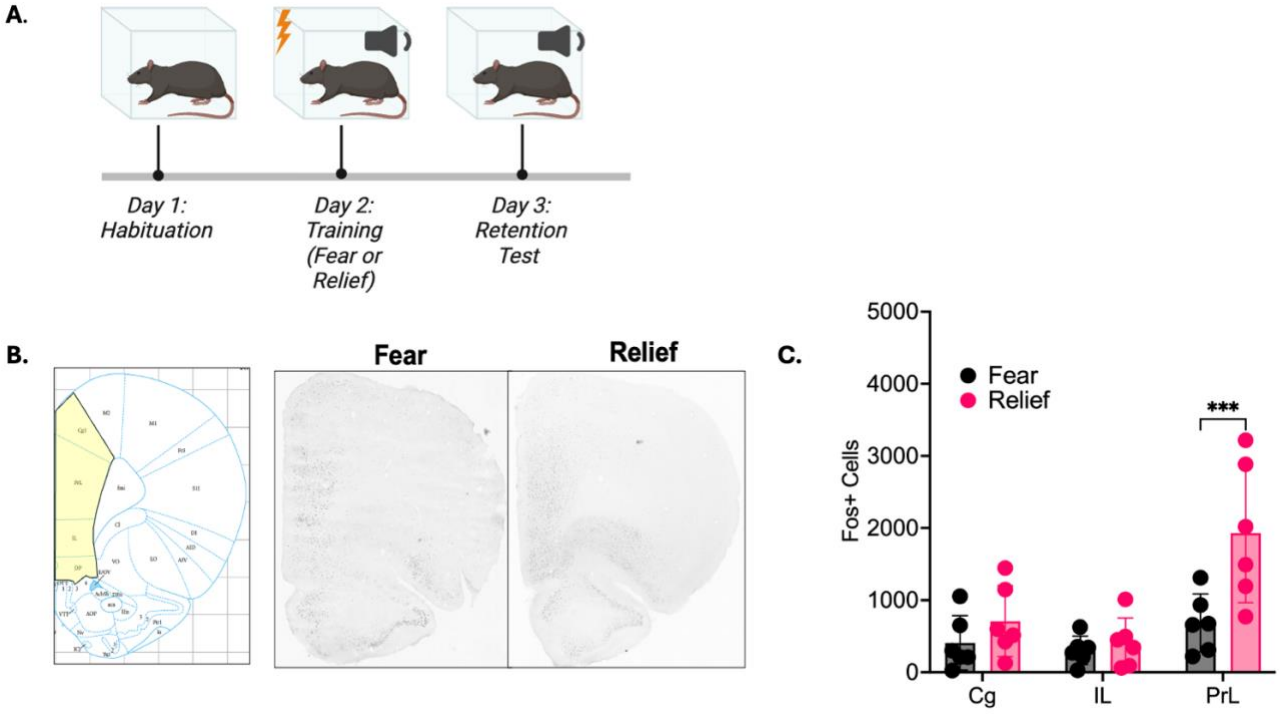


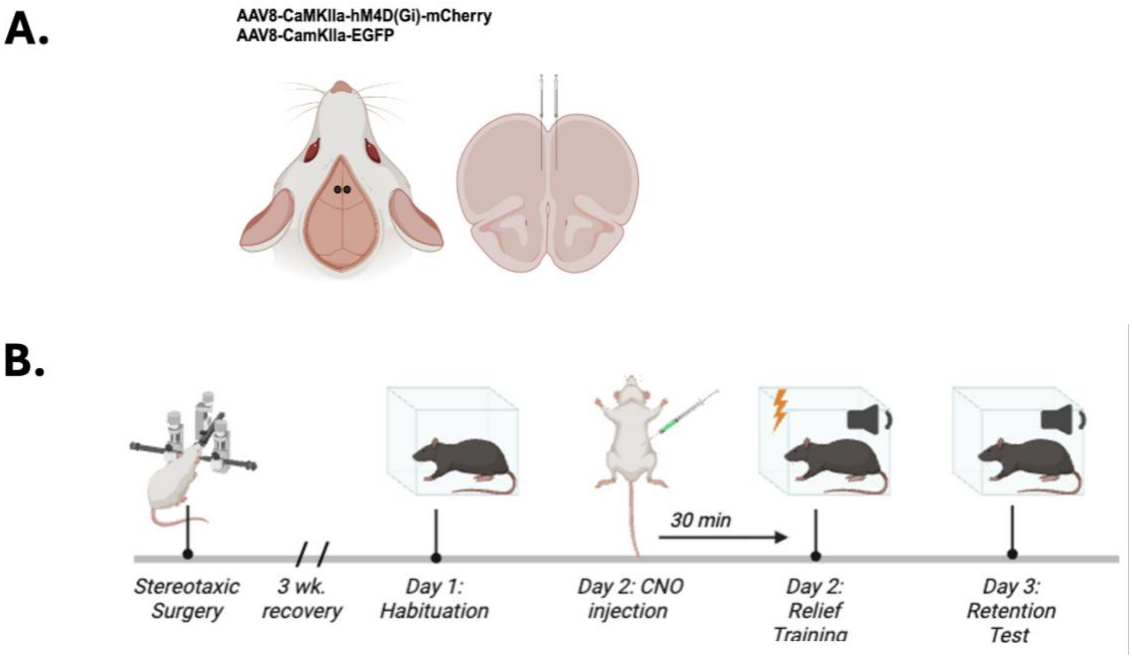
Figure 5. **A.** Experimental timeline for Experiment 2 (same animals from Experiment 1). **B.** Paxinos atlas image highlighting the mPFC and sample images taken from experiment 2 of one fear (#005) and one relief animal (#008). **C.** Quantification of Fos-positive cells in the Cg, IL, and PrL following fear- or relief-conditioning. Individual data points are shown for each animal, with bars representing group means ± SEM. Fear-conditioned

animals are shown in black, and relief-conditioned animals in pink. A significant increase in Fos expression was observed in the PrL for the relief group compared to the fear group (***$p < 0.001$), whereas no significant differences were observed in the Cg or IL.

Experiment 2 involved analysis of brain tissue from the animals used in Experiment 1, following the experimental timeline illustrated in Figure 5A. The goal of Experiment 2 was to identify brain regions selectively activated by relief versus fear conditioning, with a particular focus on the medial prefrontal cortex (mPFC), as shown in Figure 5B. To do this, c-Fos expression, a marker of neuronal activation, was quantified following a recall test in fear- and relief-conditioned rats.

A two-way ANOVA examining c-Fos expression in the medial prefrontal cortex (mPFC) revealed a significant main effect of Group ($F(1, 30) = 9.98$, $p = .004$), with higher overall c-Fos expression in the relief-conditioned group compared to the fear-conditioned group. There was also a main effect of Subregion ($F(2, 30) = 11.04$, $p < .001$), and a significant Group × Subregion interaction ($F(2, 30) = 4.11$, $p = .026$), indicating that group differences in c-Fos expression varied by mPFC subregion. As shown in Figure 5C, follow-up analyses showed that this group difference was specifically driven by increased c-Fos expression in the prelimbic cortex (PrL) in the relief group relative to the fear group ($p < .001$). No significant differences between groups were observed in the cingulate (Cg) or infralimbic (IL) subregions. These findings suggest that the prelimbic cortex is selectively recruited during relief learning.

3. Experiment 3: Effect of Chemogenetic Manipulation of the PrL on Relief Learning



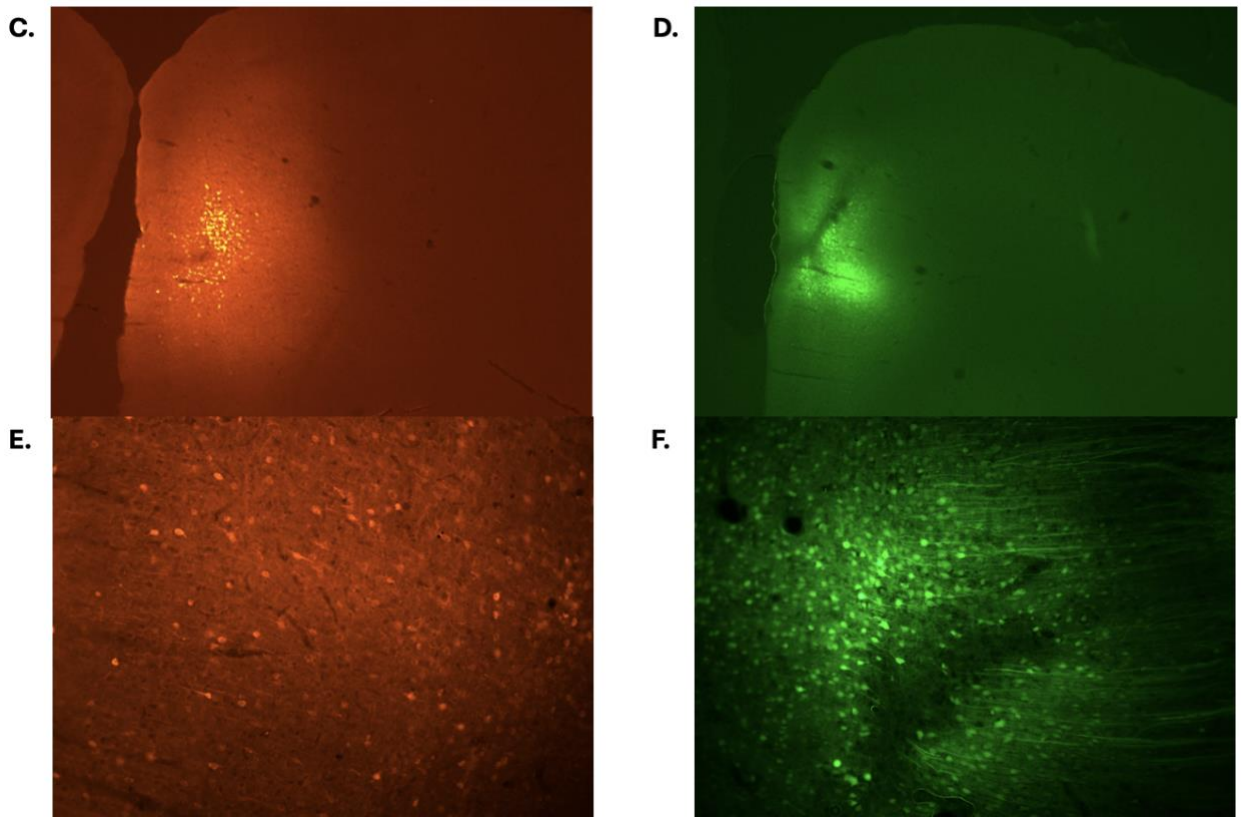


Figure 6. **A.** Diagram showing the location of viral injection. **B.** Experimental timeline of Experiment 3. **C.** Expression of mCherry-tagged cells in the prelimbic cortex. **D.** Expression of GFP-tagged cells in the prelimbic cortex **E.** 10x magnification showing mCherry-tagged cells in the PrL. **F.** 10x magnification showing GFP-tagged cells in the PrL.

Experiment 3 aimed to assess the causal role of prelimbic (PrL) cortex activity in relief learning by using chemogenetic inhibition (–DREADD) in the prelimbic cortex (Figure 6A) during relief conditioning, as shown in Figure 6B. Confocal images of mCherry and GFP tagged cells were taken to determine successful expression and location of the virus-tagged cells, as shown in Figure 6C-F. Behavioural freezing responses during recall were compared between rats with PrL inhibition and control animals to determine how silencing this region affects the expression of relief-associated defensive behaviours.

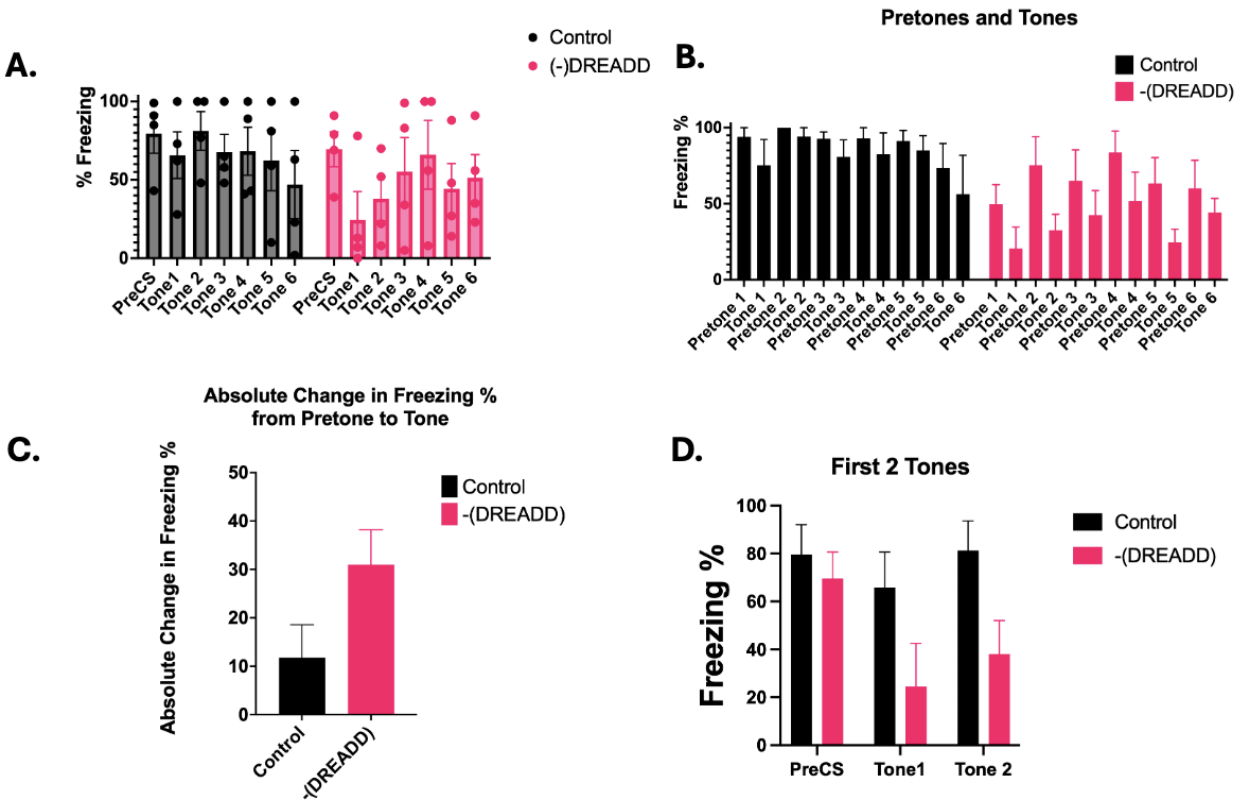


Figure 7. Effect of –DREADD on freezing behaviour across pretones and tones. **A.** Individual and mean ± SEM % freezing for Control (black) and –DREADD (pink) animals across the PreCS period and six tones. Each dot represents an individual animal. **B.** Group means ± SEM for freezing across pretones and tones. –DREADD animals show consistently lower freezing compared with Controls, though individual comparisons for each pretone or tone did not reach significance. **C.** Absolute change in freezing from pretone to tone for each group; –DREADD animals show larger mean changes, indicating altered responses to tone onset, although this did not reach significance. **D.** Freezing during the PreCS and first two tones, showing reduced freezing in –DREADD animals relative to Controls. Overall, a Welch's t-test across all pretones and tones revealed a significant reduction in freezing in –DREADD animals (Control: 84.88 ± 6.63%; –

DREADD: 51.10 ± 6.63%; difference = –33.77 ± 6.63%; Welch's $t(18.47) = 5.094$, $p < 0.0001$, $\eta^2 = 0.5842$).

To assess the effect of chemogenetic inhibition of the prelimbic cortex (–DREADD) on freezing behaviour specifically during tone presentations, a two-way mixed ANOVA was conducted with Group (Control vs. –DREADD) as the between-subjects factor and Tone (1 through 6) as the within-subjects factor. This analysis revealed no significant main effects of Group, $F(1, 42) = 3.97$, $p = 0.053$, nor Tone, $F(6, 42) = 0.77$, $p = 0.60$, and no significant Group × Tone interaction, $F(6, 42) = 0.61$, $p = 0.72$, indicating that freezing during tones alone did not differ statistically between groups, as shown in Figure 7A.

To further investigate whether chemogenetic inhibition affected freezing behaviour across the entire recall period, including both pre-tone and tone epochs, a two-way mixed ANOVA was conducted with Group (Control vs. –DREADD) as the between-subjects factor and Time (12 intervals: 6 pretones and 6 tones) as the within-subjects factor. This analysis revealed a significant main effect of Group, $F(1, 72) = 35.17$, $p < 0.0001$, $\eta^2_p = 0.28$, with –DREADD animals showing significantly lower freezing overall compared to Controls (Control mean = 84.88%, –DREADD mean = 51.10%; mean difference = 33.77%, 95% CI [22.42, 45.12]). There was no significant main effect of Time, $F(11, 72) = 1.85$, $p = 0.062$, nor a significant Group × Time interaction, $F(11, 72) = 0.86$, $p = 0.58$, indicating that the group difference in freezing was consistent across all pretones and tones. These findings suggest that chemogenetic

inhibition of the prelimbic cortex reduces overall freezing during recall, but this effect does not vary across individual pre-tone and tone periods, as shown in Figure 7B.

As shown in Figure 7C, absolute change in freezing from pretones to tones did not differ significantly between groups (Control: M = 11.75, –DREADD: M = 30.95; mean difference = –19.20 ± 9.927; $t(6) = 1.934$, $p = 0.101$), indicating comparable overall shifts in defensive behaviour.

As shown in Figure 7D, during Tone 1, freezing was numerically lower in –DREADD animals (M = 24.50, SE = 23.39) compared to Control (M = 65.75, SE = 23.39), but the difference did not reach significance ($t(6) = 1.763$, $p = 0.128$, $q = 0.453$). Tone 2 showed a similar trend (Control: M = 81.25, SE = 18.71; –DREADD: M = 38.00, SE = 18.71; $t(6) = 2.311$, $p = 0.060$, $q = 0.425$). For Tones 3–6, and the remaining pretones, differences between groups were smaller and non-significant ($p > .05$). This suggests that while –DREADD animals exhibited a consistent reduction in responding across stimuli, the effect size at each individual pretone or tone was not sufficient to survive correction for multiple testing.

In summary, when collapsing across all cues, –DREADD animals showed a statistically significant reduction in overall freezing, indicating that these circuits may contribute to the expression of defensive behaviour. Taken together, these results support a role for prelimbic mPFC circuits in modulating relief-related behaviour, although sample sizes and trial-by-trial variability limit detection of more granular effects.

4. Experiment 4: Effect of Extended Relief Learning on Conditioned Inhibition

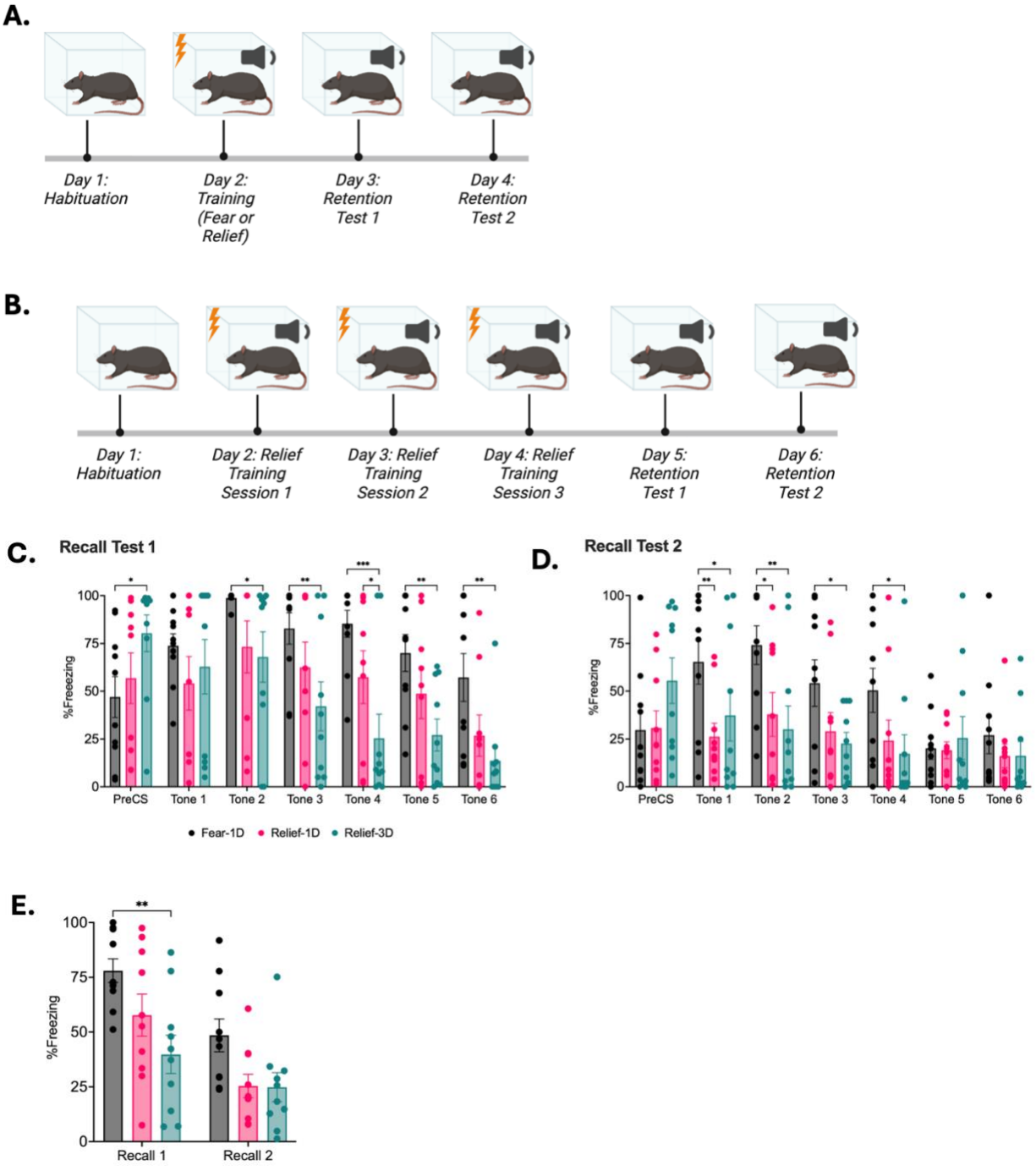


Figure 8. **A.** The experimental timeline of Experiment 4 for single day trained animals (fear and relief). **B.** The experimental timeline of Experiment 4 for three-day trained animals (relief). **C.** Percent freezing during PreCS and Tone 1–6 for Fear-1D (black),

Relief-1D (pink), and Relief-3D (teal) groups. Data are shown as mean ± SEM, with individual data points overlaid. In Recall Test 1, Fear-1D animals froze significantly more than Relief-1D and Relief-3D animals during Tones 3–5 (*$p < 0.01$; **$p < 0.001$), whereas Relief-1D and Relief-3D differed only at Tone 4 ($p < 0.05$). **D.** In Recall Test 2, group differences were reduced, with significant differences primarily observed between Fear-1D and Relief-3D at Tones 2, 3, 5, and 6 ($p < 0.05$, *$p < 0.01$), and between Fear-1D and Relief-1D at Tone 2 (*$p < 0.01$). **E.** Average freezing across all tones for Recall Tests 1 and 2. Fear-1D animals froze significantly more than Relief-3D animals in both tests (*$p < 0.01$), while differences between Fear-1D and Relief-1D were significant only in Recall Test 1. Relief-1D and Relief-3D groups did not differ significantly in either test. These results demonstrate that the Fear-1D protocol produces robust freezing, particularly in Recall Test 1, and that the 3-day relief protocol reduces freezing relative to Fear-1D**,** with effects partially maintained in Recall Test 2.

Experiment 4 aimed to investigate how extending the duration of relief conditioning across multiple days influences freezing behaviour during retention testing. Specifically, it examined whether increasing the number of shock-tone pairings by providing relief conditioning over three consecutive days, compared to a single day, would enhance conditioned freezing responses. This experimental timeline is shown in Figure 8A for 1-day trained animals and Figure 8B for 3-day trained animals. This experiment also compared relief and fear conditioning protocols with adjusted parameters to optimize learning, assessing whether prolonged training leads to stronger or more persistent behavioural effects.

4.1 Recall Test 1.

Figure 8C shows the results from Recall 1. Analysis of freezing behaviour before the first test tone (Pre-CS) revealed no significant differences between the Fear-1D and Relief-1D groups [Fear-1D: 46.97 ± 15.83 SEM, $n = 10$; Relief-1D: 56.80 ± 15.83 SEM, $n = 9$; $t(182) = 0.62$, $p = .535$], indicating comparable baseline freezing before tone onset. However, Fear-1D animals froze significantly less than Relief-3D animals during the Pre-CS period [Relief-3D: 80.32 ± 15.41 SEM, $n = 10$; $t(182) = 2.16$, $p = .032$], suggesting elevated baseline freezing in the Relief-3D group.

To assess group differences in tone-elicited freezing, a two-way mixed-design ANOVA was conducted with Tone (Tone 1–6) as the within-subject factor and Treatment (Fear-1D, Relief-1D, Relief-3D) as the between-subject factor. There was a significant main effect of Tone [$F(6, 189) = 3.32$, $p = .004$], a significant main effect of Treatment [$F(2, 189) = 8.02$, $p = .0005$], and a marginally non-significant Tone × Treatment interaction [$F(12, 189) = 1.74$, $p = .061$], suggesting that group differences in freezing were most pronounced during early tones.

Post hoc pairwise comparisons using Fisher's LSD revealed that at the PreCS period, Fear-1D rats froze significantly less than Relief-3D rats ($p = 0.0318$), whereas Fear-1D and Relief-1D did not differ. During Tone 1, no significant differences were observed between any groups. At Tone 2, Fear-1D rats exhibited significantly higher freezing than Relief-3D rats ($p = 0.0464$), and this difference persisted at Tone 3 ($p = 0.009$), Tone 4 ($p = 0.0001$), Tone 5 ($p = 0.0059$), and Tone 6 ($p = 0.0052$). Additionally, at Tone 4, Relief-1D rats froze significantly more than Relief-3D rats ($p = 0.0452$). No

other comparisons between Fear-1D and Relief-1D or between Relief-1D and Relief-3D reached significance at any tone. These results indicate that the Relief-3D group consistently exhibited reduced freezing relative to Fear-1D across most tone presentations, while Relief-1D showed intermediate freezing levels, significantly differing from Relief-3D only at Tone 4.

4.2 Recall Test 2.

Figure 8D shows the results from Recall Test 2. Analysis of freezing behaviour prior to tone onset (Pre-CS) revealed no significant differences between groups [Fear-1D: 29.65 ± 13.98 SEM, $n = 10$; Relief-1D: 30.72 ± 13.98 SEM, $n = 10$; Relief-3D: 55.47 ± 13.98 SEM, $n = 10$]. Although Fear-1D animals showed numerically lower freezing than Relief-3D, this difference did not reach significance [$t(189) = 1.85$, $p = .066$], indicating that baseline freezing levels were generally comparable across groups.

A two-way repeated-measures ANOVA was conducted to examine the effects of Time (Recall 1 vs. Recall 2) and Group (Fear-1D, Relief-1D, Relief-3D) on mean freezing. There was a significant main effect of Time, $F(1, 27) = 39.42$, $p < .0001$, reflecting a decline in freezing from Recall 1 (M = 58.51%) to Recall 2 (M = 32.92%), with a mean difference of 25.59% (95% CI [17.23, 33.95]). A significant main effect of Group was also observed, $F(2, 27) = 6.02$, $p = .0069$, as shown in Figure 8E. The Time × Group interaction was not significant, $F(2, 27) = 1.73$, $p = .196$, indicating that the relative pattern of group differences remained consistent across recalls.

Post hoc Šídák-corrected comparisons showed that during Recall 1, Fear-1D rats froze significantly more than Relief-3D rats (mean difference = 38.17%, 95% CI [12.49, 63.85]; $p = .0017$). No other group comparisons reached significance during Recall 1, although a non-significant trend was observed for Fear-1D > Relief-1D. During Recall 2, no group comparisons were significant, though the pattern of Fear-1D > Relief-1D/Relief-3D was maintained. Relief-1D and Relief-3D did not differ at either recall session. In summary, these results indicate that the strongest group differences in freezing behaviour were observed during Recall 1, with the Fear-1D group exhibiting the highest levels of freezing, particularly relative to the Relief-3D group. Overall, freezing responses declined over time across all groups, consistent with memory decay or extinction processes.

Chapter IV: General Discussion

1. Summary

The present study aimed to characterize relief learning behaviourally and identify its underlying neural substrates within the mPFC by utilizing a backward conditioning paradigm in Long-Evans rats. Across four experiments, I demonstrated that (1) relief learning produces significantly lower freezing compared to traditional fear conditioning, confirming that the paradigm effectively dissociates relief from fear learning; (2) relief learning is associated with robust recruitment of the PrL, but not the Cg or IL subregions; (3) chemogenetic inhibition of the PrL attenuates freezing responses during relief learning, suggesting a potential relationship between the PrL and the expression of relief-related behaviour; and (4) relief learning has more robust reductions in freezing after 3

days of training compared to one. Below, I discuss the behavioural evidence of relief learning, the neural correlates as shown by c-Fos expression and chemogenetic manipulations and situate these findings within the broader literature on affective neuroscience and trauma recovery.

1.1 Behavioural Differentiation of Relief Learning

The behavioural findings from Experiment 1 provided strong behavioural evidence that relief learning produces a different response to a conditioned cue compared to fear conditioning. Relief-conditioned animals consistently exhibited lower freezing during tone presentations than fear-conditioned rats. Importantly, baseline freezing before cue onset did not differ between groups, ruling out pre-existing differences in arousal or motor activity as explanations for the results. This suggests that a backward pairing of a shock followed by a tone elicits an approach-like response, supporting the notion that relief learning encodes the termination of an aversive event as a positive signal rather than a threat predictor. These results replicate and extend previous findings (Gerber et al., 2014; Kahl & Fendt, 2016; Mohammadi et al., 2014) by demonstrating robust differentiation in freezing behaviours across multiple tone trials.

The comparison between single-day and multi-day conditioning in Experiment 4 further refined this behavioural effect, showing that multiple days of relief conditioning (Relief-3D group) led to significantly reduced freezing compared to single-day fear conditioning (Fear-1D group). This suggests that three days of training creates a more stable relief memory, as the three-day protocol led to more persistent reductions in freezing at recall. The persistence of reduced freezing over two recall tests indicates that relief learning produces lasting behavioural changes. Although Relief-1D animals

showed intermediate freezing levels, these did not differ significantly from the other groups, suggesting that repeated relief training strengthens the behavioural response.

From a theoretical perspective, these results align closely with Solomon and Corbit's (1974) opponent process theory, which proposes that the relief following an aversive event constitutes an intrinsically rewarding "B-process" that grows stronger with experience. Over time, cues that predict relief may acquire motivational salience, functioning as safety or recovery signals that dampen physiological arousal and facilitate adaptive coping. This framing situates relief learning as an essential, though often overlooked, component of emotional regulation — one that transforms threat-related cues into signals of safety and recovery.

Moreover, these behavioural distinctions suggest that relief learning cannot be explained solely as extinction. Extinction involves learning that the conditioned cue no longer predicts threat, leading to a gradual suppression of fear through inhibitory learning. In contrast, relief learning involves the formation of a new positive association, a cue that signals safety after the aversive event. Thus, rather than inhibiting fear, relief learning may actively engage appetitive motivational systems, supporting approach behaviours that promote adaptive recovery after stress exposure.

1.2 Neural Correlates of Relief Learning and the Role of the Prelimbic Cortex

The c-Fos results from Experiment 2 revealed that relief conditioning selectively increases Fos expression in the PrL, with minimal activation in the Cg or IL subregions. The PrL has traditionally been associated with the generation of conditioned fear (Corcoran & Quirk, 2007). However, my results suggest that its role extends to encoding

or expressing relief learning. The absence of group differences in IL activity is notable given IL's established role in fear extinction (Milad & Quirk, 2002), suggesting that relief learning is mechanistically different from extinction. Rather than suppressing conditioned fear through inhibitory IL circuits, relief learning may recruit PrL networks that support adaptive behavioural flexibility when outcomes shift from threat to relief.

Notably, the IL did not show differential activation between groups. This lack of IL engagement reinforces the idea that relief learning is mechanistically distinct from extinction. Extinction recruits inhibitory circuits within the IL that suppress amygdala-driven fear output. Relief learning, by contrast, appears to depend on excitatory activity within the PrL, potentially facilitating top-down modulation of both limbic and striatal structures involved in motivational processing. This interpretation aligns with growing evidence that the mPFC is not functionally segregated into "fear" and "safety" regions, but instead operates as an integrated network dynamically tuned to environmental contingencies.

Experiment 3 directly tested the causal role of the PrL in relief learning using chemogenetic inhibition. Inhibitory DREADD animals showed reduced overall freezing compared to controls, though differences at individual tones did not survive correction for multiple comparisons. This pattern suggests that PrL activity contributes to the sustained expression of relief-related defensive behaviours. Considering the small sample size in this study, it is possible that a larger sample could have revealed tone-by-tone differences. These findings complement the Fos results by providing causal evidence that PrL circuits are involved in relief, but more research with larger cohorts and more

temporally precise manipulations, such as the use of optogenetics, could help to define the PrL's role in relief learning more clearly.

2. Implications and Clinical Relevance

Collectively, these findings situate relief learning as a critical and distinct process within the broader framework of defensive and appetitive learning. By implicating the PrL in relief-related processing, this study challenges the oversimplified view that the PrL exclusively drives fear while the IL mediates safety. Instead, the results point to a more dynamic role for the mPFC in evaluating outcome valence and updating behavioural strategies when environmental contingencies shift from danger to safety.

This perspective aligns with contemporary models of affective flexibility, which emphasize the brain's capacity to rapidly reconfigure threat and reward networks to promote adaptive responses (Gross, 2002; McNaughton & Corr, 2004). Relief learning may represent a neurobiological mechanism underlying this flexibility, allowing organisms to recognize when danger has passed and engage recovery-related neural systems. From an evolutionary standpoint, the ability to transition from defensive immobility to exploratory behaviour is essential for survival, conserving energy during threat yet promoting engagement once safety returns.

Translationally, these findings have important implications for understanding and treating trauma-related disorders such as PTSD. Individuals with PTSD often exhibit impaired safety and relief processing, continuing to respond defensively to cues that no longer predict harm (Jovanovic et al., 2012). If the PrL contributes to recognizing and encoding relief, dysfunction within this circuit could underlie the persistence of

pathological fear. Enhancing relief learning, either through behavioural paradigms, pharmacological facilitation, or neuromodulation, may represent a novel therapeutic avenue to restore emotional balance and promote recovery after trauma.

3. Limitations and Future Directions

While these findings provide a foundation for understanding the neural basis of relief, several limitations warrant consideration. First, the sample size in Experiment 3 was small (n = 5), limiting statistical power to detect chemogenetic effects. Larger cohorts are needed to confirm the role of the PrL and dissect the contributions of the other mPFC subregions. Second, although c-Fos mapping offers valuable anatomical resolution of neural activation, it does not directly measure circuit dynamics or neurotransmitter activity, as it is only a static snapshot of activity. Future work using in vivo electrophysiology, calcium imaging, or optogenetics could elucidate real-time neural processes during relief learning and recall phases. Future work should also examine downstream PrL targets, including the amygdala and nucleus accumbens, to delineate how relief cues influence broader defensive and motivational networks. Examining how these projections modulate defensive output could reveal whether relief learning involves a shift from amygdala-driven fear circuits to striatal reward pathways. Similarly, investigating potential interactions between the mPFC and periaqueductal gray (PAG) could help determine how prefrontal modulation influences the expression of freezing and mobility during transitions from threat to safety. Finally, extending this research to female subjects and cross-species comparisons will improve generalizability and address sex differences in affective learning and anxiety vulnerability. Female rodents often display distinct patterns of stress reactivity and prefrontal function and understanding

whether relief learning manifests similarly across sexes could have direct translational relevance for anxiety and trauma research in humans.

4. Conclusion

This thesis demonstrates that relief learning is a distinct form of affective learning characterized by reduced freezing and unique neural activation patterns. The PrL emerges as a key node in this process, showing both heightened activity and causal involvement in relief expression. By advancing the understanding of how the brain encodes the transition from threat to relief, this work lays the groundwork for future studies aimed at advancing our understanding of how the brain encodes relief from threat and harnessing relief learning mechanisms to improve mental health outcomes in anxiety and trauma-related disorders.

References

American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders* (5th ed.). https://doi.org/10.1176/appi.books.9780890425596

Alemán-Andrade, P., Witter, M. P., Tsutsui, K.-I., & Ohara, S. (2025). Dorsal-caudal and ventral hippocampus target different cell populations in the medial frontal cortex in rodents. *Journal of Neuroscience, 45*(22), e0217252025. https://doi.org/10.1523/JNEUROSCI.0217-25.2025

Ambroggi, F., Ishikawa, A., Fields, H. L., & Nicola, S. M. (2008). Basolateral Amygdala Neurons Facilitate Reward-Seeking Behavior by Exciting Nucleus Accumbens Neurons. *Neuron (Cambridge, Mass.)*, *59*(4), 648–661. https://doi.org/10.1016/j.neuron.2008.07.004

Andreatta, M., Fendt, M., Mühlberger, A., Wieser, M. J., Imobersteg, S., Yarali, A., Gerber, B., & Pauli, P. (2012). Onset and offset of aversive events establish distinct memories requiring fear and reward networks. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *19*(11), 518–526. https://doi.org/10.1101/lm.026864.112

Anglada-Figueroa, D., & Quirk, G. J. (2005). Lesions of the Basal Amygdala Block Expression of Conditioned Fear But Not Extinction. *The Journal of Neuroscience*, *25*(42), 9680–9685. https://doi.org/10.1523/JNEUROSCI.2600-05.2005

Bagot, R. C., Parise, E. M., Peña, C. J., Zhang, H. X., Maze, I., Chaudhury, D., Persaud, B., Cachope, R., Bolaños-Guzmán, C. A., Cheer, J. F., Deisseroth, K., Han, M. H., & Nestler, E. J. (2015). Ventral hippocampal afferents to the nucleus accumbens regulate susceptibility to depression. *Nature Communications*, *6*, 7062. https://doi.org/10.1038/ncomms8062

Bankhead, P. et al. (2017). QuPath: Open source software for digital pathology image analysis. *Scientific Reports*. https://doi.org/10.1038/s41598-017-17204-5

Bergado Acosta, J. R., Kahl, E., Kogias, G., Uzuneser, T. C., & Fendt, M. (2017). Relief learning requires a coincident activation of dopamine D1 and NMDA receptors within the nucleus accumbens. *Neuropharmacology*, *114*, 58–66. https://doi.org/10.1016/j.neuropharm.2016.11.022

Bergado Acosta, J. R., Schneider, M., & Fendt, M. (2017). Intra-accumbal blockade of endocannabinoid CB1 receptors impairs learning but not retention of conditioned relief. *Neurobiology of Learning and Memory*, *144*, 48–52. https://doi.org/10.1016/j.nlm.2017.06.001

Bloodgood, D. W., Sugam, J. A., Holmes, A., & Kash, T. L. (2018). Fear extinction requires infralimbic cortex projections to the basolateral amygdala. *Translational Psychiatry*, *8*(1), Article 60. https://doi.org/10.1038/s41398-018-0106-x

Bouton M. E. (2004). Context and behavioral processes in extinction. *Learning & memory, 11*(5), 485–494. https://doi.org/10.1101/lm.78804

Bradfield, L. A., & McNally, G. P. (2010). The role of nucleus accumbens shell in learning about neutral versus excitatory stimuli during Pavlovian fear conditioning. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *17*(7), 337–343. https://doi.org/10.1101/lm.1798810

Brockett, A. T. (Ed.). (2021). *What does medial frontal cortex signal during behavior?: insights from behavioral neurophysiology*. Academic Press. https://doi.org/10.1016/bs.irn.2020.11.011

Bruning, J. E. A., Breitfeld, T., Kahl, E., Bergado-Acosta, J. R., & Fendt, M. (2016).

Relief memory consolidation requires protein synthesis within the nucleus accumbens. *Neuropharmacology*, *105*, 10–14. https://doi.org/10.1016/j.neuropharm.2016.01.016

Burton, T. J., & Balleine, B. W. (2022). The positive valence system, adaptive behaviour and the origins of reward. *Emerging topics in life sciences*, *6*(5), 501–513. https://doi.org/10.1042/ETLS20220007

Chen, Y.-H., Lan, Y.-J., Zhang, S.-R., Li, W.-P., Luo, Z.-Y., Lin, S., Zhuang, J.-P., Li, X.-W., Li, S.-J., Yang, J.-M., & Gao, T.-M. (2017). ErbB4 signaling in the prelimbic cortex regulates fear expression. *Translational Psychiatry*, *7*(7), e1168–e1168. https://doi.org/10.1038/tp.2017.139

Chiaruttini, N., Castoldi, C., Requie, L. M., Camarena-Delgado, C., dal Bianco, B., Gräff, J., Seitz, A., & Silva, B. A. (2024). ABBA, a novel tool for whole-brain mapping, reveals brain-wide differences in immediate early genes induction following learning. *bioRxiv*. https://doi.org/10.1101/2024.09.06.611625

Corcoran, K. A., & Quirk, G. J. (2007). Activity in Prelimbic Cortex Is Necessary for the Expression of Learned, But Not Innate, Fears. *The Journal of Neuroscience*, *27*(4), 840–844. https://doi.org/10.1523/JNEUROSCI.5327-06.2007

Diegelmann, S., Preuschoff, S., Appel, M., Niewalda, T., Gerber, B., & Yarali, A. (2013). Memory decay and susceptibility to amnesia dissociate punishment- from relief-learning. *Biology Letters (2005)*, *9*(4), 20121171–20121171. https://doi.org/10.1098/rsbl.2012.1171

Dozois, D. J. A. (2021). Anxiety and depression in Canada during the COVID-19 pandemic: A national survey. *Canadian Psychology/Psychologie*

Canadienne, 62(1), 136-142. https://doi.org/10.1037/cap0000251

Etkin, A., Egner, T., & Kalisch, R. (2011). Emotional processing in anterior cingulate and medial prefrontal cortex. *Trends in Cognitive Sciences*, *15*(2), 85–93. https://doi.org/10.1016/j.tics.2010.11.004

Fanselow, M. S., & Bolles, R. C. (1979). Naloxone and shock-elicited freezing in the rat. *Journal of Comparative and Physiological Psychology, 93*(4), 736-744. https://doi.org/10.1037/h0077609

Fendt, M., & Fanselow, M. S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience and Biobehavioral Reviews*, *23*(5), 743–760. https://doi.org/10.1016/S0149-7634(99)00016-0

Fischer, J. A., Giniger, E., Maniatis, T., & Ptashne, M. (1988). GAL4 activates transcription in Drosophila. *Nature (London)*, *332*(6167), 853–856. https://doi.org/10.1038/332853a0

Foilb, A. R., Sansaricq, G. N., Zona, E. E., Fernando, K., & Christianson, J. P. (2021). Neural correlates of safety learning. *Behavioural Brain Research*, *396*, Article 112884. https://doi.org/10.1016/j.bbr.2020.112884

Gerber, B., Yarali, A., Diegelmann, S., Wotjak, C. T., Pauli, P., & Fendt, M. (2014). Pain-relief learning in flies, rats, and man: Basic research and applied perspectives. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *21*(4), 232–252. https://doi.org/10.1101/lm.032995.113

Goodpaster, C.M., Christensen, C.R., Alturki, M.B., & DeNardo, L.A. (2025). Prefrontal cortex development and its implications in mental illness. *Neuropsychopharmacology.* https://doi.org/10.1038/s41386-025-02154-8

Goosens, K. A., & Maren, S. (2001). Contextual and Auditory Fear Conditioning are Mediated by the Lateral, Basal, and Central Amygdaloid Nuclei in Rats. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *8*(3), 148–155. https://doi.org/10.1101/lm.37601

Gross, JJ. (2002). Emotion regulation: affective, cognitive, and social consequences. *Psychophysiology, 39*(3), 281-91. https://doi.org/10.1017/s0048577201393198

Hagihara, K. M., Bukalo, O., Zeller, M., Aksoy-Aksel, A., Karalis, N., Limoges, A., Rigg, T., Campbell, T., Mendez, A., Weinholtz, C., Mahn, M., Zweifel, L. S., Palmiter, R. D., Ehrlich, I., Lüthi, A., & Holmes, A. (2021). Intercalated amygdala clusters orchestrate a switch in fear state. *Nature (London)*, *594*(7863), 403–407. https://doi.org/10.1038/s41586-021-03593-1

Hagihara, K. M., & Lüthi, A. (2024). Bidirectional valence coding in amygdala intercalated clusters: A neural substrate for the opponent-process theory of motivation. *Neuroscience Research*, *209*, 28–33. https://doi.org/10.1016/j.neures.2024.07.003

He, Y., Huang, Y. H., Schlüter, O. M., & Dong, Y. (2023). Cue- versus reward-encoding basolateral amygdala projections to nucleus accumbens. *eLife*, *12*. https://doi.org/10.7554/eLife.89766

Heth, C. D., & Rescorla, R. A. (1973). Simultaneous and backward fear conditioning in the rat. *Journal of Comparative & Physiological Psychology*, *82*(3), 434–443. https://doi.org/10.1037/h0034124

Herry, C., Ciocchi, S., Senn, V., Demmou, L., Müller, C., & Lüthi, A. (2008). Switching

on and off fear by distinct neuronal circuits. *Nature*, *454*(7204), 600–606. https://doi.org/10.1038/nature07166

Hoover, W. B., & Vertes, R. P. (2007). Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. *Brain Structure and Function*, *212*(2), 149–179. https://doi.org/10.1007/s00429-007-0150-4

Johansen, J. P., Cain, C. K., Ostroff, L. E., & LeDoux, J. E. (2011). Molecular Mechanisms of Fear Learning and Memory. *Cell*, *147*(3), 509–524. https://doi.org/10.1016/j.cell.2011.10.009

Jovanovic, T., Kazama, A., Bachevalier, J., & Davis, M. (2012). Impaired safety signal learning may be a biomarker of PTSD. *Neuropharmacology*, *62*(2), 695–704. https://doi.org/10.1016/j.neuropharm.2011.02.023

Kahl, E., & Fendt, M. (2016). Metabotropic Glutamate Receptors 7 within the Nucleus Accumbens are Involved in Relief Learning in Rats. *Current neuropharmacology*, *14*(5), 405–412. https://doi.org/10.2174/1570159x13666150425002017

Kalinina, A., Krekhno, Z., Yee, J., Lehmann, H., & Fournier, N. M. (2021). Effect of repeated seizures on spatial exploration and immediate early gene expression in the hippocampus and dentate gyrus. *IBRO neuroscience reports*, *12*, 73–80. https://doi.org/10.1016/j.ibneur.2021.12.008

Kesner, R. P., & Churchwell, J. C. (2011). An analysis of rat prefrontal cortex in mediating executive function. *Neurobiology of Learning and Memory*, *96*(3), 417–431. https://doi.org/10.1016/j.nlm.2011.07.002

Kim, J. J., & Fanselow, M. S. (1992). Modality-Specific Retrograde Amnesia of Fear.

Science(American Association for the Advancement of Science), *256*(5057), 675–677. https://doi.org/10.1126/science.1585183

Kirov, G., Lowry, C. A., Stephens, M., Oldfield, S., O'Donovan, M. C., Lightman, S. L., & Owen, M. J. (2001). Screening ABCG1, the human homologue of the drosophila white gene, for polymorphisms and association with bipolar affective disorder. *Molecular Psychiatry*, *6*(6), 671–677. https://doi.org/10.1038/sj.mp.4000899

Kjelstrup, K. G., Tuvnes, F. A., Steffenach, H.-A., Murison, R., Moser, E. I., & Moser, M.-B. (2002). Reduced Fear Expression after Lesions of the Ventral Hippocampus. *Proceedings of the National Academy of Sciences - PNAS*, *99*(16), 10825–10830. https://doi.org/10.1073/pnas.152112399

Knapska, E., & Maren, S. (2009). Reciprocal patterns of c-Fos expression in the medial prefrontal cortex and amygdala after extinction and renewal of conditioned fear. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *16*(8), 486–493. https://doi.org/10.1101/lm.1463909

Knapska, E., Macias, M., Mikosz, M., Nowak, A., Owczarek, D., Wawrzyniak, M., Pieprzyk, M., Cymerman, I. A., Werka, T., Sheng, M., Maren, S., Jaworski, J., & Kaczmarek, L. (2012). Functional anatomy of neural circuits regulating fear and extinction. *Proceedings of the National Academy of Sciences - PNAS*, *109*(42), 17093–17098. https://doi.org/10.1073/pnas.1202087109

Kong, E., Monje, F. J., Hirsch, J., & Pollak, D. D. (2014). Learning not to fear: Neural correlates of learned safety. *Neuropsychopharmacology (New York, N.Y.)*, *39*(3), 515–527. https://doi.org/10.1038/npp.2013.191

Konorski J. 1948. Conditioned reflexes and neuron organisation. Cambridge University Press, Cambridge, UK.

Koob, G. F., Stinus, L., Le Moal, M., & Bloom, F. E. (1989). Opponent process theory of motivation: neurobiological evidence from studies of opiate dependence. *Neuroscience and biobehavioral reviews*, *13*(2-3), 135–140. https://doi.org/10.1016/s0149-7634(89)80022-3

Lai, C.-H., Park, G., Xu, P., Sun, X., Ge, Q., Jin, Z., Betts, S., Liu, X., Liu, Q., Simha, R., Zeng, C., Lu, H., & Du, J. (2024). Decoding the hidden variabilities in mPFC descending pathways across emotional states. *eLife*.14. https://doi.org/10.1101/2024.05.28.596238

Lang, P. J., Davis, M., & Ohman, A. (2000). Fear and anxiety: animal models and human cognitive psychophysiology. *Journal of affective disorders*, *61*(3), 137–159. https://doi.org/10.1016/s0165-0327(00)00343-8

Laurent, V., & Westbrook, R. F. (2009). Inactivation of the infralimbic but not the prelimbic cortex impairs consolidation and retrieval of fear extinction. *Learning & Memory,16*(9), 520–529. https://doi.org/10.1101/lm.1474609

LeDoux J. E. (2012). Evolution of human emotion: a view through fear. *Progress in brain research*, *195*, 431–442. https://doi.org/10.1016/B978-0-444-53860-4.00021-0

LeDoux, J., Cicchetti, P., Xagoraris, A., & Romanski, L. (1990). The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. *The Journal of Neuroscience*, *10*(4), 1062–1069. https://doi.org/10.1523/jneurosci.10-04-01062.1990

LeDoux, J., Iwata, J., Cicchetti, P., & Reis, D. (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. *The Journal of Neuroscience*, *8*(7), 2517–2529. https://doi.org/10.1523/jneurosci.08-07-02517.1988

Leknes, S., Brooks, J. C. W., Wiech, K., & Tracey, I. (2008). Pain relief as an opponent process: a psychophysical investigation. *The European Journal of Neuroscience*, *28*(4), 794–801. https://doi.org/10.1111/j.1460-9568.2008.06380.x

Leknes, S., Lee, M., Berna, C., Andersson, J., & Tracey, I. (2011). Relief as a reward: Hedonic and neural responses to safety from pain. *PLoS ONE*, *6*(4). https://doi.org/10.1371/journal.pone.0017870

Likhtik, E., Popa, D., Apergis-Schoute, J., Fidacaro, G. A., & Paré, D. (2008). Amygdala intercalated neurons are required for expression of fear extinction. *Nature*, *454*(7204), 642–645. https://doi.org/10.1038/nature07167

Luo, R., Uematsu, A., Weitemier, A., Aquili, L., Koivumaa, J., McHugh, T. J., & Johansen, J. P. (2018). A dopaminergic switch for fear to safety transitions. *Nature communications*, *9*(1), 2483. https://doi.org/10.1038/s41467-018-04784-7

Maren, S., Phan, K. L., & Liberzon, I. (2013). The contextual brain: implications for fear conditioning, extinction and psychopathology. *Nature reviews Neuroscience*, *14*(6), 417–428. https://doi.org/10.1038/nrn3492

Maren, S., & Quirk, G. J. (2004). Neuronal signalling of fear memory. *Nature reviews. Neuroscience*, *5*(11), 844–852. https://doi.org/10.1038/nrn1535

Maren, S., Aharonov, G., & Fanselow, M. S. (1997). Neurotoxic lesions of the dorsal

hippocampus and Pavlovian fear conditioning in rats. *Behavioural Brain Research*, *88*(2), 261–274. https://doi.org/10.1016/S0166-4328(97)00088-0

Mariotti, A. (2015). The Effects of Chronic Stress On Health: New Insights Into the Molecular Mechanisms of Brain–Body Communication. *Future Science OA*, *1*(3), FSO23. https://doi.org/10.4155/fso.15.21

Mayer, D., Kahl, E., Uzuneser, T. C., & Fendt, M. (2018). Role of the mesolimbic dopamine system in relief learning. *Neuropsychopharmacology (New York, N.Y.)*, *43*(8), 1651–1659. https://doi.org/10.1038/s41386-018-0020-1

McNaughton, N., & Corr, P. J. (2004). [Rev. of *A two-dimensional neuropsychology of defense: fear/anxiety and defensive distance*]. *Neuroscience and Biobehavioral Reviews*, *28*(3), 285–305. https://doi.org/10.1016/j.neubiorev.2004.03.005

McRae, L., O'Donnell, S., Loukine, L., Rancourt, N., & Pelletier, C. (2016). Report summary – Mood and Anxiety Disorders in Canada, 2016. *Health Promotion and Chronic Disease Prevention in Canada*, *36*(12), 314–315. https://doi.org/10.24095/hpcdp.36.12.05

McSweeney, F. K., & Murphy, E. S. (2014). *The Wiley Blackwell Handbook of Operant and Classical Conditioning* (1st ed.). Wiley. https://doi.org/10.1002/9781118468135

Meyer, H. C., Odriozola, P., Cohodes, E. M., Mandell, J. D., Li, A., Yang, R., Hall, B. S., Haberman, J. T., Zacharek, S. J., Liston, C., Lee, F. S., & Gee, D. G. (2019). Ventral hippocampus interacts with prelimbic cortex during inhibition of threat response via learned safety in both mice and humans. *Proceedings of the National*

Academy of Sciences - PNAS, *116*(52), 26970–26979. https://doi.org/10.1073/pnas.1910481116

Milad, M. R., & Quirk, G. J. (2012). Fear extinction as a model for translational neuroscience: ten years of progress. *Annual review of psychology*, *63*, 129–151. https://doi.org/10.1146/annurev.psych.121208.131631

Mohammadi, M., Bergado-Acosta, J. R., & Fendt, M. (2014). Relief learning is distinguished from safety learning by the requirement of the nucleus accumbens. *Behavioural Brain Research*, *272*, 40–45. https://doi.org/10.1016/j.bbr.2014.06.053

Mohammadi, M., & Fendt, M. (2015). Relief learning is dependent on NMDA receptor activation in the nucleus accumbens. *British Journal of Pharmacology*, *172*(9), 2419–2426. https://doi.org/10.1111/bph.13070

Moser, M.-B., & Moser, E. I. (1998). Functional differentiation in the hippocampus. *Hippocampus*, *8*(6), 608–619. https://doi.org/10.1002/(SICI)1098-1063(1998)8:6<608::AID-HIPO3>3.0.CO;2-7

Myers, K. M., & Davis, M. (2007). Mechanisms of fear extinction. *Molecular psychiatry*, *12*(2), 120–150. https://doi.org/10.1038/sj.mp.4001939

Myers, K. M., & Davis, M. (2002). [Rev. of *Behavioral and Neural Analysis of Extinction*]. *Neuron*, *36*(4), 567–584. https://doi.org/10.1016/S0896-6273(02)01064-4

Nader, K., Majidishad, P., Amorapanth, P., & LeDoux, J. E. (2001). Damage to the

Lateral and Central, but Not Other, Amygdaloid Nuclei Prevents the Acquisition of Auditory Fear Conditioning. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *8*(3), 156–163. https://doi.org/10.1101/lm.38101

Navratilova, E., & Porreca, F. (2014). Reward and motivation in pain and pain relief. *Nature Neuroscience*, *17*(10), 1304–1312. https://doi.org/10.1038/nn.3811

Ng, K. H., & Sangha, S. (2023). Encoding of conditioned inhibitors of fear in the infralimbic cortex. *Cerebral Cortex (New York, N.Y. 1991)*, *33*(9), 5658–5670. https://doi.org/10.1093/cercor/bhac450

Orsini, C. A., Kim, J. H., Knapska, E., & Maren, S. (2011). Hippocampal and Prefrontal Projections to the Basal Amygdala Mediate Contextual Regulation of Fear after Extinction. *The Journal of Neuroscience*, *31*(47), 17269–17277. https://doi.org/10.1523/JNEUROSCI.4095-11.2011

Pavlov, I. P., & Anrep, G. V. (1927). *Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex*. Oxford University Press/Humphrey Milford.

Paxinos, G., Watson, C., 2007. The Rat Brain in Stereotaxic Coordinates. Academic Press, San Diego.

Piantadosi, P. T., Yeates, D. C. M., & Floresco, S. B. (2020). Prefrontal cortical and nucleus accumbens contributions to discriminative conditioned suppression of reward-seeking. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *27*(10), 429–440. https://doi.org/10.1101/lm.051912.120

Quirk, G. J., & Mueller, D. (2008). Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology*, *33*(1), 56–72.

https://doi.org/10.1038/sj.npp.1301555

Quirk, G. J., Russo, G. K., Barron, J. L., & Lebron, K. (2000). The Role of Ventromedial Prefrontal Cortex in the Recovery of Extinguished Fear. *The Journal of Neuroscience*, *20*(16), 6225–6231. https://doi.org/10.1523/jneurosci.20-16-06225.2000

Radke, A. K., Rothwell, P. E., & Gewirtz, J. C. (2011). An anatomical basis for opponent process mechanisms of opiate withdrawal. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *31*(20), 7533–7539. https://doi.org/10.1523/JNEUROSCI.0172-11.2011

Ray, M. H., Russ, A. N., Walker, R. A., & McDannald, M. A. (2020). The Nucleus Accumbens Core is Necessary to Scale Fear to Degree of Threat. *The Journal of Neuroscience*, *40*(24), 4750–4760. https://doi.org/10.1523/JNEUROSCI.0299-20.2020

Rescorla, R. A. (1988). Behavioral studies of Pavlovian conditioning. *Annual Review of Neuroscience*, *11*(1), 329–352. https://doi.org/10.1146/annurev.ne.11.030188.001553

Rescorla, R. A. (1969). Conditioned inhibition of fear resulting from negative CS-US contingencies. *Journal of Comparative & Physiological Psychology*, *67*(4), 504–509. https://doi.org/10.1037/h0027313

Rescorla, R. A. (1969). Pavlovian conditioned inhibition. *Psychological Bulletin*, *72*(2), 77–94. https://doi.org/10.1037/h0027760

Rescorla, R. A. (1968). Probability of shock in the presence and absence of CS in fear

conditioning. *Journal of Comparative & Physiological Psychology*, *66*(1), 1–5. https://doi.org/10.1037/h0025984

Rescorla, R. A. (1971). Summation and retardation tests of latent inhibition. *Journal of Comparative and Physiological Psychology, 75*(1), 77-81. https://doi.org/10.1037/h0030694

Ressler, R. L., Goode, T. D., Evemy, C., & Maren, S. (2020). NMDA receptors in the CeA and BNST differentially regulate fear conditioning to predictable and unpredictable threats. *Neurobiology of Learning and Memory*, *174*, 107281–107281. https://doi.org/10.1016/j.nlm.2020.107281

Rodrigues, S. M., Schafe, G. E., & LeDoux, J. E. (2004). [Rev. of *Molecular Mechanisms Underlying Emotional Learning and Memory in the Lateral Amygdala*]. *Neuron*, *44*(1), 75–91. https://doi.org/10.1016/j.neuron.2004.09.014

Romanski, L., & LeDoux, J. (1992). Equipotentiality of thalamo-amygdala and thalamo-cortico-amygdala circuits in auditory fear conditioning. *The Journal of Neuroscience*, *12*(11), 4501–4509. https://doi.org/10.1523/jneurosci.12-11-04501.1992

Roth, B. L. (2016). DREADDs for Neuroscientists. *Neuron (Cambridge, Mass.)*, *89*(4), 683–694. https://doi.org/10.1016/j.neuron.2016.01.040

Royer, S., Martina, M., & Pare, D. (1999). An Inhibitory Interface Gates Impulse Traffic between the Input and Output Stations of the Amygdala. *The Journal of Neuroscience*, *19*(23), 10575–10583. https://doi.org/10.1523/jneurosci.19-23-10575.1999

Rudy, J. W., & O'Reilly, R. C. (1999). Contextual Fear Conditioning, Conjunctive

Representations, Pattern Completion, and the Hippocampus. *Behavioral Neuroscience*, *113*(5), 867–880. https://doi.org/10.1037/0735-7044.113.5.867

Rushworth, M. F. S., Noonan, M. P., Boorman, E. D., Walton, M. E., & Behrens, T. E. (2011). Frontal Cortex and Reward-Guided Learning and Decision-Making. *Neuron (Cambridge, Mass.)*, *70*(6), 1054–1069. https://doi.org/10.1016/j.neuron.2011.05.014

Salkovskis, P. M., Clark, D. M., Hackmann, A., Wells, A., & Gelder, M. G. (1999). An experimental investigation of the role of safety-seeking behaviours in the maintenance of panic disorder with agoraphobia. *Behaviour research and therapy*, *37*(6), 559–574. https://doi.org/10.1016/s0005-7967(98)00153-3

Sangha, S., Robinson, P. D., Greba, Q., Davies, D. A., & Howland, J. G. (2014). Alterations in Reward, Fear and Safety Cue Discrimination after Inactivation of the Rat Prelimbic and Infralimbic Cortices. *Neuropsychopharmacology*, *39*(10), 2405–2413. https://doi.org/10.1038/npp.2014.89

Seymour, B., O'Doherty, J. P., Koltzenburg, M., Wiech, K., Frackowiak, R., Friston, K., & Dolan, R. (2005). Opponent appetitive-aversive neural processes underlie predictive learning of pain relief. *Nature neuroscience*, *8*(9), 1234–1240. https://doi.org/10.1038/nn1527

Skinner, B. F. (1938). Behavior of organisms: An experimental analysis. New York: Appleton-Century-Crofts, Soleimanpour, E., Bergado Acosta, J. R., Landgraf, P., Mayer, D., Dankert, E., Dieterich, D. C., & Fendt, M. (2021). Regulation of CREB Phosphorylation in Nucleus Accumbens after Relief Conditioning. *Cells*, *10*(2), 238. https://doi.org/10.3390/cells10020238

Solomon, R. L. (1980). The opponent-process theory of acquired motivation: The costs of pleasure and the benefits of pain. *The American Psychologist*, *35*(8), 691–712. https://doi.org/10.1037/0003-066X.35.8.691

Sotres-Bayon, F., & Quirk, G. J. (2010). Prefrontal control of fear: more than just extinction. *Current Opinion in Neurobiology*, *20*(2), 231–235. https://doi.org/10.1016/j.conb.2010.02.005

Sotres-Bayon, F., Sierra-Mercado, D., Pardilla-Delgado, E., & Quirk, G. J. (2012). Gating of Fear in Prelimbic Cortex by Hippocampal and Amygdala Inputs. *Neuron (Cambridge, Mass.)*, *76*(4), 804–812. https://doi.org/10.1016/j.neuron.2012.09.028

Statistics Canada. (2023). *Mental disorders and access to mental health care.* (75-006-X). https://www150.statcan.gc.ca/n1/pub/75-006-x/2023001/article/00011-eng.htm

Tanimoto, H., Heisenberg, M., & Gerber, B. (2004). Experimental psychology: event timing turns punishment to reward. *Nature (London)*, *430*(7003), 983–983. https://doi.org/10.1038/430983a

Thompson, B. M., Baratta, M. V., Biedenkapp, J. C., Rudy, J. W., Watkins, L. R., & Maier, S. F. (2010). Activation of the infralimbic cortex in a fear context enhances extinction learning. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *17*(11), 591–599. https://doi.org/10.1101/lm.1920810

Urban, D. J., & Roth, B. L. (2015). DREADDs (Designer Receptors Exclusively

Activated by Designer Drugs): Chemogenetic Tools with Therapeutic Utility. *Annual Review of Pharmacology and Toxicology*, *55*(1), 399–417. https://doi.org/10.1146/annurev-pharmtox-010814-124803

VanElzakker, M. B., Kathryn Dahlgren, M., Caroline Davis, F., Dubois, S., & Shin, L. M. (2014). From Pavlov to PTSD: The extinction of conditioned fear in rodents, humans, and anxiety disorders. *Neurobiology of Learning and Memory*, *113*, 3–18. https://doi.org/10.1016/j.nlm.2013.11.014

Vervliet, B., Craske, M. G., & Hermans, D. (2013). Fear Extinction and Relapse: State of the Art. *Annual Review of Clinical Psychology*, *9*(1), 215–248. https://doi.org/10.1146/annurev-clinpsy-050212-185542

Wang, M. E., Fraize, N. P., Yin, L., Yuan, R. K., Petsagourakis, D., Wann, E. G., & Muzzio, I. A. (2013). Differential roles of the dorsal and ventral hippocampus in predator odor contextual fear conditioning. *Hippocampus*, *23*(6), 451–466. https://doi.org/10.1002/hipo.22105

Wilensky, A. E., Schafe, G. E., Kristensen, M. P., & LeDoux, J. E. (2006). Rethinking the Fear Circuit: The Central Nucleus of the Amygdala Is Required for the Acquisition, Consolidation, and Expression of Pavlovian Fear Conditioning. *The Journal of Neuroscience*, *26*(48), 12387–12396. https://doi.org/10.1523/JNEUROSCI.4316-06.2006

Wilson, K., MacDonald, S., Bauer, E., Zhang, Y., Maren, S., & MacNamara, A. (2022). P109. Backward Conditioning Indirectly Retrieves Threat Memories: Implications for Internalizing Psychopathology. *Biological Psychiatry (1969)*, *91*(9), S131–S131. https://doi.org/10.1016/j.biopsych.2022.02.343

Wu, G., Liu, G., Zhang, H., Chen, C., Liu, S., Feng, H., & Sui, J. (2015). Optogenetic stimulation of mPFC pyramidal neurons as a conditioned stimulus supports associative learning in rats. *Scientific Reports*, *5*(1), Article 10065. https://doi.org/10.1038/srep10065

Yagishita, S., Hayashi-Takagi, A., Ellis-Davies, G. C. R., Urakubo, H., Ishii, S., & Kasai, H. (2014). A critical time window for dopamine actions on the structural plasticity of dendritic spines. *Science (American Association for the Advancement of Science)*, *345*(6204), 1616–1620. https://doi.org/10.1126/science.1255514

Yarali, A., Krischke, M., Michels, B., Saumweber, T., Mueller, M. J., & Gerber, B. (2009). Genetic Distortion of the Balance between Punishment and Relief Learning in Drosophila. *Journal of Neurogenetics*, *23*(1–2), 235–247. https://doi.org/10.1080/01677060802441372

Yarali, A., Niewalda, T., Chen, Y., Tanimoto, H., Duerrnagel, S., & Gerber, B. (2008). 'Pain relief' learning in fruit flies. *Animal Behaviour*, *76*(4), 1173–1185. https://doi.org/10.1016/j.anbehav.2008.05.025

Zelikowsky, M., Pham, D. L., & Fanselow, M. S. (2012). Temporal factors control hippocampal contributions to fear renewal after extinction. *Hippocampus*, *22*(5), 1096–1106. https://doi.org/10.1002/hipo.20954

Zhang, S., Mano, H., Lee, M., Yoshida, W., Kawato, M., Robbins, T. W., & Seymour, B. (2018). The control of tonic pain by active relief learning. *eLife*, *7*, Article e31949. https://doi.org/10.7554/eLife.31949