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COMMENT LE CERVEAU INTÈGRE DES SOUVENIRS SENSORIELS ET ÉMOTIONNELS ?

Une étude du rôle de l'hippocampe dans le préconditionnement sensoriel chez le rat

HOW DOES THE BRAIN INTEGRATE SENSORY AND EMOTIONAL MEMORIES?

A study of the role of the hippocampus in sensory preconditioning in rats

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Résumé – Abstract

Comment le cerveau intègre des souvenirs sensoriels et émotionnels ? Une étude du rôle de l'hippocampe dans le préconditionnement sensoriel chez le rat

Les animaux et les humains s'adaptent à leur environnement en apprenant à prédire des événements ayant une importance motivationnelle, tels que la disponibilité de nourriture ou la présence d'un danger. Ces prédictions reposent souvent sur l'expérience directe des contingences entre différents stimuli et événements (par exemple, un son suivi de l'attaque d'un prédateur), mais elles peuvent également être générées en l'absence d'une telle expérience. Par exemple, en reliant les composantes sensorielles et émotionnelles de souvenirs distincts, il est possible de formuler des inférences à propos de stimuli jusqu'alors perçus comme inoffensifs. Ce type d'inférence soulève des questions sur la manière dont les souvenirs sont encodés et stockés dans le cerveau, ainsi que sur la façon dont ils sont intégrés pour générer des comportements adaptatifs. L'hippocampe joue un rôle essentiel dans l'encodage et l'intégration des souvenirs associatifs. Cependant, les contributions respectives de l'hippocampe dorsal et de l'hippocampe ventral, ainsi que le rôle de leur connectivité dans ces processus, restent mal compris. Cette thèse combine des paradigmes comportementaux, des inactivations pharmacologiques, des enregistrements en photométrie par fibre optique et des manipulations chémo-génétiques afin d'étudier le rôle de l'hippocampe dorsal et ventral dans l'encodage et l'intégration des souvenirs sensoriels et émotionnels chez le rat. Dans une première série d'expériences, nous avons mis en place un paradigme de préconditionnement sensoriel audiovisuel et nous avons montré que l'intégration repose sur un apprentissage médié : au moment du conditionnement, les rats réactivent des informations acquises antérieurement afin de former de nouvelles associations entre les stimuli. Ensuite, nous avons étudié le rôle causal de l'hippocampe dorsal et ventral dans les différentes étapes du préconditionnement sensoriel par inactivation pharmacologique. Nous avons montré que l'hippocampe ventral, mais non l'hippocampe dorsal, est nécessaire à la formation d'associations entre des stimuli inoffensifs, et que les deux sous-régions sont nécessaires à l'apprentissage médié. Notamment, nous avons identifié une signature de l'apprentissage médié en mettant en évidence une connectivité accrue entre l'hippocampe dorsal et l'hippocampe ventral lors de la présentation des stimuli. Cette connexion s'inscrit dans un réseau plus large soutenant l'apprentissage médié, qui inclut également le cortex périrhinal. Enfin, à l'aide d'une manipulation chémo-génétique dans un protocole de préconditionnement sensoriel gustatif, nous avons démontré que l'hippocampe joue également un rôle essentiel dans l'encodage et l'intégration des souvenirs lorsque les stimuli appartiennent à une même modalité sensorielle. Ce travail met en lumière les contributions distinctes de l'hippocampe dorsal et ventral dans la capacité des animaux à anticiper les conséquences de situations nouvelles en l'absence d'expérience directe.

Mots-clés : préconditionnement sensoriel, hippocampe, rat, conditionnement de peur, aversion gustative conditionnée

How does the brain integrate sensory and emotional memories? A study of the role of the hippocampus in sensory preconditioning in rats

Animals and humans adapt to their environments by learning to predict motivationally significant events such as the presence of food or danger. These predictions are often based on direct experience of the contingencies between different stimuli and events (e.g., a sound followed by the attack of a predator) but can also be generated in the absence of such experience. For example, by linking the sensory and emotional elements of distinct memories, we can generate novel inferences about stimuli that have only ever been experienced as innocuous. This type of inference has implications for how memories are encoded and stored in the brain; and raises the question of how they are integrated to generate adaptive behavior. The encoding and integration of associative memories are known to be hippocampus-dependent. Yet, the differential contributions of dorsal versus ventral hippocampus and their connectivity in these processes remains unclear. This thesis combines behavioral paradigms, pharmacological inactivations, fiber photometry recordings, and chemogenetic manipulations to investigate the role of dorsal and ventral hippocampus in the encoding and integration of sensory and emotional memories in rats. In the first set of experiments, we establish auditory-visual sensory preconditioning and show that integration operates via mediated learning: rats retrieve past information at the time of conditioning to form new associations between stimuli. In a second line of work, we use pharmacological inactivation to investigate the causal role of dorsal and ventral hippocampus in the different stages of sensory preconditioning. We show that ventral, but not dorsal, hippocampus is required to form innocuous stimulus-stimulus associations, and that both subregions are necessary for mediated learning. Critically, we identify the signature of successful mediated learning by evidencing higher dorso-ventral hippocampus connectivity during stimulus presentations. We show that this connection is part of a broader network supporting mediated learning, which includes the perirhinal cortex. Finally, using chemogenetic manipulation in a gustatory sensory preconditioning protocol, we demonstrate that the hippocampus is also critical to encode and integrate memories even when stimuli are unimodal. This work highlights the differential contributions of dorsal and ventral hippocampus in animals' ability to infer outcomes in the absence of direct experience when facing novel situations.

Keywords: sensory preconditioning, hippocampus, rat, fear conditioning, conditioned taste aversion

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List of abbreviations

AAV	Adeno-associated virus (viral vector)
ACC	Anterior cingulate cortex
ACSF	Artificial cerebrospinal fluid
AESOP	Affective extension of SOP (Wagner & Brandon model)
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
AP	Anteroposterior (stereotaxic coordinate)
AP5 / D-APV / DAP-V	D-2-amino-5-phosphonopentanoic acid (NMDA receptor antagonist)
AUC	Area under the curve
BL	Basolateral nucleus of the amygdala
BLA	Basolateral amygdala
BM	Basomedial nucleus of the amygdala
CA1, CA2, CA3	Cornu ammonis fields 1, 2 and 3
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CB1	Cannabinoid type 1
CB1R	Cannabinoid type 1 receptor
CeA	Central nucleus of the amygdala
CI	Confidence interval
CNO	Clozapine-N-oxide
CR	Conditioned response
CREB	cAMP response element-binding protein
CS	Conditioned stimulus
CSV	Comma-separated values (file format)
CTA	Conditioned taste aversion
DAB	3,3′-diaminobenzidine
DAPI	4′,6-diamidino-2-phenylindole
dCA1	Dorsal CA1
DFC	Dynamic functional connectivity
dH / dHPC	Dorsal hippocampus
dmPFC	Dorsomedial prefrontal cortex
DREADD	Designer Receptor Exclusively Activated by Designer Drugs
DV	Dorsoventral (stereotaxic coordinate)
ERK	Extracellular signal-regulated kinase
FDR	False discovery rate
fMRI	Functional magnetic resonance imaging
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid type A receptor
GC	Genome copies (viral titer)
GCMI	Gaussian copula mutual information
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
hM4Di	Human M4 muscarinic receptor-derived inhibitory DREADD
Hyp	Hypothalamus
i.p.	Intraperitoneal

IL	Infralimbic cortex
jGCaMP8m	Genetically encoded calcium indicator (jGCaMP8, medium kinetics variant)
LA	Lateral nucleus of the amygdala
LD1	First linear discriminant
LDA	Linear discriminant analysis
lEC	Lateral entorhinal cortex
LiCl	Lithium chloride
LM	Linear model
LOO	Leave-one-out (cross-validation)
LS mean	Least-squares mean
LTD	Long-term depression
LTP	Long-term potentiation
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
mEC	Medial entorhinal cortex
MEK	MAPK/ERK kinase
ML	Mediolateral (stereotaxic coordinate)
mPFC	Medial prefrontal cortex
mSOP	Modified SOP model
NAc	Nucleus accumbens
NaCl	Sodium chloride
NMDA	N-methyl-D-aspartate
NMDAr	N-methyl-D-aspartate receptor
NR2B	NMDA receptor subunit 2B
OFC	Orbitofrontal cortex
PB	Phosphate buffer
PBS	Phosphate-buffered saline
PBT/PBST	Phosphate-buffered saline containing Triton X-100
PFA	Paraformaldehyde
PKA	Protein kinase A
PL	Prelimbic cortex
Por	Postrhinal cortex
Prh / PRh	Perirhinal cortex
RE	Nucleus reuniens of the thalamus
REML	Restricted maximum likelihood
RFP	Red fluorescent protein
ROI	Region of interest
RSC/RSP	Retrosplenial cortex
RT	Room temperature
S-O	Stimulus-outcome (association)
S-R	Stimulus-response (association)
S-S	Stimulus-stimulus (association)
S1	First-order stimulus
S2	Second-order or preconditioned stimulus
SD	Standard deviation
SEM	Standard error of the mean
SOP	Sometimes Opponent Processes (Wagner's model)
SPC	Sensory preconditioning
SS	Sum of squares (Type III SS)
Str	Striatum
Sub	Subiculum
TA	Temporoammonic (pathway)
UR	Unconditioned response
US	Unconditioned stimulus
vCA1	Ventral CA1
vH/vHPC	Ventral hippocampus
VTA	Ventral tegmental area
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element

Chapter 1
Introduction

1. Predicting the world with and beyond direct associations

In the nineteenth century, Darwin proposed that mental processes, like physical traits, evolved through natural selection. As such, the mind came to be understood less by what it contains than by what it does: enabling organisms to adjust efficiently to changing environmental conditions. This functional perspective underpins the study of adaptive behaviour.

A fundamental adaptation is the ability to predict biologically significant events. Animals and humans alike benefit from anticipating the availability of food, the presence of danger, or other motivationally relevant outcomes, allowing them to behave appropriately before those events occur. Predicting an outcome involves understanding the relationship between distinct events, so that knowledge of one provides information about the other. In this view, prediction rests on associative memory: experiencing one event activates the representation of the other, allowing organisms to behave adequately. Associative learning is not unique to humans but is a conserved capacity observed across a wide range of species (Macphail, 1982; Pearce, 1997). As such, the ability to form associations represents a fundamental cognitive process that enables organisms to adapt their behaviour to a changing environment.

1.1. Direct predictions: from philosophical concepts to experimental science

The notion that organisms learn to anticipate events by forming associations long precedes its experimental investigation. Classical associationist philosophers, including Aristotle and later the British empiricists Locke, Berkeley, and Hume, proposed that experience is organised through associations linking sensory events that occur together in space and time. They state that expectations arise from experience, and that we infer causal relationships not because we perceive any necessary connection between events, but because their repeated encounters together lead us to expect one event following another (Hume, 1739, 1748). Hence, remembered past regularities come to guide future expectations.

The evolutionary perspective motivated the investigation of learning processes that are shared across species. A major step towards an experimental science of learning came with C. Lloyd Morgan, who argued that the study of animal behaviour should focus on observable mechanisms acquired through experience (Morgan, 1894). More than a century later, the philosophical ideas about how predictions emerge found an experimental foundation in the work of Ivan Pavlov. By repeatedly pairing an initially neutral stimulus (the sound of a metronome) with food, he showed that dogs progressively came to respond to the sound of the metronome itself, anticipating the biologically significant event it reliably preceded (Pavlov,

1927). In the terminology introduced by Pavlov, the initially neutral cue is referred to as the conditioned stimulus (CS), whereas the biologically significant event is the unconditioned stimulus (US). In short, the dog learned a CS→US association (**Figure 1**)

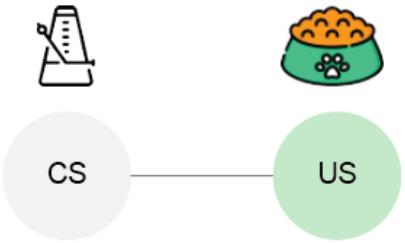


Figure 1. Pavlovian conditioning seen as a CS→US association

But is this what, in fact, occurred? The nature of the association acquired during Pavlovian conditioning has long been debated. Early associative theories proposed that conditioning establishes a direct stimulus-response (S-R) association, whereby the CS becomes linked to the motor response originally elicited by the US (Hull, 1966; Spence, 1956). Under this account, presentation of the CS would directly activate the conditioned response (CR). While this view is consistent with situations in which the CR resembles the unconditioned response (UR), it struggles to explain many observations showing that conditioned behaviour is often more flexible. In some cases, the CR differs markedly from the UR or even opposes it. For example, whereas morphine produces analgesia, a morphine-predictive cue can elicit hyperalgesia, suggesting that conditioning cannot simply consist of linking the CS to the original motor response (Siegel, 1975). The conditioned and unconditioned responses to an aversive stimulus such as brief but painful foot shock are often quite distinct: the UR involves jumping and running whereas the CR is often freezing, depending on the nature of the CS.

These findings led to the development of stimulus-outcome (S-O), or stimulus-stimulus (S–S), theories of Pavlovian conditioning (Bolles, 1972; Rescorla, 1973). According to this view, conditioning establishes an association between internal representations, or "nodes", corresponding to the CS and the specific outcome it predicts. Presentation of the CS subsequently activates the mental representation of the US, and it is this retrieved representation that gives rise to the conditioned response (**Figure 2**). Consequently, the CR does not always resemble the UR but instead varies according to the current value of the expected outcome, situational/contextual factors and the organism's motivational state.

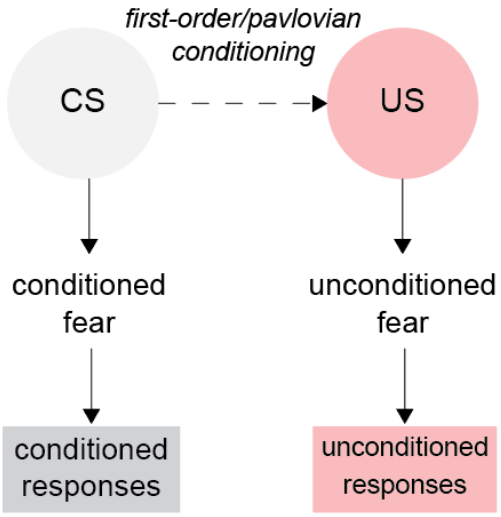


Figure 2. Conditioned and unconditioned responses can differ.

Example of fear conditioning. Adapted from Gewirtz and Davis (2000)

Strong support for this account comes from outcome devaluation studies. If the value of the US is reduced after conditioning (for example, when the US is food, by inducing satiety or pairing it with illness), responding to the associated CS is also diminished, despite the CS-US association itself remaining intact (Holland & Rescorla, 1975b; Holland & Straub, 1979). Such findings indicate that the CS does not simply trigger a learned response but evokes a representation of the specific expected outcome, whose current biological significance determines behaviour. Pavlovian conditioning therefore established that organisms learn predictive relationships between environmental events, allowing previously neutral cues to acquire behavioural significance. It also show that a US is not a unitary event: it can be decomposed into its specific sensory, motivational, temporal, response-eliciting properties, and a CS can form separable associations with each of these components (Delamater, 2012; Delamater & Oakeshott, 2007; Konorski, 1967; Wagner & Brandon, 1989). Thus, sensory-specific and value-specific associations are acquired in parallel, and the content of the association matters for what follows. Indeed, whether a CS can substitute for its outcome in new learning, or serve as a reinforcer, might depend on which components of the US it became associated with during conditioning.

If the content of Pavlovian learning is richer than the notation CS→US implies, so are the conditions under which that learning occurs at all. That is, although Pavlov's experiments are often described as demonstrating learning through stimulus pairings, subsequent work revealed that temporal contiguity alone is insufficient for learning to occur. Instead, learning depends on whether the CS provides information about the occurrence of the US. In a landmark series of experiments, Rescorla (1968) held the probability of a shock (US) during a CS constant while manipulating the probability of the US in the absence of the CS. When the

US occurred just as frequently without the CS as with it, little or no conditioning developed despite identical pairings. Conditioning emerged only when the CS improved the prediction of the US. These findings established that organisms learn about the predictive relationship between events, not simply co-occurrence.

1.2. Computational and neural basis of Pavlovian conditioning

1.2.1. Rescorla-Wagner model

Rescorla and Wagner formalised these observations in their influential model (Rescorla & Wagner, 1972) which proposed that associative learning is driven by prediction error. According to the model, change in associative strength between the CS and US during a given CS presentation depends on the discrepancy between the outcome that actually occurs and the outcome already predicted by all cues present (**Figure 3**). Mathematically, $\Delta V = \alpha\beta(\lambda - \sum V)$, where ΔV represents change in associative strength, α and β reflect the salience of the conditioned stimulus (CS) and unconditioned stimulus (US), respectively, λ corresponds to the maximum associative strength supported by the US, and $\sum V$ the summed associative strength of all cues present at the time of a CS-US pairing. Thus, learning is proportional to the prediction error $(\lambda - \sum V)$: unexpected outcomes generate large prediction errors and, thereby, large changes in associative strength; but when the outcome is fully predicted, prediction error is zero and no further learning occurs.

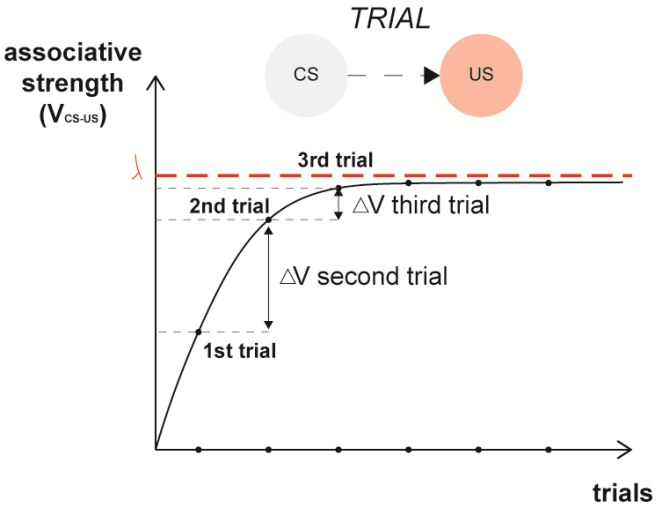


Figure 3. Gain in associative strength according to the Rescorla-Wagner model. *Across trials (e.g. three trials on the figure), as the US becomes fully predicted by the CS, the associative strength gained in the trials ($\Delta V_{CS \rightarrow US}$) becomes smaller until V reaches the theoretical asymptote.*

The Rescorla-Wagner model explained a wide range of conditioning phenomena, including overshadowing and blocking (**Figure 4**). In overshadowing, a cue acquires weaker associative strength when trained in compound with another cue than when trained alone (Mackintosh, 1976; Pavlov, 1927). In blocking, prior learning that one cue predicts the US prevents acquisition of associative strength by a novel cue presented simultaneously with it, because

the outcome is already fully expected (Kamin, 1969). Conversely, increasing the magnitude of the US restores learning to the added cue (unblocking), demonstrating that learning occurs when outcomes differ from expectation. Critically, these examples of cue-competition phenomena show that learning reflects predictive value rather than simple association: organisms preferentially allocate learning to the best available predictors of biologically significant events.

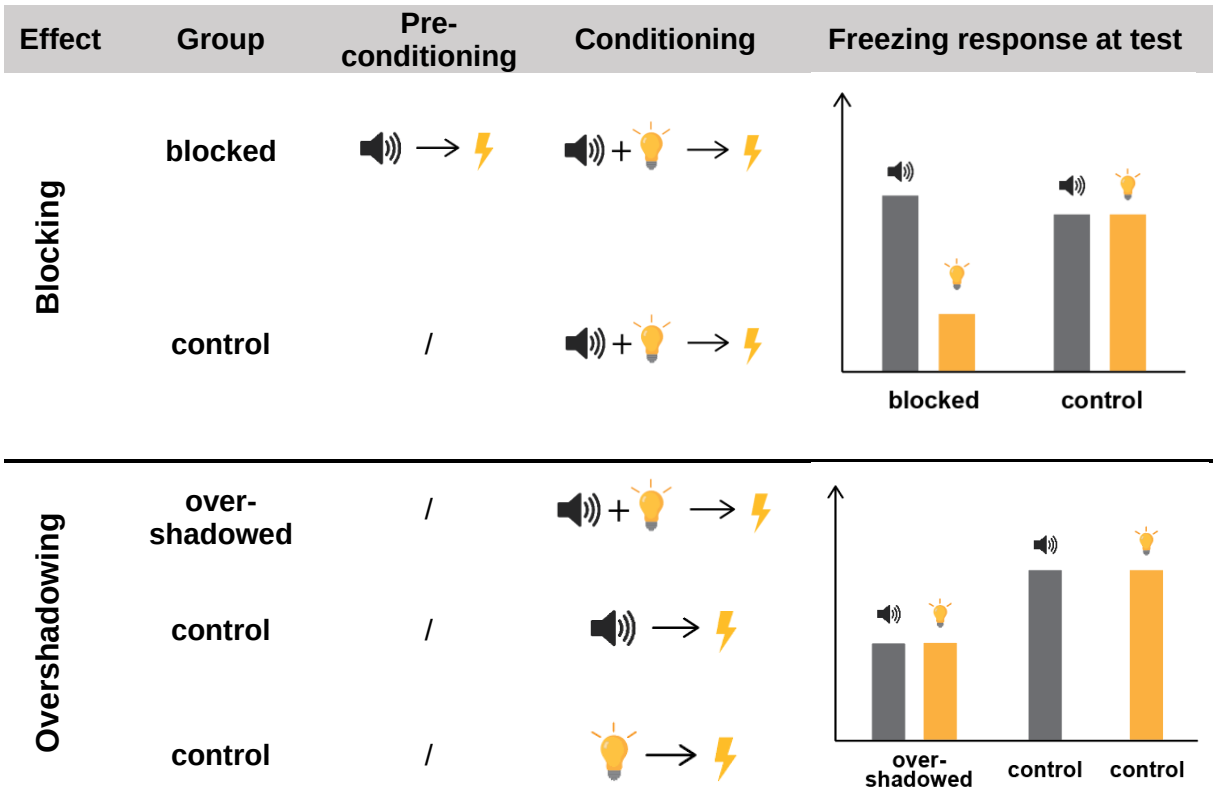


Figure 4. Overshadowing and blocking
In blocking, prior learning that a CS predicts a US reduces conditioning of a novel cue presented with this CS. In overshadowing, conditioning multiple cues results in lower levels of response to individual cues compared with conditioning to a single cue.

Although the Rescorla–Wagner model was formulated as a computational account of associative learning, subsequent work identified a neural signal matching its central prediction. Recordings from dopaminergic neurons in the ventral tegmental area and substantia nigra revealed that their phasic activity encodes reward prediction error: these neurons increase their firing to rewards that are better than expected, show little response to fully predicted rewards, and transiently reduce their activity at the time an expected reward is omitted (Schultz et al., 1997). This pattern closely mirrors the prediction-error term of the Rescorla-Wagner model and suggests that dopamine acts not simply as a signal of reward or pleasure, but as a teaching signal that updates associative learning (Schultz, 2002; Waelti et al., 2001). Importantly, this framework assumes that prediction error is expressed along the dimension of

value. Accordingly, dopamine has traditionally been viewed as signalling discrepancies in expected reward value rather than errors about the specific identity of expected outcomes. Recent findings challenge this view, suggesting that dopaminergic activity also reflects prediction errors concerning the sensory identity of expected events, independently of their value (Kahnt & Schoenbaum, 2025).

1.2.2. Neural substrates of aversive conditioning

Different forms of conditioning, such as fear, appetitive, or taste-aversion learning, exhibit distinct behavioural properties and recruit partially distinct neural circuits. Thus, while general models such as Rescorla–Wagner provide a common theoretical framework, understanding the neural basis of associative learning requires consideration of the specific conditioning paradigm. While we acknowledge an extensive literature on the behavioural and neural bases of appetitive conditioning, we thought it relevant to introduce the basis of the two aversive conditioning paradigms which are the focus of the present work: fear conditioning, and conditioned taste aversion (CTA).

In fear conditioning, an auditory, visual or contextual CS is paired with an aversive US, typically an electric shock delivered to the paws of a rat or a mouse (“footshock”) or a puff of air delivered to the eye. Following conditioning, presentation of the CS alone elicits a range of conditioned defensive responses, including preparation for fight, flight or freezing (absence of movement except breathing, (Fanselow, 1980)), as well as autonomic activation, and endocrine responses. Fear conditioning is typically dependent on a close temporal relationship between the CS and US (below the minute), and the stimuli arrangement can differ considerably between protocols. Importantly, fear conditioning does not involve a unique process elicited by the experimental procedure of a CS-US pairing (**Figure 5**). Recent work has discussed the importance of considering the multiplicity of processes and behaviours they influence in different procedures of pavlovian fear conditioning (McDannald, 2023).

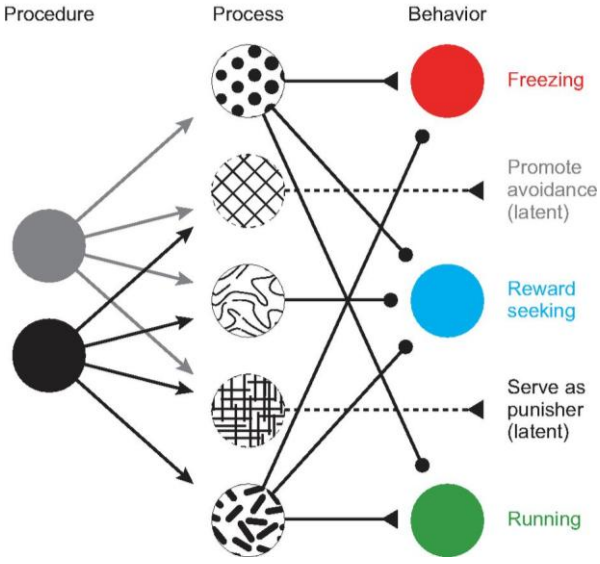


Figure 5. Pavlovian fear conditioning engages multiple processes which activate or inhibit different behaviours. From McDannald (2023).

CTA is another form of aversive learning in which an animal that consumes a novel taste (the CS) and subsequently experiences visceral malaise (US, classically induced by lithium chloride) later avoids that taste. First characterised by Garcia and colleagues, who noticed that rats made ill by radiation came to avoid flavours encountered beforehand, CTA obeys the general principles of Pavlovian conditioning (it depends on the CS-US contingency) but displays several distinctive properties. First, CTA is acquired rapidly, often in a single trial, and tolerates remarkably long intervals between the CS and the US (up to several hours) with the strength of the aversion declining gradually as the delay lengthens (Garcia et al., 1966; Smith & Roll, 1967). Second, CTA forms for certain CS-US pairings but not others in a manner that reflects an animal's evolutionary heritage. For example, in their foundational demonstration, Garcia and Koelling (1966) showed that rats readily associate tastes with internal malaise, and audiovisual cues with external pain, but not the reverse: a taste paired with nausea produces strong avoidance, whereas a light or tone paired with nausea does not. This selectivity directly contradicts the assumption that any stimulus can be associated with any outcome, an assumption which underlies general-process accounts of learning such as the Rescorla–Wagner model. It motivated the view that learning mechanisms are shaped by evolutionary pressures, showing adaptive specialisations tuned to biologically sensible relationships (Rozin & Kalat, 1971; Seligman, 1970). Finally, once acquired, taste aversions are durable and comparatively resistant to extinction, consistent with the adaptive function of avoiding foods that have previously made the animal ill.

In rodents, the circuitry supporting Pavlovian fear conditioning and CTA has been particularly well characterised. In both paradigms, the amygdala is a critical site for the formation of CS–US associations. The amygdala (**Figure 6**) comprises two interconnected subsystems contributing to fear conditioning (Fanselow, 1994; LeDoux, 1995). The basolateral complex (BLA; comprising the lateral [LA], basolateral [BL] and basomedial [BM] nuclei) receives convergent sensory inputs from cortical and subcortical pathways and is the principal site at which conditioned (CS) and unconditioned (US) information are associated. By contrast, the central nucleus (CeA) receives input from the BLA and projects to the hypothalamus, periaqueductal grey and other brainstem structures that generate autonomic, endocrine and behavioural fear responses, making it the major output pathway for conditioned fear. Accordingly, lesions of either the BLA or CeA impair both the acquisition and expression of conditioned fear (Campeau & Davis, 1995; Maren et al., 1996).

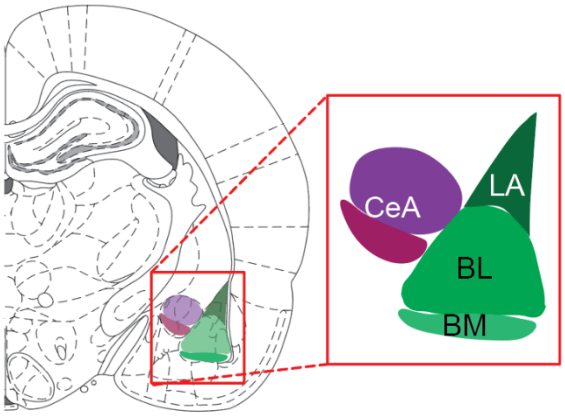


Figure 6. Representation of the rat amygdala from left hemisphere (bregma -2.6)
CeA: central amygdala nuclei; LA: lateral amygdala nuclei; BL: basolateral amygdala nuclei; BM: basomedial amygdala nuclei.

Within the BLA, the LA constitutes the sensory interface of the fear circuit (LeDoux et al., 1990). In auditory fear conditioning (**Figure 7A**), auditory CS information reaches the LA through both a rapid thalamic pathway and a slower cortical pathway via auditory association cortices, while nociceptive US information arrives from thalamic, posterior insular cortex and spino-parabrachial pathways (Doron & Ledoux, 1999; LeDoux, 2000; Shi & Davis, 1999). Plasticity at these inputs is pathway-specific: long-term potentiation develops independently at thalamic and cortical afferents but persists longer in the thalamic pathway (Doyère et al., 2003).

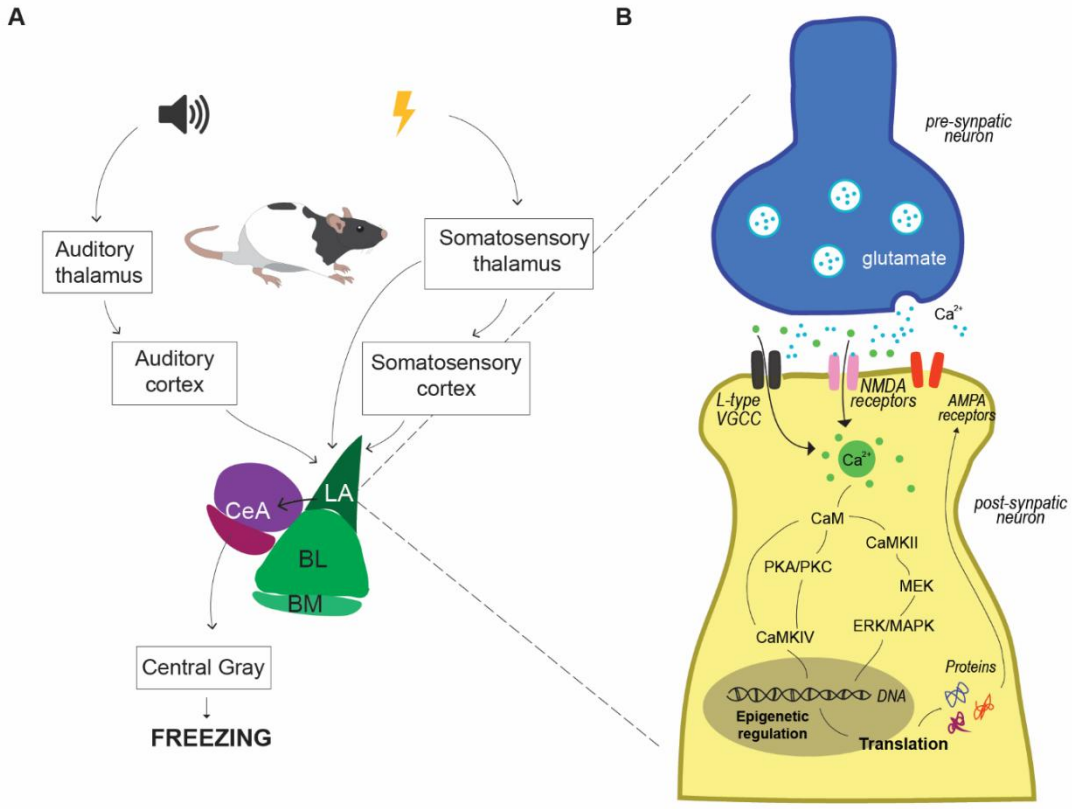


Figure 7. Fear conditioning triggers plasticity in the amygdala. *A. Auditory (CS) and shock (US) information convergence to the lateral amygdala (LA). B. NMDA receptor-dependent plasticity in the lateral amygdala.*

Current models propose that coincident CS and US activity dislodges the Mg^{2+} block in the NMDA receptor complex on LA principal neurons, allowing Ca^{2+} influx to activate CaMKII, PKA and ERK/MAPK signalling, which promotes AMPA receptor insertion and, for long-term memory, CREB-dependent transcription and protein synthesis (**Figure 7B**, Johansen et al., 2011; LeDoux, 1993; Maren, 2005). Consistent with this putative mechanism of the long-term CS-US memory, conditioning potentiates CS-evoked responses in the LA for days after training (Quirk et al., 1995; Repa et al., 2001; Rogan et al., 1997); NMDA receptor blockade in the BLA prevents acquisition but not expression of fear conditioning (Fanselow & Kim, 1994; Rodrigues et al., 2001); and experimentally inducing LTD or LTP at auditory inputs to the LA is sufficient to erase and subsequently restore fear memory (Nabavi et al., 2014). Inhibiting MEK signalling in the LA immediately after conditioning likewise prevents both long-term memory and the associated potentiation of LA responses without affecting upstream thalamic activity, demonstrating that plasticity is generated within the LA (Schafe et al., 2005; Sigurdsson et al., 2007).

By contrast, CTA relies on a distributed gustatory-visceral network including the nucleus of the solitary tract, parabrachial nucleus, gustatory thalamus, insular cortex, with each of these regions sharing reciprocal connections with the BLA (Yamamoto et al., 1994). These differences reflect the distinct sensory and motivational demands of the two paradigms: fear conditioning links exteroceptive cues with external danger, whereas CTA associates gustatory stimuli with internal physiological consequences. In spite of these differences, lesions or temporary inactivation of the BLA impair CTA acquisition, and reciprocal interactions between the BLA and insular cortex encode the affective significance of tastes (Bermúdez-Rattoni, 2004; Kayyal et al., 2019). Thus, across both fear conditioning and CTA paradigms, the BLA emerges as a common associative hub linking sensory cues with biologically significant outcomes.

1.3. Inference, or the world beyond what we experience

The Rescorla–Wagner model describes how cues that are *physically present* when an outcome occurs acquire predictive, associative strength. It does not, however, explain how cues that were only indirectly paired with some outcome also acquire predictive strength. That is, organisms routinely respond to stimuli that were never directly paired with a significant event on the basis of that stimulus' link to *other* stimuli that were paired with the significant event. The point is that behaviour is often guided by a broad web of associations among events, only some of which co-occurred with events of significance.

Everyday experience illustrates the point nicely. A child attacked by a bully at school may come to fear the shopping mall where the bully was previously encountered, though the mall

itself was never associated with harm. A hungry animal may search for food in a location where it had earlier encountered a particular smell, though the smell was never itself paired with food. In each case, an initially neutral cue (shopping mall, location of smell) comes to elicit a type of behaviour not because it predicted the outcome directly, but because it was associated with another stimulus that did. This capacity is adaptive: it allows organisms to solve novel problems, and to anticipate danger or reward in the absence of direct experience. Importantly, the elements involved are not themselves novel: the child in the example had previously visited the shopping mall, and the animal had knowledge of the location of the smell. What changes across experience is the predictive significance of these cues. Stimuli that previously signalled only neutral events now indicate the possibility of danger (attack by a bully) or reward (food). This new predictive value is not acquired through direct experience but is inferred by integrating memories acquired at different times. Contemporary work describes this process in terms of memory integration (or model-based inference, (McDannald et al., 2012; Schoenbaum et al., 2009). Rather than storing isolated stimulus-stimulus associations, organisms are thought to construct an internal model of the relationships between events (**Figure 8**), allowing previously acquired memories to be combined flexibly when making predictions about novel situations (Behrens et al., 2018; Gershman et al., 2017; Luettgau et al., 2021).

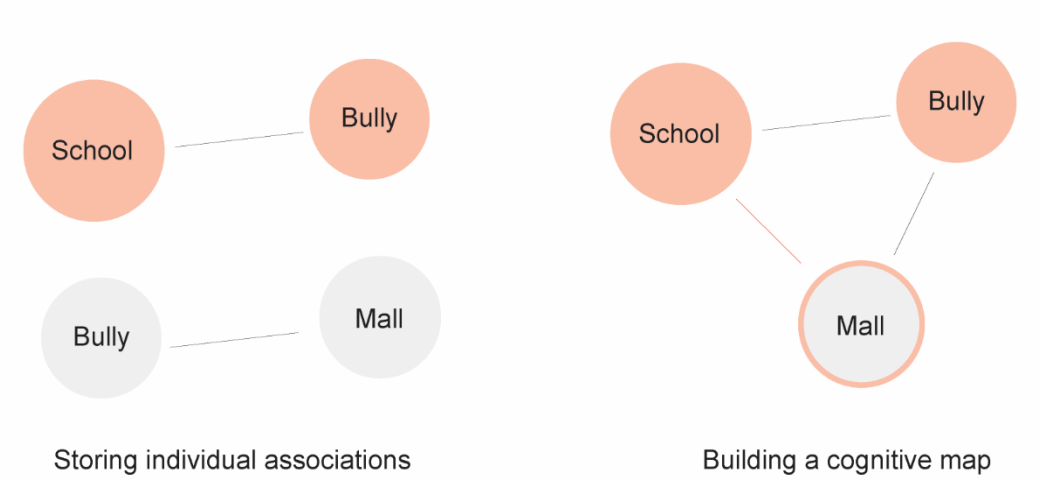


Figure 8. Example of cognitive map. *Rather than storing independently multiple associations, a cognitive map captures the relationship between events and allows inference. In the illustrated example, after having been attacked by a bully at school, a child might infer that the mall is a risky place by virtue of associations between the bully and the school, and between the bully and the mall.*

The origin of this idea traces back to Tolman's (1948) proposal that animals navigating a maze do not merely acquire a chain of reinforced movements but build a cognitive map of their environment. What such a map captures is the relational structure of the environment: the events an organism encounters, and the way one event predicts another. It is helpful to make this structure explicit. If the discrete events of the world (cues, states, and the outcomes

they lead to) are treated as representational nodes, then the learned contingencies between them are the links that connect those nodes into a network. A prediction, on this view, is not the retrieval of a single, stored quantity, but, rather, the activation of an idea (or representation) that contains information about what should happen, when it should happen and where it should happen. That is, from whatever node currently describes the animal's situation, the model reads forward along its links to recover the events that are expected to follow, together with their specific attributes – when the outcome should arrive, how likely it is, what it will look and taste like, and what it is currently worth (McDannald et al., 2012; Schoenbaum et al., 2009).

Inference implies that the organism concurrently forms a broader set of associations, including links among neutral stimuli that carry no motivational value at the time they are formed, and links between *absent* CS and present US. Learning, in other words, is not confined to what directly predicts the US. This immediately raises two questions: what, and how, is learned that allows inference?

These questions are addressed by the class of phenomena collectively known as higher-order conditioning, in which a stimulus acquires behavioural significance through its relationship with another learned stimulus rather than through direct pairing with the US. First described by Pavlov as a "reflex of the second order" (Pavlov, 1927), higher-order conditioning has since been demonstrated reliably across a wide range of species, including insects, fish, birds, rodents, non-human primates, and humans (Amiro & Bitterman, 1980; Cook & Mineka, 1987; Coureaud et al., 2013; Craddock et al., 2018; Martinez-Cervantes et al., 2022; Müller et al., 2000; Pinho et al., 2025; Tabone & de Belle, 2011; Wong & Pittig, 2022). Two experimental procedures have become the canonical models of higher-order learning: second-order conditioning, in which a novel stimulus is paired with an already conditioned stimulus, and sensory preconditioning, in which two neutral stimuli are associated before either acquires motivational significance (Brogden, 1939; Holland & Rescorla, 1975b; Rescorla & Cunningham, 1978; Rizley & Rescorla, 1972). Although both paradigms demonstrate that organisms can respond adaptively to stimuli that have never been directly paired with a biologically significant outcome, sensory preconditioning has proven particularly valuable for investigating inferential learning, because it isolates the formation and subsequent integration of associations between initially neutral stimuli and motivational ones.

1.4. The paradigm of sensory preconditioning

Sensory preconditioning allows us to study how an initially neutral stimulus can become appetitive or aversive in the absence of any direct pairing with an appetitive or aversive US. In its usual form, the procedure comprises three main stages (**Figure 9**). During an initial preconditioning (stage 1), two relatively neutral stimuli are presented in a consistent

relationship (S2→S1), allowing the animal to form an association between two cues that carry no motivational value. In a subsequent, conditioning stage (stage 2), S1 is paired with an aversive unconditioned stimulus (US), such that S1 acquires the capacity to elicit a conditioned fear response (S1→US). Critically, at test, animals express fear to S1, but also to S2, despite S2 never having been directly paired with the US. This "sensory preconditioned" fear to S2 can only arise if the animal integrates the S2→S1 and S1→US memories, making the response to S2 a readout of memory integration.

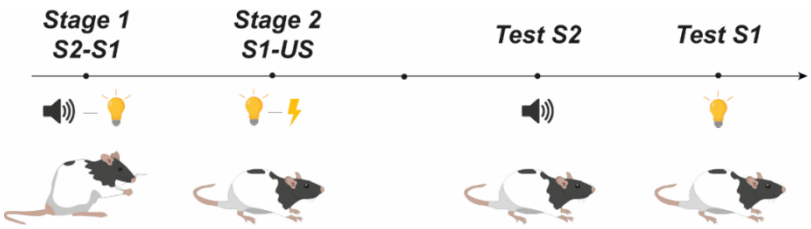


Figure 9. Sensory preconditioning protocol. *During stage 1, rats receive S2-S1 pairings. 24 hours later, during stage 2, they are presented with S1-US pairings. Tests of S2 and S1 happen respectively 48 and 72 hours later and consist of presentation of the stimulus alone.*

The theoretical value of sensory preconditioning lies precisely in the fact that responding to S2 is inferential: it requires the integration of two independently acquired associations. Unlike second-order conditioning, in which learning occurs after S1 has acquired motivational significance, sensory preconditioning pairs two initially neutral stimuli before either has been associated with the US. Consequently, any subsequent responding to S2 cannot readily be explained by a direct experienced S2-US association or by an association between S2 and an already established conditioned response. Instead, the paradigm is widely considered to provide one of the clearest demonstrations of stimulus-stimulus learning (Rizley & Rescorla, 1972), even though this learning has no observable behavioural consequence until S1 is later paired with the US. This is reminiscent of Tolman's demonstrations of latent learning, in which knowledge acquired without reinforcement remained behaviourally silent until a motivational reason to express it arose (Tolman & Honzik, 1930). In the sensory preconditioning protocol, that reason is supplied by the direct fear conditioning of S1, which makes the protocol well suited for investigating the neural mechanisms underlying the formation, storage, and subsequent integration of associative memories (Gewirtz & Davis, 2000).

Factors	Examples	Influence on learning
Stimulus type	Auditory, visual, tactile, olfactory, gustatory, appetitive, aversive…	Associability influences the strength of sensory preconditioning
Stimulus arrangement	Serial, simultaneous	Simultaneous arrangement produces stronger sensory preconditioning than serial
Stimulus similarity	Unimodal, polymodal	Spatial similarity and unimodality promotes sensory preconditioning
Stimulus order	Forward (S2→S1), backward (S1→S2)	Forward classically used, demonstration with backward exist

Factors	Examples	Influence on learning
Trial number	Conditioned aversion: 1 trial Aversive conditioning: 4 trials Appetitive: from 2 to 200	Appetitive designs require more trials; sensory preconditioning classically obtained with 8 S2-S1 pairings

Table 1. Factors influencing sensory preconditioning. Adapted from Gostolupce et al (2022).

Sensory preconditioning can be implemented with stimuli from different sensory modalities or from a single modality, and the nature of the stimuli influences the nature of the representations formed during preconditioning, how the stage 1 and stage 2 memories are combined, and which circuits are recruited for the combination (Gostolupce et al., 2022; Holmes, Wong, et al., 2022). Examples of variations in the procedure and their influence on learning are detailed in **Table 1**.The present thesis uses two complementary versions of the sensory preconditioning paradigms, specifically, an auditory-visual protocol and a gustatory protocol, to study processes of memory encoding, storage and integration in distinct sensory domains.

In the auditory-visual protocol, the two neutral cues are a tone and a light (S2 and S1), presented in a serial relationship in stage 1 (S2→S1); S1 (the light) is then paired with footshock in stage 2, and fear to the S2 at test provides the readout of integration (**Figure 9**). Freezing is used as a proxy for fear (Fanselow, 1980).

In the gustatory protocol (**Figure 10**), the two neutral cues are two tastes presented simultaneously as a single compound solution (AX), rather than as two temporally separated events. Stage 1 is therefore a case of within-event (or within-compound) learning: the association is formed between elements of one perceptual episode. This unimodal, within-event structure changes the demands on the system.

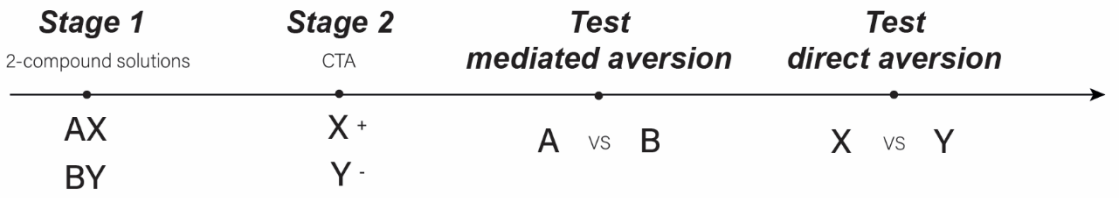


Figure 10. Gustatory preconditioning protocol. During stage 1, rats receive two-compound solutions AX and BY. During stage 2, taste X is paired with gastric malaise (typically induced by lithium chloride) to produce a conditioned taste aversion (CTA), while Y is paired with saline. At test, the mediated aversion is reflected in reduced consumption of A compared with B, and the direct aversion is reflected in reduced consumption of X compared with Y.

Taken together, the two protocols probe integration under contrasting conditions: polymodal versus unimodal, temporally contiguous versus within-event, fear versus taste aversion. As such, the two protocols provide different windows through which we can study the architecture of sensory preconditioning and its neural substrates.

2. Behavioural and neural bases of sensory preconditioning

The fear or aversion for the stimulus never directly paired with the US comes from the integration of the initial sensory memory acquired in stage 1 and the subsequent emotional memory acquired in stage 2. These memories can be acquired in at least two different ways (Holmes, Wong, et al., 2022). The first is through associative chaining at the time of testing with the S2. The second is through mediated learning about the S2 during conditioning of the S1. These two mechanisms of integration are reviewed in the next section.

2.1. How are the sensory and emotional memories integrated?

To extract new information about the relationship between the two memories acquired in sensory preconditioning, the organism must retrieve and then manipulate the encoded associations based on their content. The content of the new learning, and the relative timing of each of these processes, are usually conceptualized into two mechanisms. The first reflects the successive retrieval of two independently stored associations at the moment of test, when S2 is finally presented alone. This mechanism is also referred to as “chaining”. The second reflects integration processes that happened earlier, during conditioning, here referred to as “mediated learning” (**Figure 11**). What should be flagged is that the two mechanisms are not mutually exclusive. Both the animal learning literature (Holmes, Wong, et al., 2022) and the human decision-making literature (Shohamy & Daw, 2015) have converged on the view that the routes operate concurrently, with the balance between them set by the training protocol and its parameters.

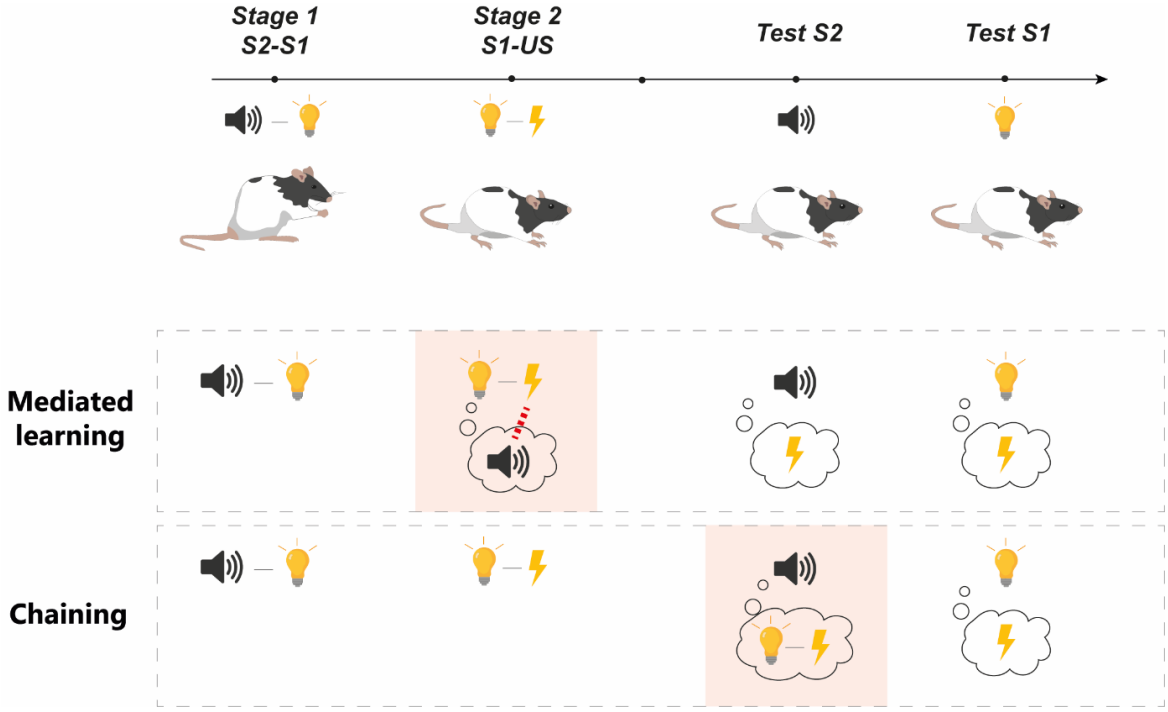


Figure 11. Mediated learning and chaining integration mechanisms in sensory preconditioning.

2.1.1. The "chaining" account: integration at test

a. Content of learning

Before studying the integration process, one should ask about the content being integrated. Unlike first-order conditioning, sensory preconditioning begins with the pairing of two neutral stimuli, before either has acquired motivational significance. The critical question is therefore what kind of representation is established between these stimuli and later retrieved to support inference. A series of experiments by Sharpe and colleagues suggests that this representation preserves the *sensory* identity of the associated stimulus rather than its value (Sharpe, Batchelor, et al., 2017; Sharpe, Chang, et al., 2017).

Their demonstration relied on conditioned reinforcement, a procedure used to determine whether a Pavlovian cue has itself acquired motivational value. In this procedure, animals are given the opportunity to perform a novel instrumental action, such as pressing a lever, to obtain presentation of a cue, but not the reward itself. If the cue itself has become desirable through learning, animals will act to obtain it. Sharpe and colleagues compared two types of cues. One was a second-order cue (**Figure 12**). That is, rats first received a cue B paired with a reward, and a non-reinforced cue D, before being presented with cue A followed by B, and cue C followed by D. The other tested cue was a preconditioned cue (**Figure 13**). That is, rats first received A followed by B and C followed by D, before receiving B paired with reward and the non-reinforced D. Thus, in both cases, cue A could evoke anticipatory responding during Pavlovian testing, but only the second-order cue had ever been paired with a stimulus carrying motivational significance during learning. In both designs, conditioned reinforcement was used to associate a response (pressing lever) with the delivery of cues A (R1→A) and C (R2→C). Probe test consisted of presentations of cues A and C to measure the time spent in the magazine. The authors found that rats readily learned to press to obtain the second-order cue (**Figure 12**C), whereas they showed little response to obtain the preconditioned cue (**Figure 13C**), despite the fact that in both cases cue A elicited appetitive responding when presented alone (**Figure 12**D and **Figure 13D**). Hence, the preconditioned cue supports adaptive responding without acquiring reinforcing properties. Rather than becoming valuable, S2 appears to retrieve the sensory representation of S1, which in turn predicts the outcome. In other words, preconditioning allows S2 to signal what is about to occur without making S2 itself motivationally significant.

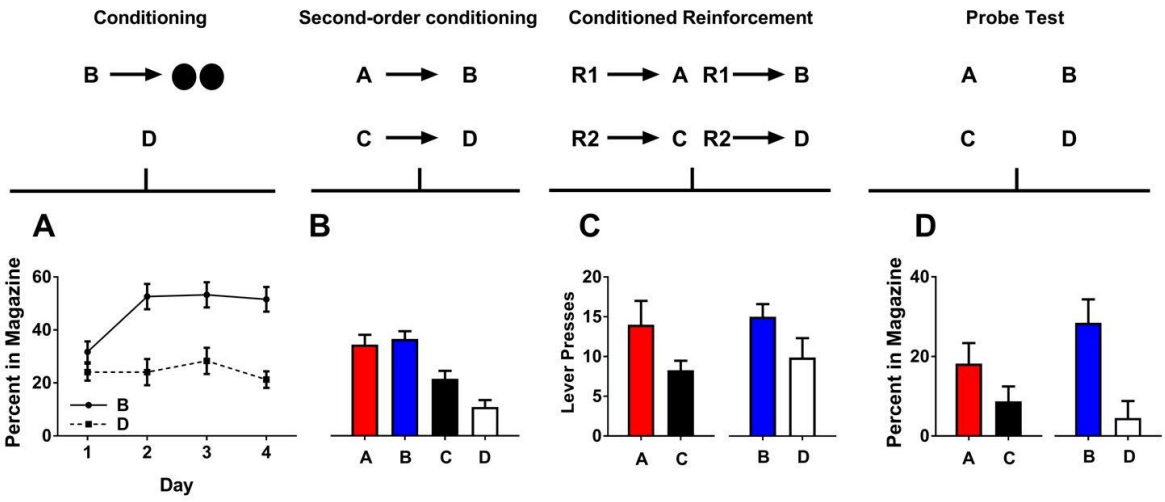


Figure 12. Second-order cue acts as a reinforcer. From Sharpe et al. (2017)

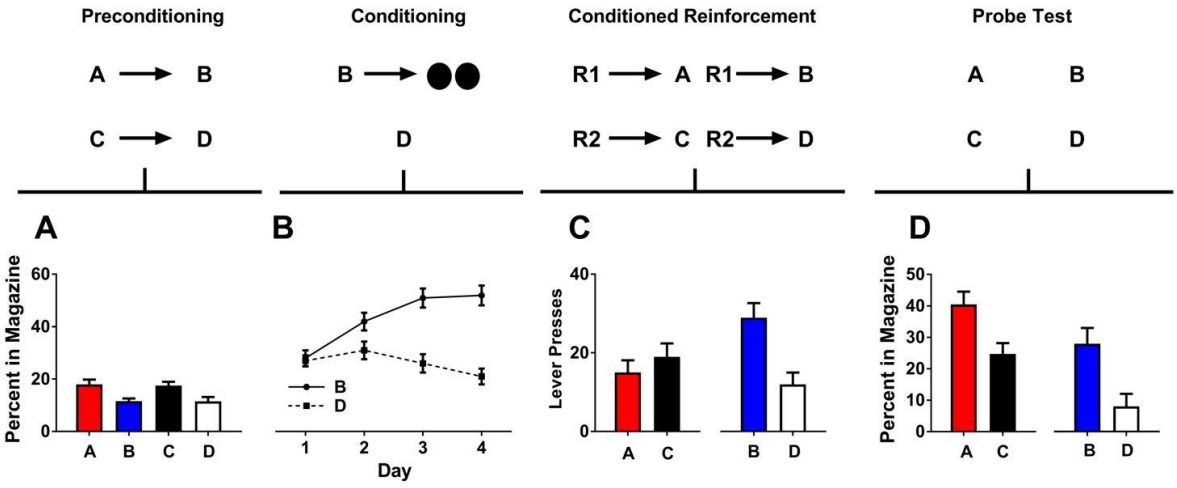


Figure 13. Sensory preconditioned cue does not act as a reinforcer. From Sharpe et al. (2017)

In a separate series of experiments, Sharpe and colleagues showed that the S2-S1 association formed during stage 1 of preconditioning is subject to blocking, which is the standard way of showing that it is, in fact, a form of predictive learning. Animals first learned that one neutral cue A predicted cue X. They were then presented with different compounds: one containing this established predictor A and a novel neutral cue (say, D), followed by X, so that animals received AD → X pairings; and another with two novel cues (say, E and F) followed by X (EF→X). In a subsequent stage, X was paired with reward, before rats were tested with D and F. As in standard Pavlovian blocking, little response was obtained with D. In other words, because D provided no additional information about the occurrence of X already fully predicted by A, it was not associated with X. This finding indicates that, during preconditioning, X was treated as a sensory-specific event whose occurrence can be predicted

by A, rather than as a source of motivational value, since neither stimulus has acquired value at that stage.

The Sharpe et al. studies suggest that what animals learn in preconditioning is a representation of the specific relationship between the innocuous cues (S2 and S1), not a transfer of motivational significance across the protocol. That is, under this account, the data do not evidence that rats learn a direct predictive link between S2 and the US. Instead, S2 simply predicts the occurrence of S1. This indicates what might be retrieved when S2 is presented during the test of sensory preconditioning, and is an argument for the first mechanism of integration: chaining of the S2-S1 and S1-US associations at test. Of note, this demonstration has been realized with appetitive conditioning. Whether the same conclusion applies for aversive conditioning has not been examined.

b. Chaining integration

"Chaining" at test holds that the two memories are stored independently throughout stages 1 and 2 of the sensory preconditioning protocol, and are brought together only when S2 is presented alone at test. On being encountered, S2 retrieves S1, S1 in turn retrieves the US, and the animal responds to S2 because it now anticipates the sequence that leads to the motivationally significant event: S2→S1→US (**Figure 11**). Holmes et al. (2022) call this memory chaining; Shohamy and Daw (2015) describe the corresponding decision-time process as prospective integration, the relevant associations are combined "just in time," as an inference drawn on the spot when a choice is faced.

The behavioural signature of this mechanism is the dependence of S2 responding on the status of S1 at test. If responding to S2 requires an intact chain that passes through S1, then breaking the S1 link should abolish it. This is what Rizley and Rescorla (1972) first demonstrated in a conditioned-suppression protocol: extinguishing S1 after stage 2 of sensory preconditioning reduced defensive responding to S2 in the subsequent test. Miller and colleagues subsequently extended the chaining account to show that what is chained is not only the identity or current status of S1 but temporal information about the US. Across a series of experiments varying the temporal relation between S1 and the US, responding to S2 tracked the expected time to the US (that is, the sum of the S2-S1 and S1-US intervals) rather than the level of conditioning to S1 as such. Strikingly, backward S1-US pairings (i.e., the US presented before S1) could support stronger preconditioning of S2 than forward pairings, an outcome that no purely value-based chain can accommodate (Arcediano et al., 2003; Barnet et al., 1997; Matzel et al., 1988; Polack et al., 2013). Although the neural substrates will be developed later in this chapter, it is worth noting that signatures of chaining integration can also be found at the neural level. Indeed, the temporal fingerprint of sequential retrieval has

been recorded directly: Sadacca et al. (2018) found that orbitofrontal activity evoked by a preconditioned S2 peaked after S2 had terminated, at the moment the food would have arrived. This is consistent with S2 activating a representation of S1 which in turn activated the food representation, rather than with S2 carrying its own precomputed value. Complementary evidence implicates the orbitofrontal cortex in this chaining more generally (Jones et al., 2012; Wang, Howard, et al., 2020). A corollary of associative chaining is that reaction times at test would be longer compared to integration via mediated learning, because it would involve retrieving and linking multiple associations rather than a single one (see Nicholas et al., 2025 for a comparison of reaction times in the different integration mechanisms in humans).

2.1.2. The "mediated learning": integration during conditioning

The two memories acquired in sensory preconditioning can be combined before the test is ever given. This possibility was referred to as (representational-)mediated learning by Ward-Robinson and Hall (1996) and Holland (1981). Specifically, mediated learning happens during stage 2 while S1 is being paired with the US. When S1 is then presented with the US, it reactivates a representation of the now-absent S2, and this reactivated representation is available to associate with the US. As a result, conditioning establishes an S2–US association even though S2 was never actually paired with the US – a "false" association in the sense that it reflects inferred rather than experienced contingencies (Bae et al., 2015; Lingawi et al., 2018). Holmes et al. (2022) refer to this as mediated conditioning; Shohamy and Daw (2015), approaching the same phenomenon from decision neuroscience, describe it as retrospective integration, because motivational significance is attributed to the stimulus of interest before any decision is faced.

Importantly, Holland's own work revealed some of the conditions under which mediated learning does and does not occur. In studies of representation-mediated taste aversion, in which a cue previously associated with food was later paired with illness, the retrieved food representation acquired aversive properties only when the initial cue–food association had received relatively little training (approximately 16–20 pairings). After more extensive training (around 40 pairings), the same procedure no longer produced representation-mediated learning (Holland, 1981, 1998). This finding suggests that a cue's ability to retrieve and update the representation of its associate diminishes as the association becomes highly familiar or overtrained. Familiarity therefore appears to determine whether this mechanism of integration is prioritised, and has important implications for sensory preconditioning. As the number of S2–S1 pairings increases during the preconditioning stage, the association between the two cues may become less amenable to representation-mediated updating. At the same time, repeated experience changes *what* is learned about a cue. For example, pre-exposure to a cue retards conditioning, but enhance timing (Bonardi et al., 2016). Moreover, repeated exposures allow

animals to learn not only that the stimuli are associated but also the directionality of that association - that S2 predicts S1 rather than simply co-occurring with it. Under these conditions, integration may increasingly rely on an alternative mechanism based on associative chaining, in which presentation of S2 successively retrieves the representation of S1 and then that of the US (Holmes, Wong, et al., 2022).

In contrast to associative chaining, the behavioural signature of mediated learning is its independence of S2 responding to changes in the status or value of S1. If the critical S2–US link was encoded during stage 2, then subsequent manipulation involving S1 should leave responding to S2 intact, because that responding no longer depends on S1. Several lines of evidence show that this is often the case. Coureaud and colleagues (2013) disrupted the reconsolidation of a directly conditioned stimulus memory (A-US) in newborn rabbits by administering the protein-synthesis inhibitor anisomycin. At test, conditioned responding to the A itself was reduced, yet responding to the preconditioned stimulus B was intact (**Figure 14**). This implies that the aversion for B did not rely on an intact A-US memory. Instead, it must rely on predictive strength that B had acquired across the training in stage 2.

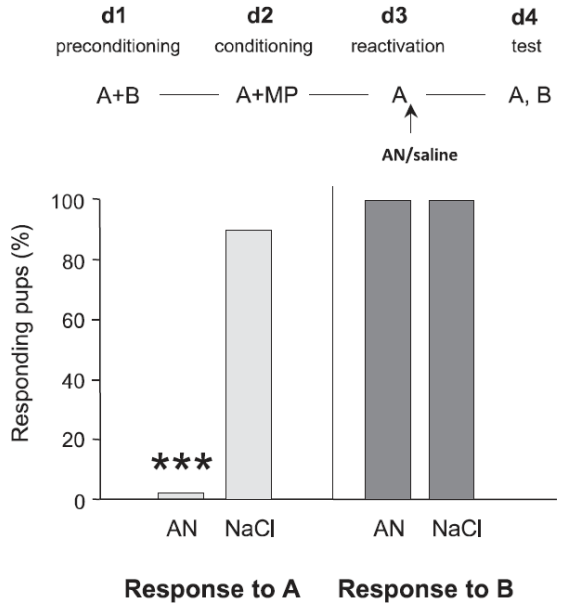


Figure 14. Disruption of consolidation of the directly conditioned stimulus A leaves aversion to the preconditioned stimulus B intact. *From Coureaud et al (2013)*

Another source of evidence for mediated learning comes from studies that have interfered with neural activity during stage 2 of sensory preconditioning. Specifically, in auditory-visual sensory preconditioning, the perirhinal cortex encodes the S2-S1 association that forms in stage 1. Importantly, temporary inactivation of this region across the session of S1–US pairings (Wong et al., 2019) spares the directly conditioned S1–US memory but abolishes subsequent responding to S2. Since the first-order memory is left intact, the

disruption cannot be attributed to a failure of retrieval at test. Instead, the integrative event must have occurred during conditioning, which is to say that the integration must have taken the form of a mediated S2-US association in stage 2.

Interestingly, work in gustatory preconditioning protocols show that integration during the conditioning stage may not proceed exclusively via the representation-mediated learning described above. For example, in a study by (Lin et al., 2017), rats were exposed to two flavour compound solutions AX and BY in stage 1, followed by X-US and Y-US pairings in stage 2. During the subsequent tests, rats displayed greater aversion to the trained compounds AX and BY than to the new configurations BX and AY, suggesting that conditioning accrued to the *whole* configural AX and BY memories rather than to just A and B alone.

Importantly, this variation of mediated conditioning is possible when the learning in stage 1 produces a configural (S2S1) rather than a directional (S2 signals the S1) representation, such as when S2 and S1 are presented simultaneously in stage 1 (Rescorla & Cunningham, 1978; Rescorla & Durlach, 1981). Rescorla and Durlach argued that these simultaneous conditioning protocols produce "within-event" rather than "between-event" associations. Gustatory preconditioning classically falls in this category. In stage 1, the two stimuli A and X are bound into a single, unitary AX representation, which is retrieved via pattern-completion when X is presented in stage 2. The US then changes the value of that whole representation rather than the value of just A alone (Honey et al., 2014; Iordanova et al., 2008; Lin et al., 2016). Hence, when rats are subsequently tested with the A alone, the whole AX representation is activated via pattern completion, resulting in maximal expression of conditioned taste avoidance.

2.2. Computational framework

The accumulated evidence suggests that the mechanism of sensory preconditioning [i.e., whether it is due to associative chaining or mediated learning] depends on the conditions under which learning takes place. Factors such as whether S2 and S1 are presented simultaneously or serially, the amount of training, and the familiarity of the stimuli and testing context can all determine whether responding to a sensory preconditioned stimulus is supported by chaining or mediated learning (Holmes, Wong, et al., 2022). However, it is important to note that associative chaining and mediated learning are not two competing explanations of sensory preconditioning. Instead, they are complementary processes whose relative contribution to sensory preconditioned responding depends on the specific features of the experimental protocol. The question, then, is which mechanism should predominate under which set of experimental conditions.

This question can be addressed with reference to associative learning models, even though they were not originally proposed to explain sensory preconditioning. By applying these models to sensory preconditioning, we can derive predictions about the experimental conditions under which integration should occur and the form that it should take. These models differ from the Rescorla-Wagner model which cannot, on its own, explain why a stimulus that was never paired with the US comes to elicit a conditioned response. Indeed, its learning rule updates associative strength only for stimuli that are physically present on a trial, which is not the case for S2 in stage 2 of sensory preconditioning. What is needed is a mechanism by which an *absent* stimulus can participate in learning. Holland's (1983) extension of Wagner's "Sometimes Opponent Processes" [SOP] theory supplies this mechanism, and does so in a way that speaks directly to the question of how the sensory and emotional memories are integrated.

2.2.1. Wagner's SOP and its extension

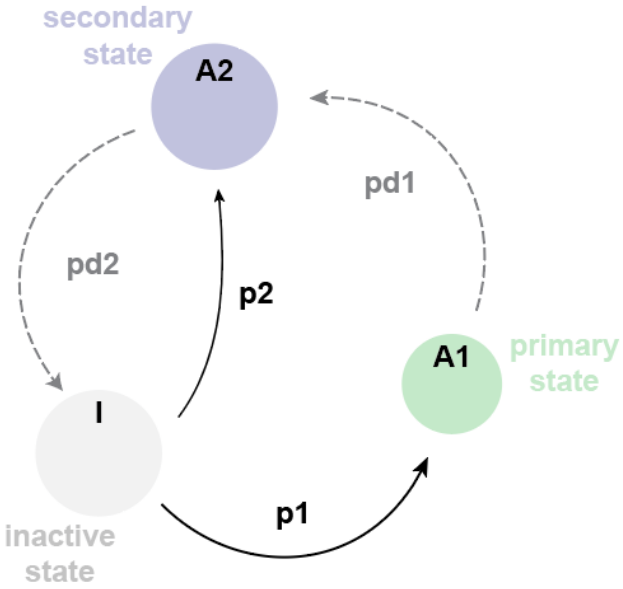


Figure 15. SOP model. *Elements of stimulus representations can occupy and move from one of three activation states to another: a highly active primary state (A1), a less active secondary state (A2), and an inactive state (I). Presentation of a stimulus activates a proportion of its elements, which transition from the inactive state (I) to A1. As activation decays, these elements move from A1 to A2 at different proportions before eventually returning to the inactive state. Importantly, this progression is unidirectional: elements cannot move directly from A2 back into A1. The quantities p and pd respectively represent the proportion of elements primed and decaying into a state.*

"Sometimes Opponent Process" is an automatic memory-processing model of Pavlovian learning that explains behaviour in terms of the moment-to-moment activation of stimulus representations in memory (Wagner, 1981). Its central assumption is that each stimulus is represented by a node composed of many elements, and that these elements can occupy one of three activation states (**Figure 15**): a highly active primary state (A1), a less active secondary state (A2), and an inactive state (I). The capacity of A1 is limited, so that only

a small number of representations can occupy this focal state simultaneously, whereas A2 can accommodate many more partially active representations. As a result, A1 and A2 can be seen as corresponding to the focus and periphery of attention, respectively. The model assumes that stimulus representations move through these states in a fixed sequence (Figure 15). It is useful to state these dynamics in the model's quantities, because the success of sensory preconditioning depends on them. Let pA1(x) and pA2(x) denote the proportion of a node's elements currently in A1 and A2. Physical presentation of a stimulus drives a fixed proportion *p1* of its elements from I into A1; decay then moves elements from A1 to A2 at rate *pd1*, and from A2 back to I at rate *pd2*. Associative activation is the crucial term for sensory preconditioning: a physically present stimulus X drives its associate Y into A2 (never into A1) by an amount proportional to V(x→y)·pA1(x), where V is the associative strength linking the two nodes.

Given this architecture, Wagner went on to specify that the association that forms between two stimulus representations depends on the degree and pattern of their overlapping elements in memory. An excitatory link forms between two nodes when their elements overlap in A1 (both stimuli concurrently in focal activity); an inhibitory link forms when one node's elements are in A1 while the other's are in A2; and there is no associative product when elements are jointly in A2 or in I (this rule will be modified under Dickinson and Burke's revision, 1996).

2.2.2. Mediated learning viewed via the SOP prism

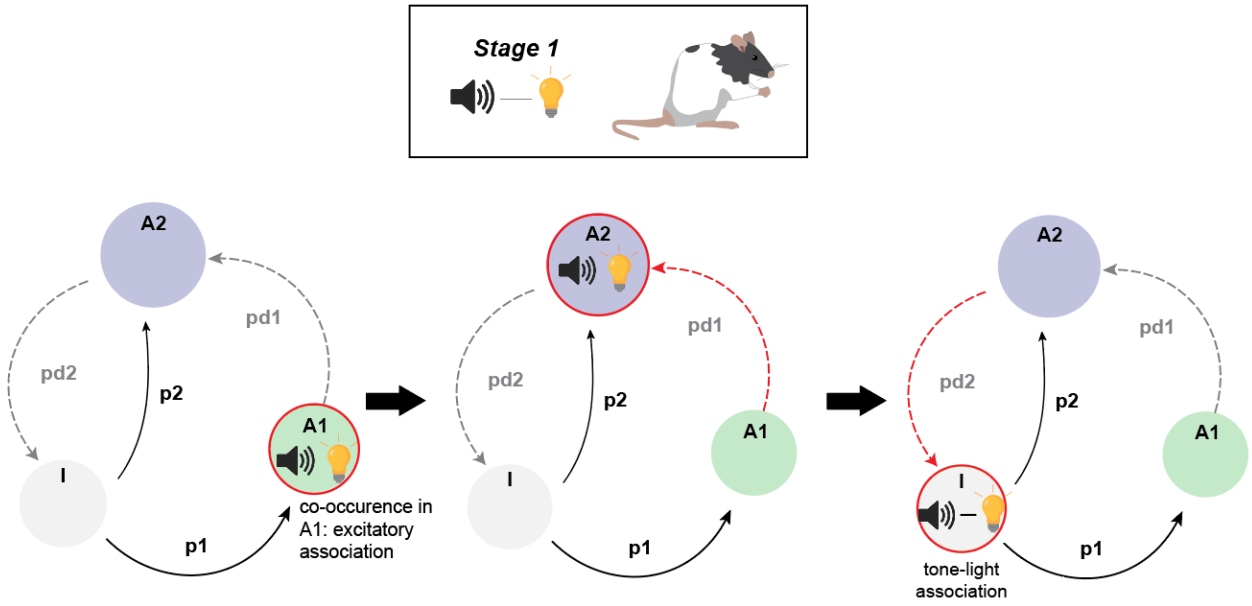


Figure 16. Repartition of stimuli in stage 1 of sensory preconditioning.
***Left**: Tone and light representations are first processed in A1, where co-occurrence leads to excitatory association between the stimuli. **Middle**: A proportion pd1 of stimuli elements of the representations then decay in A2 before going into the inactive state (**Right**).*

In Wagner's original formulation, there can be no excitatory learning between two nodes unless elements of each are in A1. Hence, SOP explains sensory preconditioning in stage 1 as elements of the S2 and S1 co-occur in the A1 state, which builds an excitatory S2-S1 association before the elements decay to A2 and then to I (**Figure 16**).

However, the model fails to explain the dynamics of mediated learning in stage 2 when the retrieved S2 is activated in A2 while the US is processed in A1. Following the original rules, this pattern of activation and processing should result in an inhibitory, not excitatory, association between S2 and the US. Yet mediated learning, or representation-mediated conditioning, had already been demonstrated in the few years after the inception of Wagner's SOP (Holland, 1981, 1983). Recognizing this dilemma, Holland (1983) modified the learning rules in Wagner's (1981) SOP model to explicitly allow for mediated learning (**Figure 17**).

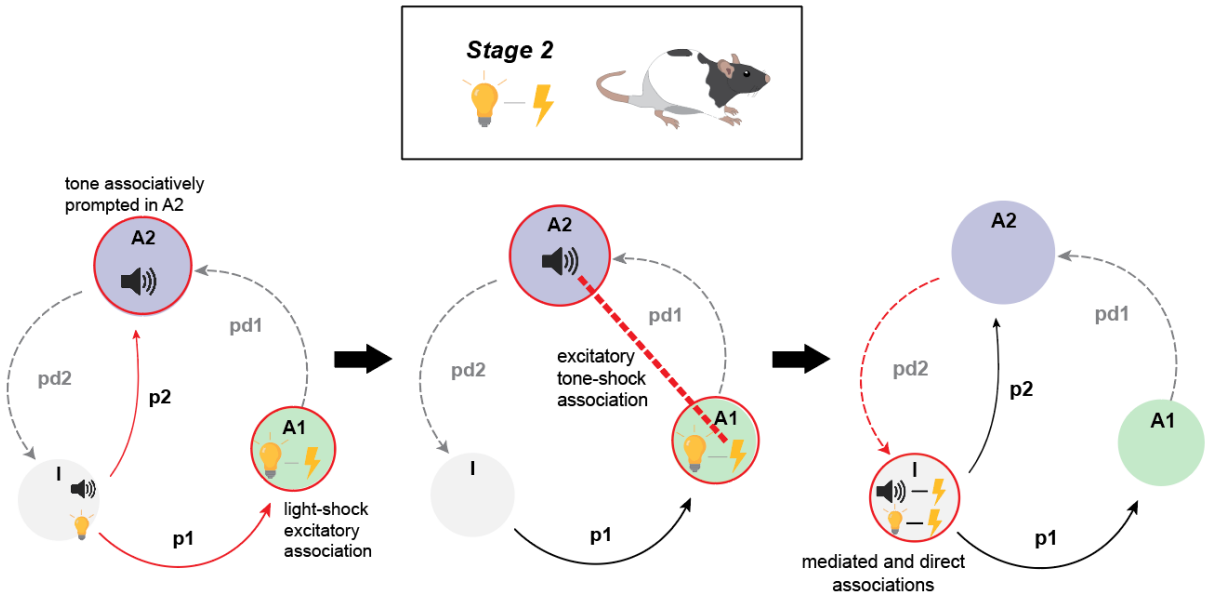


Figure 17. Repartition of stimuli during stage 2 of sensory preconditioning allowing mediated learning. ***Left:*** *The presence of the light in A1 allows representation of the tone to be primed into A2.* ***Middle****: Under Holland's modification of the SOP model, the product of mediated learning is explained by an excitatory link between elements in A2, such as the tone, and elements in A1, such as the shock, leading to the formation of a tone-shock memory. Essentially, Holland's modification allowed an excitatory increment* $\Delta V(S2 \rightarrow US) \propto pA2(S2) \cdot pA1(US)$. ***Right****: Eventually, all stimuli and associative products decay into I.*

In this modified model (mSOP), during the S2-S1 pairings of stage 1, the excitatory S2-S1 link forms: $V(S1 \rightarrow S2)$ and $V(S2 \rightarrow S1)$. During the S1-US pairings of stage 2, the presented S1 (in A1) both associates directly with the US and, through the stage-1 association, drives a proportion of the absent S2 elements into A2. With the US in A1 and S2 in A2, an excitatory association forms between S2 and the US: $\Delta V(S2 \rightarrow US) \propto pA2(S2) \cdot pA1(US)$.

Since the US is physically present in stage 2, pA1(US) is fixed by the reinforcement schedule; the amount of mediated learning therefore reduces to how many elements of S2 are in A2

while the US is active (that is, to the quantity pA2(S2)). This quantity is itself set by the associative-priming term, namely how strongly the current S1 can elicit S2 via its previously acquired association: $pA2(S2) \propto V(S1 \rightarrow S2) \cdot pA1(S1)$.

The mSOP model grounds one of the boundary conditions for sensory preconditioning that Holland himself identified. The strength with which S1 can evoke S2 depends on how well-established the S2-S1 link is and how much of S2 remains available to be primed. In other words, the amount of stage 1 training determines the value of $V(S1 \rightarrow S2)$ and, thereby, how much of S2 is activated to A2 during conditioning of the S1. Empirically, in serial S2→S1 presentations, mediated learning is favoured at moderate levels of stage 1 training and declines with overtraining (because $V(S1 \rightarrow S2)$ decreases against $V(S2 \rightarrow S1)$), consistent with Holland's finding that mediated aversions appeared after sixteen to twenty pairings of the relevant stimuli in stage 1 but were absent after around forty such pairings.

2.2.3. Chaining viewed via SOP prism

The same A2 dynamics can also account for memory integration occurring at retrieval (via associative chaining) rather than during encoding (via mediated learning). Under this account, stage 2 establishes only the direct S1–US association, leaving the original S2–S1 association unchanged. The two memories therefore remain stored independently. At test, presentation of S2 (processed in A1) retrieves the representation of S1 through the stage-1 association. Although this retrieved representation occupies the weaker A2 state, it is nevertheless sufficient to activate the associated US representation in A2, allowing the animal to infer that S2 predicts the outcome through the sequence S2→S1→US (**Figure 18**). Responding to S2 therefore does not rely on a stored S2–US association but, instead, occurs through the reconstruction of the associative chain each time S2 is encountered.

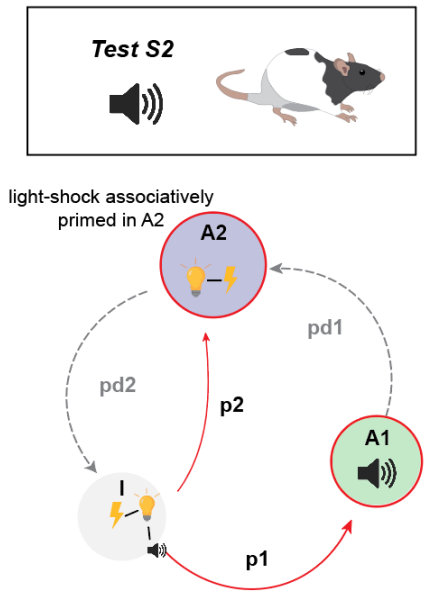


Figure 18. Repartition of stimuli representations during testing of S2 with chaining integration.
The light is associatively primed in A2 via presentation of the tone processed in A1. The light itself activates the shock representation in A2, leading to freezing via a chaining integration.

SOP accommodates this retrieval route because A2 representations, although much less effective than A1 representations at driving associative activation (Wagner estimated their influence to be roughly one hundredth that of A1), are not functionally silent. The model explicitly allows associatively activated A2 elements to retrieve their own associates, a feature Wagner introduced specifically to account for sensory preconditioning. This chaining account therefore predicts that because responding to S2 depends on traversing the S2→S1→US chain at test, manipulations that weaken or degrade the S1 representation after conditioning should also reduce or abolish responding to S2.

2.2.4. Bases and limits

Although SOP was not developed to explain sensory preconditioning, it provides a remarkably coherent framing for the current empirical literature showing how subtle procedural variations determine whether behaviour reflects online integration or retrieval-based inference (Wong et al., 2019, 2025, 2026). SOP's principal strength lies in its economy. A single set of state-transition and learning rules in a single memory architecture is sufficient to generate both integration mechanisms and to understand the conditions under which one or the other predominates. Because the distinction between A1 and A2 naturally captures the difference between focal and peripheral processing, SOP also provides a conceptual bridge between the behavioural learning literature and the attentional framework used to interpret the functionally distinct and dissociated neural substrates of sensory preconditioning (Wong et al., 2026).

Despite these strengths, two issues concerning sensory preconditioning motivate the development of the next model. According to the encoding interpretation of SOP, mediated learning during stage 2 establishes a direct S2–US association that is stored independently of S1. Once this association has formed, responding to S2 should survive later extinction of S1, a pattern seen in second-order conditioning (Rizley & Rescorla, 1972) and in some but not all sensory-preconditioning protocols. Yet the same appeal to associatively-activated representations means that, because presenting S1 alone reactivates S2 into A2, the S2–US association becomes eligible for mediated extinction (Holland & Forbes, 1982; Ward-Robinson & Hall, 1996). In general, or empirically, the impact of S1 extinction on performance to S2 depends on whether S1-alone presentations reactivate S2 together with the US (or not), and which revision of the A2-learning rule is assumed. The base model does not adjudicate between them.

The second issue is an empirical failure. Introducing a trace interval between S1 and the US weakens conditioning to S1 itself. Under both the mediated and chaining interpretations of SOP, responding to S2 ultimately depends on the strength of the S1–US association. The model therefore predicts that trace conditioning should reduce sensory-preconditioned

responding to S2. Experimentally, however, the opposite is observed: inserting a trace interval enhances responding to S2 (Lin & Honey, 2011; Ward-Robinson & Hall, 1998). Within SOP's element-based architecture, in which activation spreads through A1 and A2 states, a weaker terminal association can only reduce the strength transmitted through the chain, never increase it. This finding therefore falls outside the explanatory scope of the model.

The limitations of SOP motivated a series of refinements and extensions, such as Pearce's configural model (Pearce, 1994) and the Honey–Dwyer model (Honey et al., 2020) that sought to account for phenomena lying beyond the scope of the original framework. Collectively, these frameworks converge on the idea that the mechanisms supporting sensory preconditioning are complex and determined by environmental factors. The neural substrates underlying these mechanisms are also complex and have the same environmental dependency.

2.3. Neural substrates of SPC

The neural circuitry supporting sensory preconditioning is not fixed but depends on the nature of the information that must be integrated. The sensory modality of the stimuli, as well as their temporal arrangement during learning, influence which brain regions are recruited to support memory encoding and integration. For example, higher-order processes involving audiovisual cues do not necessarily rely on the same neural substrates as learning involving olfactory or gustatory stimuli.

Across paradigms, sensory preconditioning is supported by a distributed network that includes several cortical and limbic regions, notably the perirhinal cortex (Holmes et al., 2013; Nicholson & Freeman Jr, 2000; Wong et al., 2025), orbitofrontal cortex (Hart et al., 2020), retrosplenial cortex (Fournier et al., 2020), and dorsomedial prefrontal cortex (Gu & Johansen, 2025), together with hippocampal subregions discussed below. However, the contribution of each structure has been evidenced in different ways. The following sections summarize the current evidence for the neural substrates of sensory preconditioning in rodents, organised according to stimulus modality and experimental design. Unless otherwise stated, the evidence derives primarily from stage-specific temporary inactivation studies, which have been instrumental in identifying the distinct contributions of individual brain regions to sensory learning, emotional learning, and memory integration.

2.3.1. Stage 1

Protocol/Cue features	Brain regions	Citations
Auditory-visual, serial, neutral context	PRh, RSP, dmPFC	Gu & Johansen, 2025; Holmes et al., 2013; Nicholson & Freeman Jr, 2000; Robinson et al., 2011, 2012, 2014; Talk et al., 2002 (all auditory)
Auditory-visual, serial, dangerous context	BLA	Holmes et al., 2013, 2018; Kikas et al., 2021
Auditory-visual, serial, appetitive context	OFC, VTA, NAc	Hart et al., 2020; Sadacca et al., 2018; Sharpe, Chang, et al., 2017
Auditory-visual, simultaneous compound	DH (CB1 receptors on GABAergic interneurons)	Busquets-Garcia et al., 2018; Pinho et al., 2025
Context-stimulus	PRh	Iordanova et al., 2009
Context-stimulus-time	DH	Iordanova et al., 2011
Odour-taste (within-event)	DH (CB1 receptors)	Busquets-Garcia et al., 2018; Wheeler et al., 2013

Table 2. Brain regions necessary in stage 1 of sensory preconditioning. *PRh: perirhinal cortex; RSP: retrosplenial cortex; dmPFC: dorsomedial prefrontal cortex; BLA: basolateral amygdala; OFC: orbitofrontal cortex; VTA: ventral tegmental area; NAc: nucleus accumbens; DH: dorsal hippocampus; CB1: cannabinoid type 1.*

Most of our understanding of the neural substrates supporting learning of the S2-S1 association in stage 1 comes from sensory preconditioning paradigms using auditory and visual cues presented either serially or simultaneously. Across these protocols, a consistent picture has emerged: the formation of stimulus-stimulus associations relies primarily on cortical regions implicated in relational memory (**Table 2**). This is particularly true of the perirhinal cortex, whose established role in object recognition and representation of stimuli (Kealy & Commins, 2011; Lim & Lee, 2024) makes it well suited to encode relationships between initially neutral events. Accordingly, disrupting activity in the perirhinal or retrosplenial cortex during S2–S1 pairings prevents later responding to S2 while leaving first-order conditioning to S1 intact. This behavioural dissociation indicates that these cortical regions are specifically required to establish and store the neutral association acquired during preconditioning, rather than for subsequent emotional learning or behavioural expression.

Within the perirhinal cortex, several molecular mechanisms are involved to encode the S2-S1 association. Briefly, formation of the S2–S1 association requires NMDA receptor activation (Holmes et al., 2013), whereas its consolidation depends on ERK/MAPK signalling and de

novo protein synthesis in the Prh (Qureshi et al., 2023). More recently, dopaminergic signalling has also been implicated in this stage of learning. Blocking D1 or D2/D3 receptors before preconditioning selectively abolishes later responding to S2 without affecting conditioning to S1 (Roughley et al., 2021), consistent with the emerging view that dopamine acts as a teaching signal not only for reward and punishment but also for learning predictive relationships between motivationally neutral events (Seitz et al., 2021).

The neural substrates of stage 1 are not, however, invariant across sensory modalities. When preconditioning involves gustatory and olfactory stimuli, the dorsal hippocampus rather than the perirhinal cortex becomes critical. Using compound odour–taste pairings, Busquets-Garcia et al. (2018) showed that mice later avoided an odour associated with a taste that had subsequently been paired with lithium chloride, demonstrating successful formation of an odour–taste association during stage 1. This learning was abolished by systemic or intra-hippocampal blockade of CB1 receptors, by selective deletion of hippocampal CB1 receptors, or by silencing hippocampal GABAergic neurons expressing these receptors. Importantly, these manipulations spared first-order taste-aversion learning and were effective only when administered during preconditioning, indicating that endocannabinoid signalling within the dorsal hippocampus is specifically required for encoding, rather than retrieving or expressing, the neutral association.

Although lesion and pharmacological studies have identified the brain regions necessary for stage-1 learning, more recent work has begun to reveal how these associations are represented at the level of neuronal populations. Recording from the dorsomedial prefrontal cortex during an aversive sensory preconditioning task, Gu and Johansen (2025) found that pairing auditory and visual cues progressively recruited a population of multisensory co-responsive neurons that came to respond to both stimuli. These neurons emerged only after paired, but not unpaired, cue presentations, and remained stable throughout subsequent conditioning, consistent with a multi-step tag-and-capture process where the co-responsive neurons are initially “tagged” before being recruited during aversive learning (“capture”). While the broader dmPFC population encoded multiple task features, neurons projecting to the amygdala selectively represented the inferred cue–danger relationship and were specifically destabilised when the inferred association was extinguished. Together, these findings suggest that the neutral association established during stage 1 may ultimately be represented by distributed neuronal ensembles linking the two sensory cues, providing a cellular counterpart to the regional and molecular mechanisms identified by earlier studies.

2.3.2. Stage 2

Protocol/Cue features	Brain regions	Citations
Audio-visual serial to shock	PRh (retrieval of the stored S2; consolidation of the mediated link), BLA (activity, but no plasticity), dmPFC (whole population; inferred association only)	Gu & Johansen, 2025; Holmes et al., 2018; Wong et al., 2019
Context to shock (immediate-shock protocol)	DH (retrieves the pre-exposed context), BLA (associates it with shock)	Huff & Rudy, 2004; Lingawi et al., 2018; Matus-Amat et al., 2004
Context-stimulus-time to shock	DH	Iordanova et al., 2011
Odour-mediated taste aversion (O to LiCl)	DH	Wheeler et al., 2013

Table 3. Brain regions necessary in stage 2 of sensory preconditioning. *PRh: perirhinal cortex; dmPFC: dorsomedial prefrontal cortex; BLA: basolateral amygdala; DH: dorsal hippocampus.*

Unlike stage 1, stage 2 is where emotional significance is introduced, and is the critical stage where mediated learning happens. In the auditory-visual protocol, studies using temporary inactivation show that this integration depends critically on the perirhinal cortex (**Table 2**). Silencing the perirhinal cortex during S1–shock conditioning selectively abolishes subsequent responding to S2 while leaving conditioning to S1 intact (Wong et al., 2019). The same dissociation is observed when protein synthesis is blocked immediately after conditioning or when the perirhinal cortex is inactivated at test, indicating that this region contributes not only to encoding the mediated association but also to its consolidation and subsequent retrieval.

At the molecular level, mediated learning requires NMDA receptor activation within the perirhinal cortex: blocking these receptors during S1–US conditioning selectively prevents later responding to S2 while sparing conditioning to S1. However, this mechanism is engaged only under specific circumstances. One determining factor is the amount of preconditioning. After relatively few S2–S1 pairings, when behavioural evidence indicates that integration occurs online during stage 2, both perirhinal NMDA receptors and communication between the perirhinal cortex and basolateral amygdala are required. After more extensive preconditioning, however, altering these processes in stage 2 no longer impairs responding to S2, consistent with the idea that the task is instead solved through retrieval-based chaining at test (Wong et al., 2025). A second determinant is stimulus familiarity. When S2 is already familiar at the time of conditioning, mediated learning again depends on NMDA receptor activation within the perirhinal cortex. In contrast, when S2 is novel and first encountered within the S2–S1–US

sequence, conditioning instead relies on NMDA receptor activation in the basolateral amygdala (Wong et al., 2026). These findings closely parallel the behavioural models discussed above, suggesting that the neural substrates recruited during stage 2 depend on which state of attention the stimuli are in.

Recent electrophysiological studies have begun to reveal how these integrated memories are represented once established. Recording single neurons in the dorsomedial prefrontal cortex throughout sensory preconditioning, Gu and Johansen (2025) found that the neuronal ensembles linking the preconditioned and directly conditioned cues persisted beyond learning and could be modified independently. Extinguishing the inferred cue selectively weakened its representation and destabilised the multisensory co-responsive ensemble linking the two cues. In contrast, extinguishing the directly conditioned cue left the inferred representation largely intact. Together, these results suggest that once memory integration has occurred, the inferred association becomes an independent representation that can subsequently be updated without requiring the original S1–US association from which it arose.

2.3.3. Test

Protocol/Cue features	Brain regions	Citations
Audio-visual serial (aversive)	PRh, dmPFC-to-BLA projection neurons	Gu & Johansen, 2025; Wong et al., 2019
Context (aversive)	DH, BLA	Iordanova et al., 2009; Matus-Amat et al., 2007
Context-stimulus-time	DH	Iordanova et al., 2011
Audio-visual serial (appetitive)	OFC	Jones et al., 2012; Sadacca et al., 2018; Wang, Schoenbaum, et al., 2020

Table 4. Brain regions necessary during testing of sensory preconditioning. *PRh: perirhinal cortex; dmPFC: dorsomedial prefrontal cortex; BLA: basolateral amygdala; DH: dorsal hippocampus; OFC: orbitofrontal cortex.*

The retrieval of inferred fear engages a network that is functionally distinct from the one supporting direct fear conditioning (**Table 4**). At the regional level, the basolateral amygdala and the perirhinal cortex make complementary contributions. Activity in the basolateral amygdala is required for the expression of conditioned fear regardless of how that fear was acquired. Accordingly, inactivating the BLA with muscimol before test abolishes freezing to both the directly conditioned S1 and the inferred S2. This lack of selectivity is consistent with the idea that the amygdala represents the aversive significance of the current cue, independently of the learning history through which that value was acquired. The perirhinal

cortex, by contrast, is selectively required for responding to the inferred stimulus. Its inactivation leaves freezing to S1 intact but abolishes responding to S2, indicating that the integrated S2-US memory is retrieved by the same region which encoded the S2-S1 memory in stage 1 and integrated it with the S1-US memory in stage 2.

Circuit-level studies have begun to identify how this retrieved information reaches the amygdala. Rather than depending on a single brain region, expression of fear to S2 appears to rely on interactions between cortical areas that recover the neutral association and subcortical structures that generate defensive behaviour. In particular, optogenetic silencing of dorsomedial prefrontal cortex projections to the basolateral amygdala selectively disrupts freezing to S2 while sparing responding to S1, identifying the dmPFC→BLA pathway as a key route through which inferred value gains access to the amygdala (Gu & Johansen, 2025). This selectivity closely parallels that observed following perirhinal inactivation but localises it to a specific projection rather than an entire region. Together, these findings support the view that fear to a stimulus never paired with danger depends on a distributed circuit in which the cortices maintain the integrated memory, and projections to the amygdala allow the expression of defensive behaviour.

2.3.4. General remarks

Sensory preconditioning is supported by a distributed network of brain regions, with each of these regions making a different and distinct contribution to its underlying representations and computations. Cortical regions (including the perirhinal, retrosplenial, orbitofrontal, anterior cingulate, and dorsomedial prefrontal cortices) and the hippocampus encode, maintain, and retrieve representations of sensory events for their subsequent integration. These regions are extensively interconnected, receive input from higher-order sensory association cortices, and converge onto the hippocampal formation through the entorhinal cortex, forming part of the cortico-hippocampal relational memory system described by Ranganath & Ritchey (2012) and Ritchey et al. (2015). Downstream, structures such as the basolateral amygdala retrieve the representation of the aversive stimuli, guiding defensive behaviour.

This functional organisation lends itself to analysis in terms of Wagner's (1981) SOP model. Cortical and hippocampal regions appear to support the retrieval and maintenance of absent stimulus representations, which resembles the function of Wagner’s peripheral A2 state, whereas structures such as the amygdala process stimuli that have become behaviourally relevant and translate them into appropriate responses, resembling the function of Wagner’s focal A1 state. Although the correspondence is not exact, it provides a useful conceptual framework linking computational theories of associative learning to their neural implementation.

Within this framework, the hippocampus occupies a particularly strategic position. Its extensive reciprocal connections with cortical association areas allow it to integrate information across sensory modalities and across time, while its interactions with motivational systems enable retrieved representations to acquire behavioural significance. This anatomical and functional organisation is consistent with the well-established role of the hippocampus in relational memory, flexible memory expression, and inferential reasoning. It therefore places the hippocampus in an ideal position to support the higher-order integration processes required during sensory preconditioning.

3. The hippocampus and integration

3.1. Anatomy and connectivity of the hippocampus

The hippocampus is a bilateral, elongated structure located within the medial temporal lobe. In the rat, it forms a curved, banana-shaped structure extending from the septal region dorsally to the temporal lobe ventrally (Amaral & Lavenex, 2007). Its principal anatomical axis, referred to as the septotemporal, dorsoventral, or longitudinal axis, runs along this extent, whereas the transverse (or proximodistal) axis extends from the dentate gyrus towards the entorhinal cortex (**Figure 19**). Strictly speaking, the term *hippocampus* refers only to the "cornu ammonis" (CA) fields: CA1, CA2 and CA3 (Lorente de Nó, 1934).

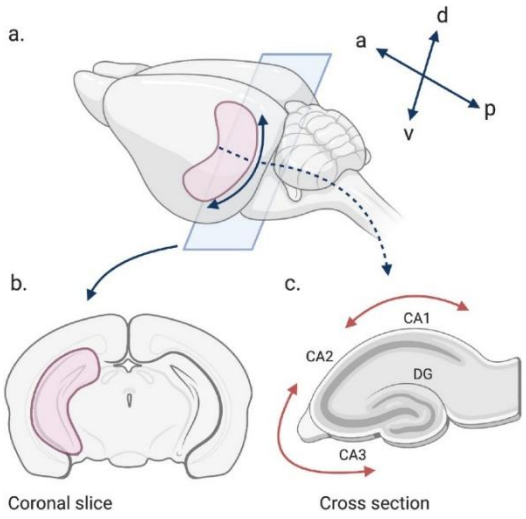


Figure 19. **Schematic representation of the rat hippocampus.**
a: external view, b:coronal slice; c:cross section. From Gunnarsdóttir et al., 2022

Each subregion of the hippocampal formation is organised around a single principal excitatory cell layer. In the dentate gyrus, this organisation comprises an outer molecular layer, a densely packed granule cell layer containing the principal excitatory neurons, and a deeper polymorphic layer, which contains mossy cells and a variety of interneurons. In contrast, the principal excitatory neurons of the CA3, CA2, and CA1 subfields are pyramidal cells, whose

cell bodies form the pyramidal layer. Across the hippocampal formation, GABAergic interneurons represent only about 10-15% of the neuronal population but comprise a remarkably diverse set of cell types. By targeting different dendritic and somatic compartments of principal neurons, they exert precise control over hippocampal activity and information processing (Pelkey et al., 2017; Roux & Buzsáki, 2015).

The canonical organisation of the hippocampal formation is traditionally described as the trisynaptic circuit (Andersen et al., 1971). Information enters the hippocampus through the *perforant path*, whereby neurons in layer II of the entorhinal cortex project to the dentate gyrus and, to a lesser extent, directly to CA3 (**Figure 20**). Granule cells of the dentate gyrus then project to CA3 pyramidal neurons via the mossy fibres, whose large, specialized synaptic boutons provide a small number of excitatory contacts. Finally, CA3 pyramidal cells project to CA1 through the *Schaffer collateral pathway*, terminating on both the apical and basal dendrites of CA1 pyramidal neurons.

Several anatomical features influence hippocampal function. First, the intrinsic connections are largely unidirectional. As originally noted by Ramón y Cajal (1893), information flows from the dentate gyrus to CA3 and then to CA1, without reciprocal projections between successive stages. This organisation contrasts with the predominantly reciprocal connectivity observed between cortical areas (Felleman & Van Essen, 1991) and establishes a preferred direction of information processing within the hippocampus. Second, the trisynaptic pathway represents only one component of a broader hippocampal network. In parallel, neurons in layer III of the entorhinal cortex project directly to CA1 and the subiculum through the *temporoammonic (TA) pathway*, bypassing the dentate gyrus and CA3 (**Figure 20**, Xu et al., 2015). CA1 projects to the subiculum, and both structures send outputs back to the deep layers of the entorhinal cortex, thereby closing the cortico-hippocampal loop (Amaral & Lavenex, 2007). Consequently, although the trisynaptic circuit remains the canonical description of hippocampal organisation, it represents only one element of a more complex and highly interconnected network (Aggleton & Christiansen, 2015).

Importantly, the intrinsic circuitry is conserved along the entire dorsoventral axis of the hippocampus (and is preserved in the homologous poles of the hippocampus across mammalian species, Strange et al., 2014). However, the entorhinal inputs supplying this circuit are themselves topographically organised. Distinct dorsoventral bands of the entorhinal cortex preferentially project to dorsal, intermediate, or ventral levels of the hippocampus (Burwell, 2000; Dolorfo & Amaral, 1998), and this topographic organisation is maintained throughout the intrinsic hippocampal circuitry (for a review, see Fanselow & Dong, 2010).

Although the intrinsic circuitry is repeated along the dorsoventral axis, its organisation is not strictly lamellar. Instead, hippocampal connectivity is highly distributed along the longitudinal axis. Entorhinal inputs can influence a large proportion of the dentate gyrus, mossy cells redistribute granule cell output across much of the longitudinal axis, and single CA3 pyramidal neurons send extensive axonal projections spanning a substantial fraction of CA1 (Amaral & Lavenex, 2007; Amaral & Witter, 1989; Ishizuka et al., 1990; Li et al., 1994).

The cellular architecture also changes gradually along the dorsoventral axis. Granule cells are more densely packed dorsally, whereas CA3 pyramidal cells become progressively more numerous ventrally, producing marked changes in the population ratios (Gaarskjaer, 1978). Thus, the hippocampus is better viewed as a three-dimensional network of convergent and divergent connections capable of integrating information across much of its dorsoventral extent (Amaral & Lavenex, 2007).

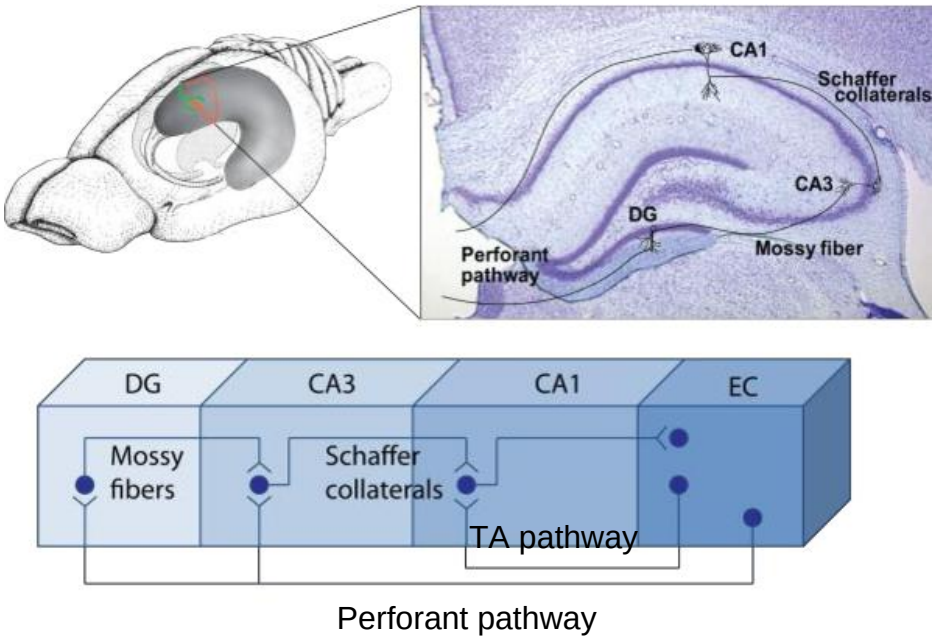


Figure 20. Intrinsic connectivity of the hippocampus. *From Xu et al (2015).*

The major distinction between dorsal and ventral hippocampus lies in their external connectivity (**Figure 21**). The dorsal hippocampus is embedded primarily within cognitive and spatial networks. It receives visuospatial information through the postrhinal-to-medial entorhinal pathway (Agster & Burwell, 2013; Burwell, 2000) and projects extensively to the dorsal subiculum (Amaral et al., 1991), retrosplenial cortex, and anterior cingulate cortex (Cenquizca & Swanson, 2007; Van Groen & Wyss, 2003). It also communicates with restricted regions of the lateral septum and dorsal striatum (Groenewegen et al., 1996; Naber & Witter, 1998). Notably, it has little direct connectivity with the amygdala, medial prefrontal cortex, hypothalamus or other structures involved in emotional regulation. In contrast, the ventral hippocampus is embedded within limbic, motivational and neuroendocrine networks. It

receives preferential olfactory, visceral and gustatory inputs through lateral entorhinal cortex (Burwell, 2000; Insausti et al., 1998) together with direct inputs from the basolateral amygdala (Pitkänen et al., 2000). Its outputs target the amygdala, medial prefrontal cortex (Hoover & Vertes, 2007), nucleus accumbens shell (Naber & Witter, 1998), bed nucleus of the stria terminalis, hypothalamus and olfactory regions (Canteras & Swanson, 1992; Cenquizca & Swanson, 2007). Through these connections, the ventral hippocampus is positioned to regulate emotional behaviour, stress responses and motivated action (Fanselow & Dong, 2010; Moser & Moser, 1998a). The "intermediate" hippocampus displays a genuinely intermediate connectivity profile. It receives inputs from the intermediate entorhinal cortex (Burwell, 2000) and projects moderately to medial prefrontal cortex while maintaining indirect interactions with the amygdala through the intermediate subiculum (Cenquizca & Swanson, 2007; Kishi et al., 2006). This position has led to the proposal that the intermediate hippocampus links accurate spatial representations with behavioural control systems (Bast, 2007; Bast et al., 2009).

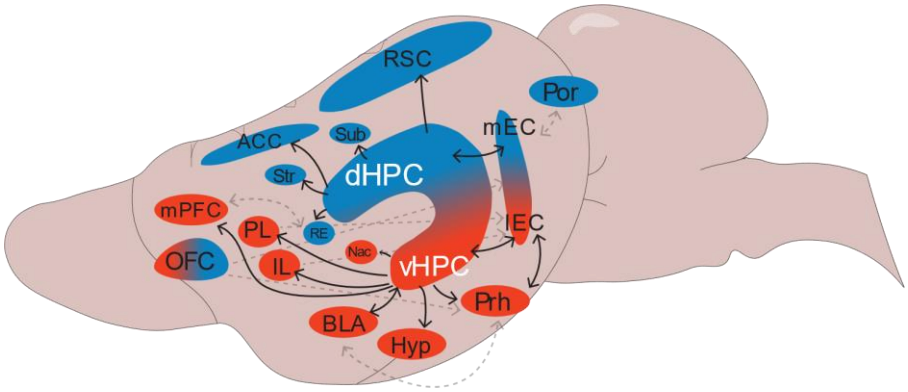


Figure 21. Connectivity profile of dHPC and vHPC.

ACC: anterior cingulate cortex; RSC: retrosplenial cortex; Sub: subiculum; Str: striatum; mPFC: medial prefrontal cortex; OFC: orbitofrontal cortex; PL: prelimbic cortex; IL: infralimbic cortex; BLA: basolateral amygdala; Nac: nucleus accumbens; RE: nucleus reuniens; Hyp: hypothalamus; Prh: perirhinal cortex; mEC: medial entorhinal cortex; lEC: lateral entorhinal cortex; Por: postrhinal cortex. Bold: direct projections. Dashed: indirect projection from or to the dHPC or vHPC.

Despite their distinct connectivity, the dorsal and ventral poles do not always operate independently. Multiple pathways allow communication between them. The perirhinal and postrhinal cortices provide one interface, integrating ventral hippocampal output with multimodal cortical and amygdalar information before it reaches dorsal hippocampal networks (Fanselow & Dong, 2010). The nucleus reuniens forms another important relay by linking medial prefrontal cortex, hypothalamus and the hippocampus. More recently, disynaptic pathways have been described in which dorsal CA3 reaches prefrontal cortex through the

ventral hippocampus and lateral septum, effectively making the ventral pole a relay for dorsal information rather than an entirely separate processing stream.

Overall, the anatomical evidence supports both functional specialisation and functional integration. The distinct extrinsic connectivity of the dorsal and ventral poles predicts differences in behaviour: dorsal hippocampus is expected to contribute preferentially to spatial and contextual processing, whereas ventral hippocampus should play a greater role in emotion, anxiety and neuroendocrine regulation. At the same time, the anatomy argues against viewing the dHPC and vHPC as two independent structures. Indeed, most transitions in connectivity are gradual rather than abrupt, intrinsic projections distribute information across large portions of the longitudinal axis, and multiple anatomical pathways permit communication between dorsal and ventral regions. Consequently, a strict dissociation between dorsal and ventral manipulations without considering potential interaction between the two poles might hide a part of how computation is performed, especially when studying the integration of different information.

3.2. The integrative functions of the hippocampus

The preceding section described the anatomy of the hippocampus and the organisation of its connectivity along the dorsoventral axis. However, a structure may receive convergent inputs from multiple sources without necessarily integrating them, and the relationship between connectivity and computation must therefore be established empirically. To understand the hippocampal contribution to integrative functions, it is useful to turn to paradigms in which the operation to be performed is well defined.

The term integration is used to describe several related but distinct operations. These can be distinguished along two dimensions: what is bound together, and when the elements to be bound are experienced (**Figure 22**). When the relevant elements are experienced *within the same event*, integration consists of forming a unified representation of co-occurring information. This may involve the binding of multiple sensory cues into a configural representation (within-event learning), such as encoding an audiovisual compound (AX) as a representation distinct from A or X presented alone, or the binding of the different components of an experience into an episodic representation comprising what happened, where, and when. By contrast, other forms of integration require combining information acquired across *separate* experiences. In these cases, the memories share one or more common elements but are separated in time. Trace conditioning (whereby the CS and US are separated by a temporal gap) provides a simple example, but memories acquired days or even weeks apart can later be integrated through overlapping representations, allowing organisms to infer relationships that were never directly experienced. The key distinction does not lie in the binding process

itself but in the nature of what is bound, and the occurrence of the joint elements (co-occurrence, where content differs but timing and context are the same; vs separated experiences, where content, and sometimes context, overlap but timing differs).

Importantly in this approach, hippocampal lesions do not impair the formation of direct associations between individual elements of an experience. What they disrupt is the formation of representations that bind those elements into a unified whole (Iordanova et al., 2011; Jarrard, 1993). Any account of hippocampal function must therefore explain not associative learning per se, but the computations that transform individual associations into integrated representations.

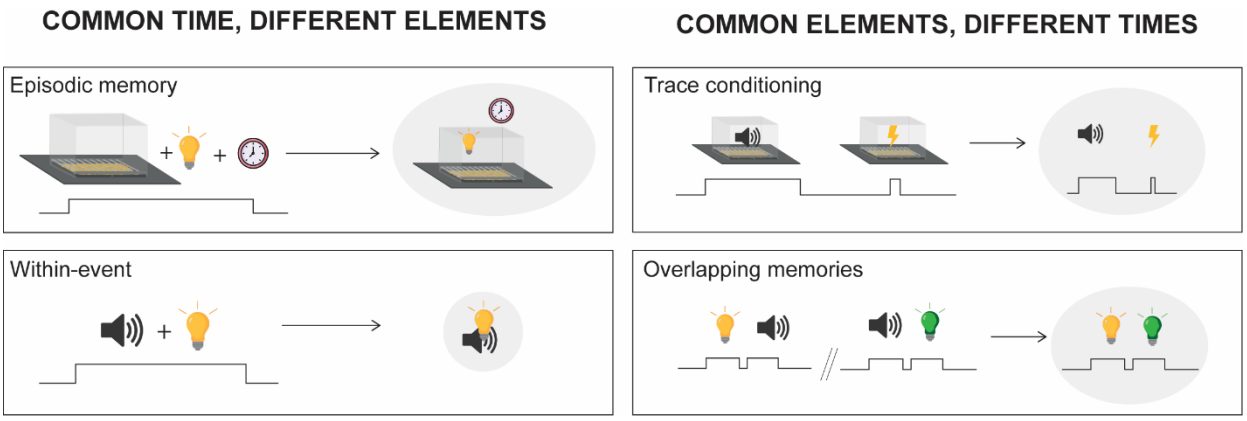


Figure 22. Integrative functions differ by the content and timing of experienced elements to be bound.

3.2.1. Episodic memory

Episodic memory is classically defined as the integration of what happened with where and when it happened (Tulving, 1983, 2002). Rodent paradigms, most often based on spontaneous object exploration, have led to the broad conclusion that rats and mice also display episodic-like memory (for a review see Chao et al., 2020). The hippocampus contributes not only by storing the individual elements of an experience, but by binding them into a coherent representation (Veyrac et al., 2015). This conclusion is supported by lesion studies showing that the elements themselves remain available while the relationships between them are lost. Fortin and colleagues (2002), for example, found that rats with hippocampal lesions readily recognised recently encountered odours but were severely impaired at remembering the order in which they had occurred. Because the task contained no spatial component, the deficit reflected the loss of temporal organisation rather than spatial memory. Likewise, hippocampal lesions impair discrimination of longer elapsed intervals, and this deficit persists despite extensive post-operative training (Sabariego et al., 2020). Together, these findings indicate that the hippocampus contributes to the relational structure that binds the components of an episode.

A second feature of episodic memory is particularly relevant to sensory preconditioning: episodes are often encoded incidentally, without any expectation that they will later be used. This has been demonstrated in foraging experiments (Zhou et al., 2012). Rats were allowed to forage freely under conditions in which encounters with food or its absence carried no immediate behavioural relevance. Temporary inactivation of CA3 selectively impaired later memory for these *incidentally* encoded events while sparing memory for events that were *intentionally* encoded to solve the task. Hippocampal processing was therefore specifically required when information acquired without immediate significance was later retrieved for an unforeseen purpose. This closely parallels stage 1 of sensory preconditioning, where the S2-S1 association is learned incidentally, particularly in aversively motivated protocols where animals are neither food- nor water-deprived during stage 1.

These behavioural findings align with the organisation of the hippocampal circuit. The medial and lateral entorhinal cortices provide complementary spatial and non-spatial inputs (Knierim et al., 2006). Within the hippocampus, the dentate gyrus performs pattern separation, CA3 rapidly binds distributed elements into a conjunctive representation and later retrieves it from partial cues, and CA1 integrates this output with direct entorhinal input before relaying it back to cortex (Rolls, 2017).

3.2.2. Configural process (within-event)

The proposal that the hippocampus forms configural representations was formalised by O'Reilly and Rudy's conjunctive representation theory, and later complemented by indexing theory according to which the hippocampus stores an index to distributed cortical representations rather than the representations themselves (for a review, Rudy, 2024). Contextual fear conditioning became the principal test of this account because it separates configural encoding from associative learning. Although fear of a context where shock occurred is a Pavlovian association, successful conditioning first requires exploration during which the features of the context are bound into a unified representation. Rats given insufficient pre-exposure show little contextual fear (Fanselow, 2000), and blocking hippocampal NMDA receptors or protein synthesis before, but not after, context pre-exposure prevents later conditioning (Barrientos et al., 2002; Stote & Fanselow, 2004). These findings indicate that the critical hippocampal computation occurs during the formation of the contextual representation. After pre-exposure, conditioning proceeds by retrieving that stored representation and associating it with shock, making contextual fear conditioning an example of representation-mediated learning in which the retrieved representation is configural rather than elemental (Rudy et al., 2002).

This computation is generally attributed to the dentate gyrus-CA3 circuit. The dentate gyrus separates similar experiences into distinct representations, whereas the recurrent connectivity of CA3 supports pattern completion, allowing partial cues to reactivate stored configurations (Leutgeb et al., 2007; Rolls, 2017). Consistent with this account, selectively disrupting NMDA receptor function in CA3 impairs the ability to link events represented within the same ensemble (Nomoto et al., 2023).

However, the strict dependence on the hippocampus for configurational-learning tasks can be refined. This point is made in two studies. First, Lin et al. (2016) showed that hippocampal lesions abolish configural pattern completion without eliminating performance altogether. In the critical experiment of this study, rats were exposed to AX and BY audiovisual compounds in stage 1, received fear conditioning of X but not Y in stage 2 and, finally, were tested with AX and BX compounds in stage 3. Whereas sham-operated rats showed greater fear to AX than BX, lesioned animals showed equivalent fear to the two test compounds. This was taken to mean that lesioned animals responded according to an elemental A–X–US chain, consistent with stronger learning about X when configural retrieval is unavailable. The important point is that pattern completion can therefore proceed through distinct configural and elemental routes, only the former depending on the hippocampus. The second relevant study showed that hippocampal lesions do not consistently impair reinforced configural discriminations (Coutureau et al., 2002; Gallagher & Holland, 1992; Saksida et al., 2006). Rather than supporting configural learning in general, the hippocampus appears to be specifically required when behaviour depends on retrieving configural representations formed during incidental, non-reinforced experience (Lin et al., 2016). This distinction is directly relevant to sensory preconditioning where stage 1 involves largely incidental learning, and predicts that hippocampal involvement should be greatest under conditions favouring configural encoding, such as simultaneous rather than serial S2-S1 presentations.

3.2.3. Temporal binding: the case of trace conditioning

The hippocampus is required for associative formation when the conditioned stimulus (CS) and unconditioned stimulus (US) are separated by a temporal gap but not when they are presented contiguously (as in delay conditioning), a dissociation observed across species (McEchron et al., 1999; Puhger et al., 2024; Solomon et al., 1986). Although several mechanisms have been proposed to explain this dependence, such as maintenance of a CS representation across the interval, contextual bridging, temporal coding, and interval discrimination, subsequent work suggests that the hippocampus contributes specifically to linking temporally discontinuous events rather than to learning the predictive relationship itself. Indeed, Bangasser et al. (2006) showed that restoring temporal contiguity by briefly re-presenting the CS together with the US rescued learning in rats with hippocampal NMDA

receptor lesions, despite leaving the predictive CS→US relationship unchanged. These findings indicate that the hippocampal requirement arises from the temporal discontinuity between events rather than from associative learning per se.

Recent work further suggests that the mechanisms supporting this integration differ along the dorsoventral axis. In dorsal CA1, calcium imaging revealed little sustained activity during the trace interval but robust post-shock activity accompanied by sharp-wave ripples. Disrupting this post-outcome activity impaired subsequent memory (Puhger et al., 2024). This led the authors to propose that dorsal hippocampus bridges temporal gaps retrospectively, through replay that reactivates the CS once the US has occurred. By contrast, recordings from ventral CA1 revealed sustained representations of the expected outcome spanning the interval between cue and outcome, consistent with a prospective mechanism that maintains predictive information until reinforcement (Biane et al., 2023). Although this sustained activity depended on task conditions, it suggests that dorsal and ventral hippocampus may bridge temporal gaps through distinct computations.

In other words, the hippocampus can integrate events separated in time, but this function may rely on different processes along its dorsoventral axis. While trace conditioning is not directly of interest in the present thesis, this dorsoventral dissociation raises the possibility that different hippocampal subregions also make complementary contributions to integrating memories acquired across separate experiences.

3.2.4. Integrating memories acquired at different times

In sensory preconditioning, the integration is of memories acquired days or weeks apart. In humans, it has been studied extensively using overlapping paired-associate paradigms, in which participants first learn an AB association, then an overlapping BC association, before being tested on the never-experienced AC relationship. Neural activity during encoding of the overlapping BC pair reliably predicts later success on the inferential AC test, indicating that integration occurs while the second association is being learned rather than only when probed for the AC relationship at test. Coupled hippocampal-midbrain activity during encoding predicts subsequent generalisation (Shohamy & Wagner, 2008), medial temporal lobe activity during BC learning predicts inferential performance (Zeithamova & Preston, 2010), and this effect localises particularly to CA1, where activity during encoding predicts later inference and is reinstated when participants infer the AC relationship (Schlichting et al., 2014). Importantly, integration does not overwrite the original memories. Instead, reactivation of an existing memory during new learning predicts its subsequent retention, suggesting that linking experiences stabilises rather than weakens earlier representations (Kuhl et al., 2010). Lesion evidence further shows that integration is dissociable from both encoding and retrieval:

patients with medial temporal lobe damage learned and retained the directly trained AB and BC associations normally, yet failed to improve on inferential AC trials across repeated learning cycles (Pajkert et al., 2017). The same dissociation emerges when reactivation itself is disrupted. Acute stress reduced hippocampal reactivation of the A representation during BC learning, impaired subsequent AC inference, and increased the representational separation of the overlapping memories AB and BC, while leaving both intact (Schüren et al., 2026). Thus, integration depends specifically on reactivating prior memories during new learning.

The temporal dynamics of this process are equally informative. In humans, integration is not always immediate. Inferential judgments can be behaviourally absent shortly after learning and emerge only after an offline delay, even though memory for the directly trained pairs is unaffected (Ellenbogen et al., 2007). The same delay-dependence is visible neurally: representational similarity among overlapping memories increases across a week in both medial prefrontal cortex and posterior hippocampus, and participants showing the largest post-encoding increase in medial prefrontal–anterior hippocampal connectivity subsequently show the greatest inferential performance (Tompary & Davachi, 2017). Hippocampal replay seems to play a role in this type of integration. Indeed, disrupting hippocampal replay prevents predictive representations from emerging (Liu et al., 2023), whereas replay during spatial exploration can construct representations of locations the animal has never directly experienced relative to a newly discovered landmark (Bakermans et al., 2025). Replay therefore not only stabilises existing representations (Roux et al., 2017) but also generates the relational structure that later supports inference.

Finally, integration appears to preserve the identity of constituent memories even as their associations are updated. Biane et al. (2023) found that CA1 odour representations remained relatively stable after learning and across changes in odour–outcome associations. Distinct odours remained discriminable even when they predicted the same outcome, and the representation of a given odour was preserved when its associated outcome was reversed from sucrose to shock or vice versa. These findings suggest that new learning can modify the relationship between a cue and its outcome without completely overwriting the neural representation of the cue itself. This stability was not absolute, however, as representations exhibited some inter-session drift and, in vCA1, odour representations were sensitive to changes in the current value associated with the odour.

Overall, the integrative functions of the hippocampus operate at different scales. Across paradigms, they occur whenever separate pieces of information must be integrated. Conjunctive coding supports the formation of integrated representations from co-occurring

elements, whereas representation-mediated reinstatement allows previously stored memories to be reactivated and linked to new experiences.

3.3. The hippocampus in sensory preconditioning

Extensive work has investigated the involvement of the hippocampus in sensory preconditioning in humans and rodents. During the first stage, the hippocampus may contribute to encoding the relationship between two initially neutral stimuli through the same conjunctive processes described for episodic and configural learning. During conditioning and test, that stored representation can be reactivated and integrated with a newly acquired emotional memory, consistent with the retrospective nature of hippocampal integration observed across timescales.

The hippocampus has therefore shown importance at every stage of the protocol (cf Appendix table). Collectively, these studies establish that the hippocampus is necessary for successful sensory preconditioning. They do not, however, reveal how exactly it contributes to learning and integration. This is not because the findings are contradictory, but because the available studies differ in almost every important methodological dimension: the behavioural protocol, the arrangement of the stimuli, the sensory modalities involved, the hippocampal subregion targeted, and the stage and type of neural manipulation employed. As a result, the existing literature identifies hippocampal involvement without isolating the computation it supports or the specific conditions under which it is required. Three features of the literature are particularly limiting.

First, most studies rely on permanent lesions. Lesions performed before training affect all stages of the task simultaneously, making it hard to determine at which stage and, therefore, for which process the hippocampus is required. Second, the hippocampus has largely been treated as a single structure. Most rodent studies either used complete hippocampal manipulations or selectively targeted the dorsal hippocampus. Only one study has examined the ventral hippocampus in sensory preconditioning (Pinho et al., 2025), and no study has assessed the interaction between the two poles. This reflects the historical emphasis on a strict dorsoventral dichotomy, despite growing evidence that hippocampal function is distributed along a gradient and that cognition emerges from coordinated activity across the longitudinal axis. Consequently, it remains unclear whether memory integration relies on dorsal and ventral hippocampus performing distinct computations, complementary functions, or coordinated processing. Third, behavioural protocols differ in ways that make the existing findings difficult to compare. One example illustrates the problem. Silencing the dorsal hippocampus during preconditioning abolishes later responding to the preconditioned cue while sparing direct conditioning, both after chemogenetic inhibition of excitatory neurons (Pinho et al., 2025) and

after disruption of CB1 signalling on hippocampal interneurons (Busquets-Garcia et al., 2018). In both studies, however, the neutral cues were presented simultaneously. The impairment could therefore reflect the hippocampal role in forming configural representations of stimulus compounds, or a more general role in encoding incidental associations between neutral events independently of their modality or arrangement. These limitations consequently leave three major questions unresolved, which form the basis of the work in this thesis.

4. Objectives

The aim of this thesis was to determine the contribution of the hippocampus to the integration of sensory and emotional memories acquired at different times. To this end, we used aversive sensory preconditioning in rats, a paradigm in which fear of a stimulus that was never paired with danger provides a direct behavioural readout of memory integration. Building on the limitations of the existing literature identified above, we combined the analytical tools of associative learning theory with stage-specific circuit manipulations and in vivo recordings to investigate the hippocampal mechanisms supporting sensory preconditioning. This work is organised around three main questions, each addressed in a dedicated experimental chapter.

The first question asks **which integration mechanism predominates in an auditory-visual sensory preconditioning protocol**. Because the different stages of sensory preconditioning recruit distinct learning processes, identifying when hippocampal activity is required provides a way to infer the computations it supports. This is, of course, predicated on understanding which mechanism of integration is engaged by the behavioural protocol itself. Chapter 2 therefore begins by establishing an auditory-visual sensory preconditioning procedure in the two laboratories where this thesis was conducted (University of New South Wales, Australia, and University of Bordeaux, France). Because the original protocol did not transfer directly between the two sites, its parameters were systematically adapted, and this optimisation, together with its validation in both males and females, is reported. The chapter then determines whether the adapted protocol relies primarily on mediated learning or associative chaining by testing whether responding to S2 remains dependent on the S1–US memory. To this end, shock intensity during conditioning was manipulated in one experiment, and S1 was extinguished before test in another. Together, these experiments establish the behavioural framework required to interpret the hippocampal manipulations described in the following chapters.

Building on the demonstration in Chapter 2 that the auditory-visual protocol predominantly engages mediated learning, the second question addresses the central issue of this thesis: **what process does the hippocampus support during the integration of sensory and emotional memories, and does this contribution differ along the dorsoventral axis?** With

the exception of Pinho et al. (2025), rodent studies have focused almost exclusively on the dorsal hippocampus. This emphasis reflects the traditional dorsoventral dichotomy, despite growing evidence that hippocampal function is organised along a gradient and that behaviour may depend on interactions between dorsal and ventral subregions. Whether memory integration relies on distinct computations within each pole or on coordinated activity between them has never been directly examined. Chapter 3 addresses this question using the auditory-visual protocol characterised in Chapter 2. The dorsal and ventral hippocampus were inactivated separately with muscimol, either before preconditioning in stage 1 or before conditioning in stage 2, to determine when each subregion is causally required. Because functional specialisation does not preclude cooperation, calcium activity was then recorded simultaneously from both regions using dual-site fibre photometry throughout the two learning stages, and dorsoventral functional coupling was related to subsequent expression of preconditioned and directly conditioned fear. Finally, c-Fos expression was quantified in the dorsal hippocampus, ventral hippocampus, perirhinal cortex, and basolateral amygdala to place any intra-hippocampal coordination within a broader network of brain regions that support sensory preconditioning.

The final question asks **whether the hippocampal contribution generalises across sensory modalities**. Excitotoxic hippocampal lesions spared sensory preconditioning in a purely gustatory paradigm (Ward-Robinson et al., 2001), whereas positive findings have largely come from cross-modal protocols. Because these studies also differed in the type of the manipulation (permanent lesions vs temporary perturbation) and, in the gustatory case, lesions of the entorhinal cortex produced a similarly negative result (Ward-Robinson et al., 2005), it remains unclear whether hippocampal involvement reflects a general mechanism of memory integration or is particularly important when information from different sensory modalities must be combined. Chapter 4 addresses this question using a unimodal gustatory version of the paradigm, in which two tastes are experienced together as a compound (AX) before one of them is paired with gastric malaise, and the mediated aversion to the other is measured as reduced consumption at test. Chemogenetic inhibition (hM4Di) of the dorsal and ventral hippocampus was used to disrupt hippocampal activity either during preconditioning or at test, thereby dissociating encoding of the taste-taste association from retrieval of the mediated aversion, with the direct aversion to the conditioned taste serving as an internal control. These experiments determine whether the hippocampal contribution identified in the cross-modal protocol extends to a within-event, single-modality form of sensory preconditioning, and therefore whether it reflects a modality-independent mechanism of memory integration.

Together, these three questions build on one another. Establishing the integration mechanism engaged by the behavioural protocol provides the framework for interpreting hippocampal manipulations. Determining when and where the hippocampal contribution lies within the protocol then identifies the computations supported by its dorsal and ventral subregions. Finally, testing the same principles in a unimodal preparation assesses the generality of the hippocampal mechanisms beyond cross-modal sensory preconditioning.

Chapter 2

Demonstration and characterization of sensory preconditioning

Significance statement

Sensory preconditioning is a protocol that allows to study how memories acquired at different times (an S2-S1 association and an S1-US association) are integrated to infer new relationships between events. This chapter demonstrates a protocol of auditory-visual sensory preconditioning for the remainder of the thesis and determines which integration mechanism it engages.

What was done. An auditory–visual sensory preconditioning protocol was transferred from the laboratory in which it was developed (host laboratory 1, UNSW, Australia) to the second host laboratory (Bordeaux, France). When direct transfer failed, the protocol was adapted and revalidated, testing males and females separately. Two further experiments then dissociated the two candidate integration mechanisms (mediated learning versus chaining) by (i) lowering shock intensity during first-order conditioning and (ii) extinguishing fear to S1 before test.

What was found. The original protocol produced robust preconditioned fear at the original site but none at the second site, despite equivalent first-order conditioning. A combined set of five changes (background white noise, lower shock intensity, sexes tested separately, twice as many S2–S1 pairings, interleaved rather than blocked unpaired controls) restored the effect in males; but females showed no reliable preconditioned fear despite successful direct conditioning. Mechanistically, the two memories proved doubly dissociable. A 0.4 mA shock supported normal fear to S1 while abolishing fear to S2, whereas extinction of fear to S1 left fear to S2 intact, suggesting that fear to S2 relies on mediated learning.

Why it matters. First, these results identify the mechanism by which integration occurs in this protocol: an integrated S2–shock representation is formed during conditioning rather than inferred by successive retrieval at test. **Knowing the predominant integration mechanism fixes the stage at which the circuit-level manipulations of the following chapters must be targeted**, and the terms in which their effects can be interpreted. Second, the double dissociation evidenced in the shock-intensity reduction and the extinction experiments **provides an independent behavioural convergence on the mediated-learning account**, and does so in the direction predicted by Wagner's SOP model for learning about an associatively activated (A2) representation. Third, documenting that a widely used **higher-order conditioning protocol occupies a narrow parametric window**, does not survive a change of site, and can be recovered by an explicit set of adaptations is of direct practical use to other laboratories and is rarely reported. Finally, the **sex difference in sensory preconditioning further questions whether females engage the same associative process**.

Introduction

A central aim of this thesis is to investigate the neural circuits supporting the integration of separate memories into an association that can guide adaptive behaviour. Sensory preconditioning is a protocol that can be used to study this integration. In its usual form, the protocol involves three main stages (**Figure 23**). During the initial stage of preconditioning (stage 1), two neutral stimuli are presented in a consistent relationship (S2→S1), allowing the animal to form an association between two cues that carry no explicit motivational value. During the subsequent stage of direct conditioning (stage 2), S1 is paired with an aversive unconditioned stimulus (US), such that S1 acquires the capacity to elicit a conditioned fear response. Critically, at test, animals express fear to both S1 and S2, even though the latter was never directly paired with the US or danger of any sort. This "sensory preconditioned" fear to S2 can only arise if the animal integrates the S2→S1 and S1→US memories, making the response to S2 a readout of memory integration.

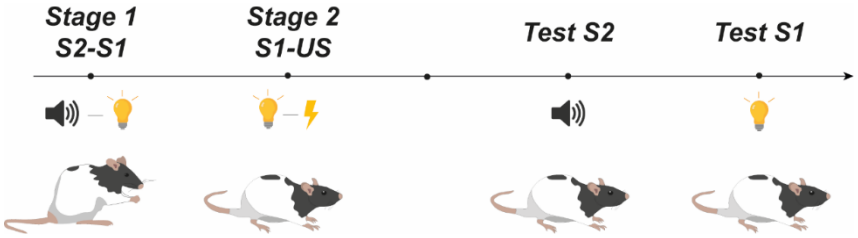


Figure 23. **Auditory-visual sensory preconditioning protocol**

The work presented in this chapter pursued two objectives. The first objective was methodological and reproducibility-oriented: to establish a reliable auditory-visual sensory preconditioning procedure across the two laboratories in which this thesis was conducted (host laboratory 1 at the University of New South Wales in Australia, and host laboratory 2 at the University of Bordeaux in France). As will become apparent, transferring an ostensibly well-established protocol between laboratories required a systematic adaptation of the procedure. We thought it relevant to present a summary of these adaptations alongside the behavioural demonstrations.

Several considerations motivated the choice of the auditory-visual sensory preconditioning protocol. First, the procedure does not require food or water deprivation. Appetitive and instrumental variants of sensory preconditioning necessarily engage animals in a state of heightened motivational drive, which may change rats' attentional state and their processing of individual stimuli, including the associative strategies recruited during preconditioning. The aversive, non-deprived protocol used here instead allows the S2→S1 association to be

acquired incidentally, without an explicit task demand, more closely approximating latent learning. Second, the procedure benefits from an existing body of circuit-level work: perirhinal cortex and basolateral amygdala have already been implicated in the online integration process underlying it, and the sensitivity of the engaged mechanism to parametric variation has likewise been characterised (for reviews, see Holmes, Wong, et al., 2022; Holmes, Fam, et al., 2022). Lastly, this choice anticipates the structure of the thesis. A gustatory analogue of sensory preconditioning is introduced in a later chapter to test whether the hippocampal contribution identified here extends across sensory modalities. Establishing a well-characterised auditory–visual protocol of sensory preconditioning in the present chapter therefore provides the necessary reference point for that comparison.

The second objective of this chapter was conceptual: to identify the psychological mechanism supporting fear to S2 in the adapted protocol deployed in the second host laboratory. As detailed in the Introduction, two mechanisms, *mediated learning* and *chaining*, can in principle account for sensory preconditioning, and these are distinguished by whether or not fear to S2 remains dependent on the integrity of the S1→US memory. The mechanisms do not operate at the same time in the protocol but they are not mutually exclusive and may involve different neural substrates. Establishing which mechanism is engaged was therefore a prerequisite for any subsequent interrogation of the neural circuits involved in memory integration, and constituted a necessary first-step for the thesis.

1. Demonstration of sensory preconditioning across laboratories

1.1. Replication of an auditory-visual sensory preconditioning protocol

We first sought to reproduce a well-characterised sensory preconditioning protocol, that established by Parkes, Holmes and colleagues (Holmes et al., 2013, 2018; Parkes & Westbrook, 2010). This protocol (here referred to as the original protocol) has been used to study the behavioural and neural bases of sensory preconditioning in host laboratory 1 (Holmes et al., 2018; Holmes & Westbrook, 2017; Wong et al., 2019, 2026). We attempted to replicate the sensory preconditioning effect using the same overall design, procedures and behavioural readout in the same rat strain (Long Evans). Because no study had previously identified sex effects in sensory preconditioning, both males and females were included in the replication in host laboratory 2.

MATERIAL AND METHODS – ORIGINAL PROTOCOL

Subjects (Host laboratory 1).

Subjects were 24 naïve male Long-Evans adult rats (>10 week-old) obtained from a colony maintained by the Biological Resources Centre at the University of New South Wales. Rats were housed in plastic cages (40 cm width x 22 cm height x 67 cm length), with between two and four rats per cage.

Subjects (Host laboratory 2).

Subjects were 12 male and 12 female naïve Long Evans adult rats aged (>8 weeks) obtained from Centre d'Elevage Janvier, France. Rats were housed in pairs in plastic cages in a colony room. Only male rats were used. In pilot work, the sensory preconditioning protocol did not yield reliable preconditioned freezing in females (see **Figure 25**), we therefore restricted the present study to males for all remaining experiments.

Apparatus (Host laboratory 1). Experiments were conducted in 8 identical chambers (30 x 25 x 21 cm) located within the same room. The ceiling, front door, and back wall were clear polycarbonate, and the side walls were aluminium. The floor consisted of 19 stainless-steel rods, each 2 mm in diameter and spaced 13 mm apart (centre-to-centre), positioned above a stainless-steel waste tray filled with corncob bedding. The stainless-steel floor was setup so that it could deliver mild foot shocks. At the end of each session, the chambers were cleaned with 70% ethanol and water. Each chamber was positioned inside a light- and sound-attenuating fibreboard cabinet with a loudspeaker at the top of the back wall. Each chamber was illuminated with an infrared light source and a camera mounted on the back wall of each cabinet was used to record the behaviour of each rat. The cameras were connected to a monitor and recording device located in the experimental room. Experimental events were controlled and recorded by a computer equipped with the Med-PC V software.

Apparatus (Host laboratory 2). Experiments were conducted in conditioning chambers (40 × 35 × 30 cm, Imetronic) made of gray polyvinyl chloride on three sides and transparent Perspex on the opening face (Esclassan et al., 2009a). A grid floor (27 parallel 0.5-cm-diameter stainless-steel bars, 1.5 cm apart) above a sawdust tray could deliver mild foot shocks. The chamber was located inside a sound-attenuating cubicle with a loudspeaker at the top of the back wall. The ceiling contained a 10 cm circular opening to allow cable access for fiber photometry recordings. An infrared camera was placed on the

ceiling. Experimental events were controlled and recorded by a computer equipped with the POLY software (Imetronic, France).

Stimuli. For all experiments, the auditory stimulus was a pure tone (1,000 Hz, 70 dB) and the visual stimulus was a pair of flashing lights (3.5 Hz, 57 lux) delivered by two horizontally aligned LEDs placed on a side wall of the chamber. Each LED was separated by at least 15cm. The tone and light were used as S2 and S1. Each S1 presentation was 10 s and each S2 presentation was 30 s. The US was a 0.8 mA, 0.5 s foot shock (Michalscheck et al., 2021).

Procedures. The procedure was administered over 7 consecutive days (**Figure 24A**).

Context exposure. On Days 1 and 2, rats received two 20-min exposures to the chambers, one in the morning and the other approximately 3hr later in the afternoon. Context exposure aims to familiarize the rats with the training context and eliminate any neophobic reactions.

Sensory preconditioning. On Day 3, all rats received eight presentations of the S2 (tone) and eight of the S1 (light). The first stimulus presentation occurred 5 min after rats were placed into the chambers. For rats that received paired presentations, the offset of S2 co-occurred with the onset of S1 and the interval between each paired presentation was fixed at 5 min. For rats that received unpaired presentations, interleaved presentations of S2 and S1 were 2 min and 30 s apart. After the last stimulus presentation, rats remained in the chambers for an additional 2 min. They were then returned to their home cages.

Direct conditioning. On Day 4, all rats received four presentations of the S1 and four presentations of the US. The first S1 presentation occurred 5 min after rats were placed in chambers. For rats receiving paired presentations, each S1 presentation lasted for 10 s and co-terminated with foot shock, and the interval between light-shock pairings was 5 min For rats that received unpaired presentations, the four S1 presentations were presented as a block with 2 min and 30 s between each stimulus presentation; and this was followed by four US presentations with the same interstimulus interval. Rats remained in the chambers for an additional 1 min after the final stimulus presentation and were then returned to their home cages.

Context extinction. On Day 5, rats received two 20-min exposures to the chambers, one in the morning and the other approximately 3hr later, in the afternoon. These exposures were intended to extinguish any freezing elicited by the context and thereby provide a measure of the level of freezing to the tone and the light un-confounded by context-elicited freezing. On

Day 6 (Day 7 for fiber photometry), rats received a further 10 min extinction exposure to the context.

Test. On Day 6, approximately 2 hr after the context-extinction session, rats were tested for their levels of freezing to the preconditioned tone S2. On Day 7 (Day 8 for fiber photometry), rats were tested for their levels of freezing to the conditioned light. For each test, the first stimulus presentation occurred 2 min after rats were placed in the chambers. Each test consisted of eight stimulus alone presentations, with a 3 min interval between each presentation. Each tone presentation was 30 s in duration and each light was 10 s. Rats remained in the chamber for an additional minute after the final stimulus presentation in each test.

Scoring and statistics. The sessions of training (stages 1 and 2), and testing sessions were recorded. Freezing, defined as the absence of all movements except those required for breathing (Fanselow, 1980), was used as a measure of conditioned fear. Freezing data were collected using a time-sampling procedure in which each rat was scored as either 'freezing' or 'not freezing' every 2 s by an observer blind to the rat's group allocation. A second blind observer scored a randomly selected 25% of the data. The correlation between the scores were high (Pearson > 0.9). Freezing was scored during the stimulus presentations. The baseline level of freezing was measured during the first 2 minutes of the test session prior to any stimulus delivery. All freezing data were converted to a percentage of the total number of observations that were made during stimulus presentations and during the baseline period. The percent freezing data were then analysed using a set of planned orthogonal contrasts (Hays, 1963) with the type 1 error rate controlled at $\alpha = 0.05$. The 95% confidence intervals (CIs) were reported for significant results. Non-orthogonal follow-up comparisons were Bonferroni-corrected. The required number of rats per group was determined during the design stage of the study. It was based on our prior experience in running sensory preconditioning studies: experiments of this sort typically require an average of 8 subjects per group to provide sufficient statistical power.

To demonstrate sensory preconditioning, rats were allocated to 3 groups (**Figure 24A**). Rats in the group of interest [PP] were exposed to S2-S1 (tone-light) pairings in stage 1 and S1-US (light-shock) pairings in stage 2. They were expected to show fear responses when presented with the S2 [tone] alone at test. To confirm that these fear responses reflect the encoding and integration of associations formed in stages 1 and 2, we used two control groups. The first received the same training in stage 2 and testing but was exposed to explicitly unpaired presentations of the S2 and S1 in stage 1 [Group UP]. The second received the same training in stage 1 and testing but was exposed to explicitly unpaired presentations of the S1

and US in stage 2 [Group PU]. If fear of the S2 in Group PP is associatively mediated, it should be greater in this group than in either of the two controls where the integration of memories is not possible.

In host laboratory 1 (UNSW, Australia), sensory preconditioning was successful (**Figure 24C**). The baseline levels of freezing at test were low (<5% for all groups) and did not differ between the groups. During test presentations of the tone, Group PP froze significantly more than Groups UP and PU ($F(1,21) = 10.914$; $p=0.003$; 95% CI: [6.986;30.723]), while Groups UP and PU did not differ from each other ($F=0.001$, $p=0.975$). This shows that freezing to the tone is not due to generalisation from the light or context. Indeed, the pattern of freezing across each of the groups during testing with the light was completely different and reflective of their training history; Groups PP and UP exhibited higher levels of freezing than Group PU ($F(1,21) = 79.825$; $p<0.001$; 95% CI: [-72.040;-44.835]), without differing from each other ($F=1.157$, $p=0.294$). This pattern of results was also evident across conditioning in stage 2 (**Figure 24B**), where Groups PP and UP acquired freezing to the light at a faster rate than Group PU ($F(1,21) = 66.36$; $p < 0.0001$; 95% CI: [-88.26;-52.363]), without differing from each other ($F=0.098$, $p=0.757$).

In host laboratory 2, the same protocol resulted in successful direct conditioning of the light but failed to produce detectable sensory preconditioned fear of the tone. Indeed, while the pattern of results across conditioning of the light in stage 2 and its testing in stage 3 was as expected (**Figure 24E** and **24G**); Groups PP and UP froze more than Group PU in stage 2, $F(1,21)=30.65$, $p<0.001$, 95% CI[-59.325;-26.925] and during the light test, $F(1,21)=58.7$, $p<0.001$, 95% CI[-78.104; -44.753]), there were no significant differences in freezing between the groups during the tone test (**Figure 24F**, largest $F(1,21)=0.339$, $p=0.567$).

These results revealed a clear dissociation between the two sites. In host laboratory 1 (UNSW), the procedure reproduced the expected sensory preconditioning effect: animals displayed robust conditioned freezing to S2 at test, confirming successful integration of the S2→S1 and S1→US memories. In host laboratory 2, however, the same procedure failed to produce sensory preconditioned fear to S2 despite comparable levels of first-order fear to S1. This dissociation indicates that the sensory preconditioning effect, although behaviourally reproducible, is sensitive to the specific experimental conditions of a given laboratory (e.g., conditioning chamber features, housing conditions...), and that the protocol needed to be adapted to produce the phenomenon in host laboratory 2.

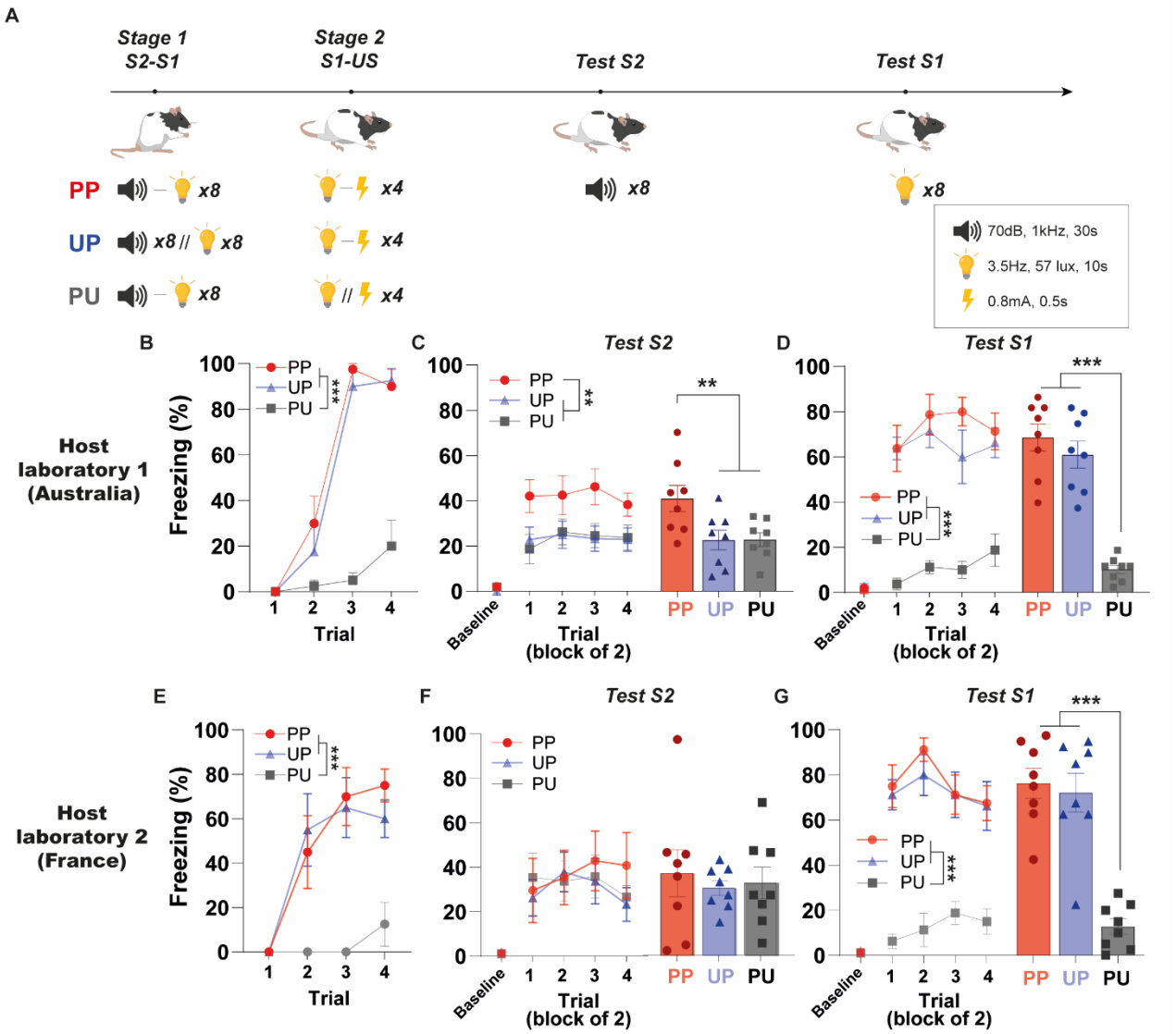


Figure 24. **Identical protocol of sensory preconditioning resulted in site-related differences**

(A) Experimental design of the original sensory preconditioning task, replicated across two host laboratories (Host laboratory 1, Australia; Host laboratory 2, France). In stage 1, Groups PP and PU received paired presentations of the tone (S2) and light (S1), while Group UP received unpaired presentations. In stage 2, Groups PP and UP received paired presentations of the light (S1) and foot shock (US), while Group PU received unpaired presentations. All groups were subsequently tested for freezing to the tone (Test S2) and light (Test S1). **(B–D)** Results in host laboratory 1 (Australia). **(B)** Mean (±SEM) freezing during the four S1 presentations in stage 2. **(C, D)** Mean (±SEM) freezing across the 8 test presentations (blocks of 2 trials and average over all trials) during S2 test (tone; C) and S1 test (light; D). Individual data points are shown in bar graphs. Error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Baseline freezing was measured before the first stimulus presentation at test and was never different between groups. For test levels of freezing to the tone, the first contrast compared Group PP versus the weighted average of Groups PU and UP; the second contrast compared Group PU versus Group UP. For test levels of freezing to the light, the first contrast compared the weighted average of Groups PP and UP versus Group PU, and the second contrast compared Group PP versus Group UP. **(E–G)** Results for host laboratory 2 (France). Same design and analyses as in (B–D), with n = 4 male + 4 female rats.

1.2. Adaptation of the original protocol and validation of the modified protocol

The failure of sensory preconditioning in host laboratory 2 prompted a systematic reconsideration of the parameters that could be varied in order to generate the phenomenon at this site. In a series of experiments, we adapted a range of factors in a step-by-step fashion. While adapting a single factor did not rescue sensory preconditioning on its own, the combination of changes did rescue the effect. Specifically, we (i) tested males and females separately rather than pooling the sexes, (ii) introduced a background white noise in the experimental chambers, (iii) increased the number of S2 and S1 presentations during the preconditioning stage, (iv) interleaved (rather than blocked) unpaired stimulus presentations to minimise non-associative contributions to responding, and (v) decreased the shock intensity used during first-order conditioning. The rationale and references for each of these modifications, as well as the direction of the change relative to the original protocol, is summarised in **Table 1**.

MATERIAL AND METHODS – ADAPTED PROTOCOL					
Material and Methods	**Parameter**	**Original protocol**	**Adapted protocol**	**Rationale for change**	**References**
Apparatus	Background white noise	Absent	Present	Reduce influence of movements and events outside the chambers	(Bergstrom, 2020; Curzon et al., 2009)
	Shock intensity	0.8 mA	0.6 mA	Reduce fear generalisation	(Baldi et al., 2004; dos Santos Corrêa et al., 2019; Dunsmoor et al., 2017)
Subjects	Sex	Pooled 12 males 12 females	Separated 24 males 24 females	Avoid masking of effects by sex-dependent variability; allow sex to be assessed as a factor	(Day & Stevenson, 2020; Pinho et al., 2025)
Procedure	Number of S2 andS1 presentations in stage 1	8 over 1 session (1 day)	16 over 2 sessions (2 days)	Greater opportunity for learning about the paired and unpaired stimulus-stimulus relations	(Holmes, Fam, et al., 2022; Prewitt, 1967)
	Unpaired presentations in stage 1	Blocked	Interleaved	Strengthen discrimination between S2 and S1 in unpaired rats, minimise stimulus generalisation	(Mackintosh, 2009; Rescorla, 1967)

Table 1. Changes in the adapted protocol parameters and their rationale

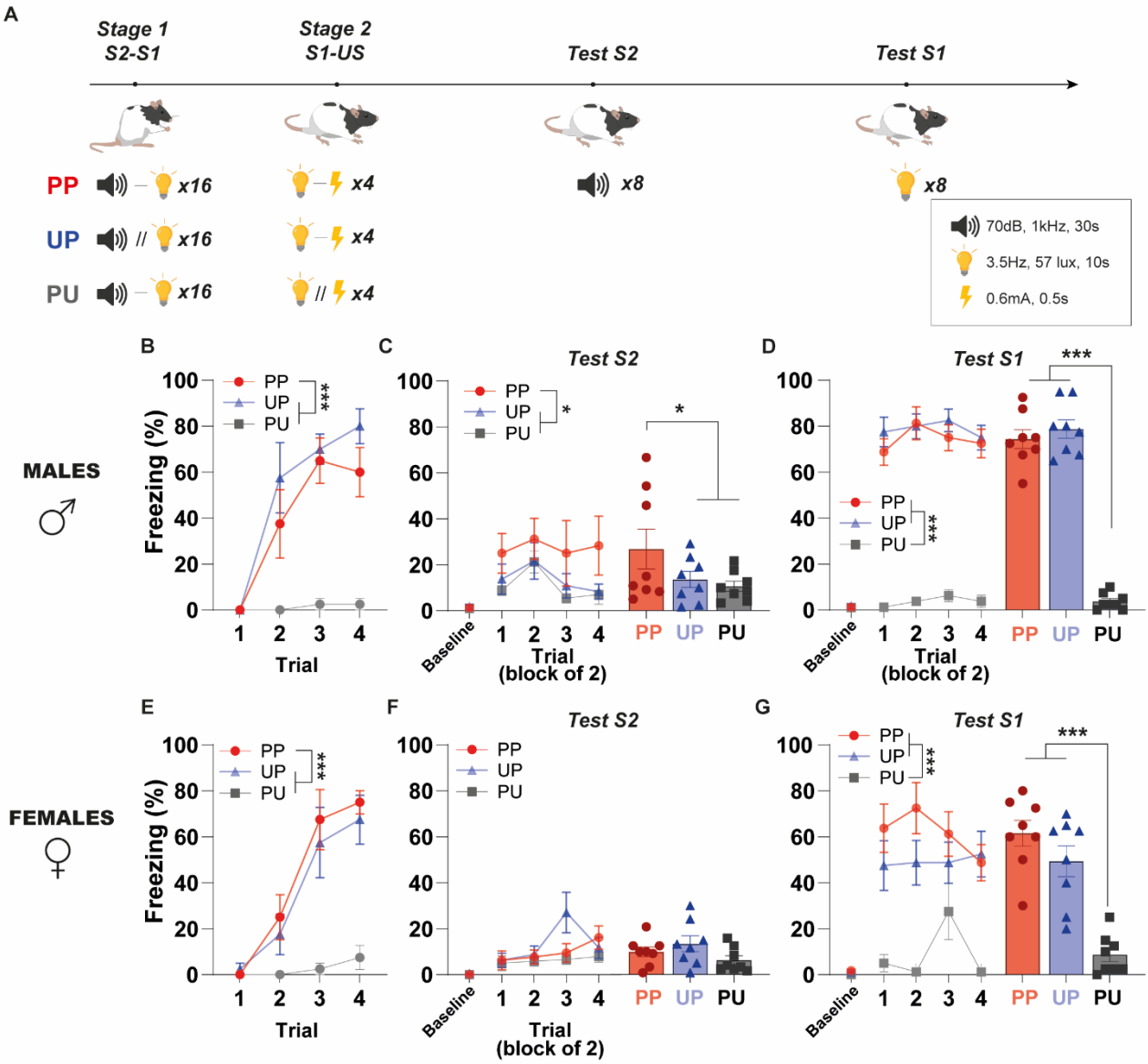


Figure 25. **Sex-differences in sensory preconditioning**

(A) Experimental design of the sensory preconditioning task, tested on males and females in host laboratory 2, using Long Evans rats aged >8 weeks (n = 8 per group per experiment). In stage 1, Groups PP and PU received paired presentations of the tone (S2) and light (S1), while Group UP received interleaved unpaired presentations. In stage 2, Groups PP and UP received paired presentations of the light (S1) and foot shock (US), while Group PU received blocked unpaired presentations. All groups were subsequently tested for freezing to the tone (Test S2) and light (Test S1). **(B–D)** Results with male rats. **(B)** Mean (±SEM) freezing during the four S1 presentations in Stage 2. **(C, D)** Mean (±SEM) freezing across the 8 test presentations (blocks of 2 trials and averaged across all trials) during Test S2 (tone; C) and Test S1 (light; D). Individual data points are shown in bar graphs. Error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001. Baseline freezing was measured before the first stimulus presentation at test and was never different between groups. For test levels of freezing to the tone, the first contrast compared Group PP versus the weighted average of Groups PU and UP; the second contrast compared Group PU versus Group UP. For test levels to the light, the first contrast compared the weighted average of Groups PP and UP versus Group PU, and the second contrast compared Group PP versus Group UP. **(E–G)** Results with female rats. Same design and analyses as in (B–D).

Using the adapted protocol, we assessed whether sensory preconditioning was expressed comparably in the two sexes in host laboratory 2. Except for the changes listed in **Table 1**, the same design and procedure as described in the original material and methods section was used for this demonstration (**Figure 25A**).

In average, sensory preconditioning was successful in the male rats. Baseline levels of freezing at test were low (<5% for all groups) and did not differ between the groups. During the test of the tone, Group PP froze significantly more than Groups UP and PU (**Figure 25C**, $F(1,21) = 4.689$; $p=0.042$; 95% CI: [0.584;28.925]) while Groups UP and PU did not differ from each other ($F=0.147$, $p=0.705$). This pattern of results shows that freezing to the tone is not due to generalisation from the light or context. Indeed, the pattern of freezing across the groups during testing with the light was different and reflective of their training history; Groups PP and UP exhibited higher levels of freezing than Group PU (**Figure 25D**, $F(1,21) = 303.05$; $p<0.001$; 95% CI: [0.932;19.068]), without differing from each other ($F=0.823$, $p=0.375$). This pattern was also evident in stage 2 (**Figure 25B**), where Groups PP and UP acquired freezing to the light at a faster rate than Group PU ($F(1,21) = 42.920$; $p<0.001$; 95% CI: [-56.801 ;-33.199]), without differing from each other ($F=1.347$, $p=0.259$).

In the female rats, the same protocol resulted in successful direct conditioning of the light but failed to produce sensory preconditioning of the tone. That is, the pattern of results across conditioning of the light in stage 2 and its testing in stage 3 was as expected (**Figure 25E** and **25G**): Groups PP and UP froze more to the light than Group PU in stage 2 ($F(1,21) = 24.078$; $p<0.001$) and again during testing of the light: ($F(1,21) =60.582$; $p<0.001$). However, there were no significant differences between the groups in freezing to the tone at test (**Figure 25F**, $F(1,21)=0.156$, $p=0.697$).

The adapted protocol produced sensory preconditioned fear to S2 in male rats but failed to do so in female rats. Whether this reflects genuine sex differences in the acquisition and/or expression of sensory preconditioning, or differences in the parameters required to reveal integration in females, cannot be resolved from the present data and is considered further in the Discussion. For the purposes of the subsequent experiments in this thesis, which require a stable behavioural baseline against which to assess neural manipulations, we elected to proceed with males only.

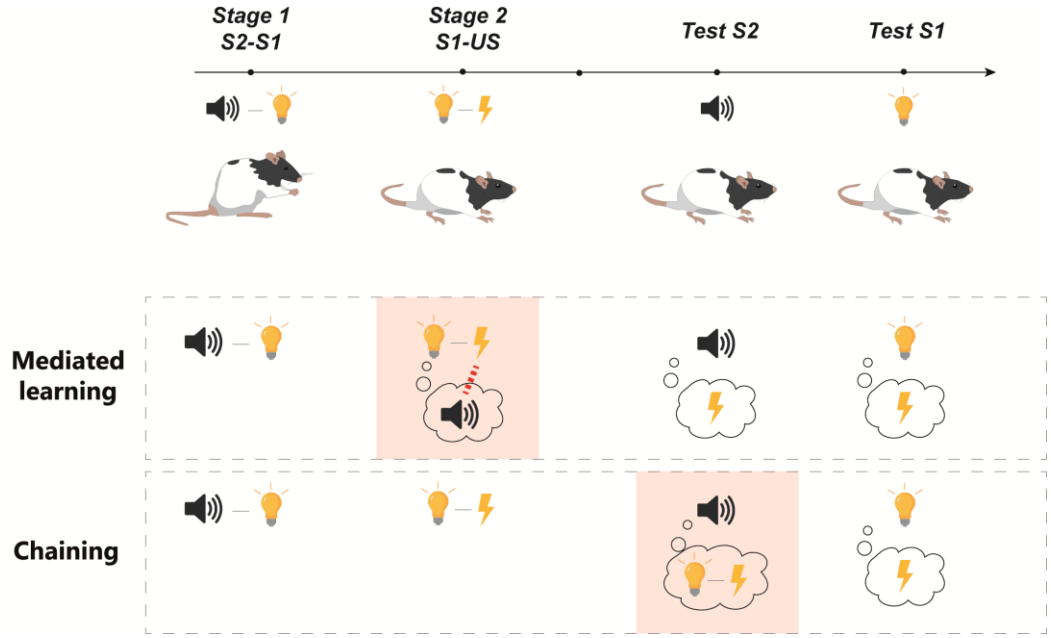
Together, these experiments met the first objective of the chapter: a reproducible sensory preconditioning effect was established in both host laboratories in male rats, although this required a substantial, coordinated adaptation of the original protocol (rather than a direct transfer) in host laboratory 2.

2. Identification of the mechanism of integration in the modified protocol

2.1. Two candidate mechanisms

The demonstration of sensory preconditioned fear to the tone in male rats shows that they can integrate the S2-S1 and S1-US memories formed in training. It does not, by itself, specify *how* the two memories are integrated. Two dissociable mechanisms, presented in the Introduction and summarised in Box 1, have been proposed.

BOX 1. MECHANISMS OF MEMORY INTEGRATION IN SENSORY PRECONDITIONING



Two mechanisms of memory integration can support sensory preconditioned fear:

- According to the ***mediated learning*** account, integration occurs in stage 2. When S1 is paired with the US, S1 associatively activates the representation of S2, and a new S2–US association is formed. On this view, fear to S2 at test is supported by a direct but "false" S2–US memory that is *independent* of whether S1 elicits fear.
- According to the ***chaining*** account, integration occurs during testing with S2 in stage 3 via a serial retrieval process: S2 retrieves a representation of S1, which in turn retrieves the S1→US association and, thereby, generates the fear response.

Modifications of the protocol can change the way memories are integrated (Holmes, Wong, et al., 2022). Namely, in a version with an extended number of S2-S1 pairings in stage 1 (i.e., 32 instead of 8), the mechanism of integration switches from mediated learning in stage 2 to chaining at test (i.e., manipulation of perirhinal cortex in stage 2 has no effect on sensory preconditioned fear to S2 at test, Wong et al., 2019). Because the number of S2-S1

presentations in stage 1 of the adapted protocol has been increased from 8 to 16 compared with the original protocol, it cannot be assumed that the modified procedure engages the same underlying integration mechanism.

2.2. Identifying the mechanism in the modified protocol

We reasoned that, if fear to the tone in the adapted protocol is supported by mediated learning rather than chaining, it should be possible to affect fear to S2 without affecting fear to S1; and, conversely, to affect fear to S1 without affecting fear to S2. Demonstrating that the two memories can be independently manipulated and are doubly dissociable would establish that S2 fear does not depend on the S1→US memory, and hence that integration does not rely on chaining at test but rather on mediated learning during stage 2. We therefore conducted two complementary experiments. In the first of these experiments, we manipulated the shock intensity during first-order conditioning. We expected that the reduction (from 0.6 mA to 0.4 mA) would maintain direct fear to the light (first-order) but abolish sensory preconditioned fear of the tone as the level of US processing during conditioning would be insufficient to support the formation of a mediated tone–US association. Hence, if fear to the tone is affected by a manipulation during stage 2 (when mediated learning is supposed to occur) but fear to the light is spared, then sensory preconditioned fear observed at test (in the higher-intensity shock condition) cannot be a mere by-product of retrieving the light→US memory at test.

The design of this experiment is shown in **Figure 26A**. We replicated the modified protocol in Groups $PP_{0.6}$ and $UP_{0.6}$ and added two other groups, Group $PP_{0.4}$ and Group $UP_{0.4}$. During stage 2, Groups $PP_{0.6}$ and $UP_{0.6}$ received four presentations of the light paired with a 0.6 mA foot shock, whereas Groups $PP_{0.4}$ and $UP_{0.4}$ received the same presentations but the light was paired with a 0.4 mA foot shock. When we tested rats with the tone (**Figure 26C**), Group $PP_{0.6}$ froze significantly more than Group $UP_{0.6}$ ($F(1,28)=5.530$, $p=0.026$, 95%CI=[-22.024; -1.517]) while levels of freezing for Groups $PP_{0.4}$ and $UP_{0.4}$ did not differ ($F(1,28)=1.31$, $p=0.262$), indicating sensory preconditioning can be observed when the shock is 0.6 mA but not when it is 0.4 mA. Critically, there was no effect of pairing, intensity, or pairing x intensity interaction in the analysis of freezing to the light (**Figure 26D**, $F(1,28)=1.646$, $p=0.21$), indicating that the reduction in shock intensity from 0.6 to 0.4 mA had minimal impact on the direct conditioning of this stimulus. These results first replicate the previous sensory preconditioning effect, and second, suggest that with a low-intensity shock US, direct conditioning is preserved but sensory preconditioning is disrupted. This result is consistent with our hypothesis and points to mediated learning as the mechanism of integration in the adapted sensory preconditioning protocol.

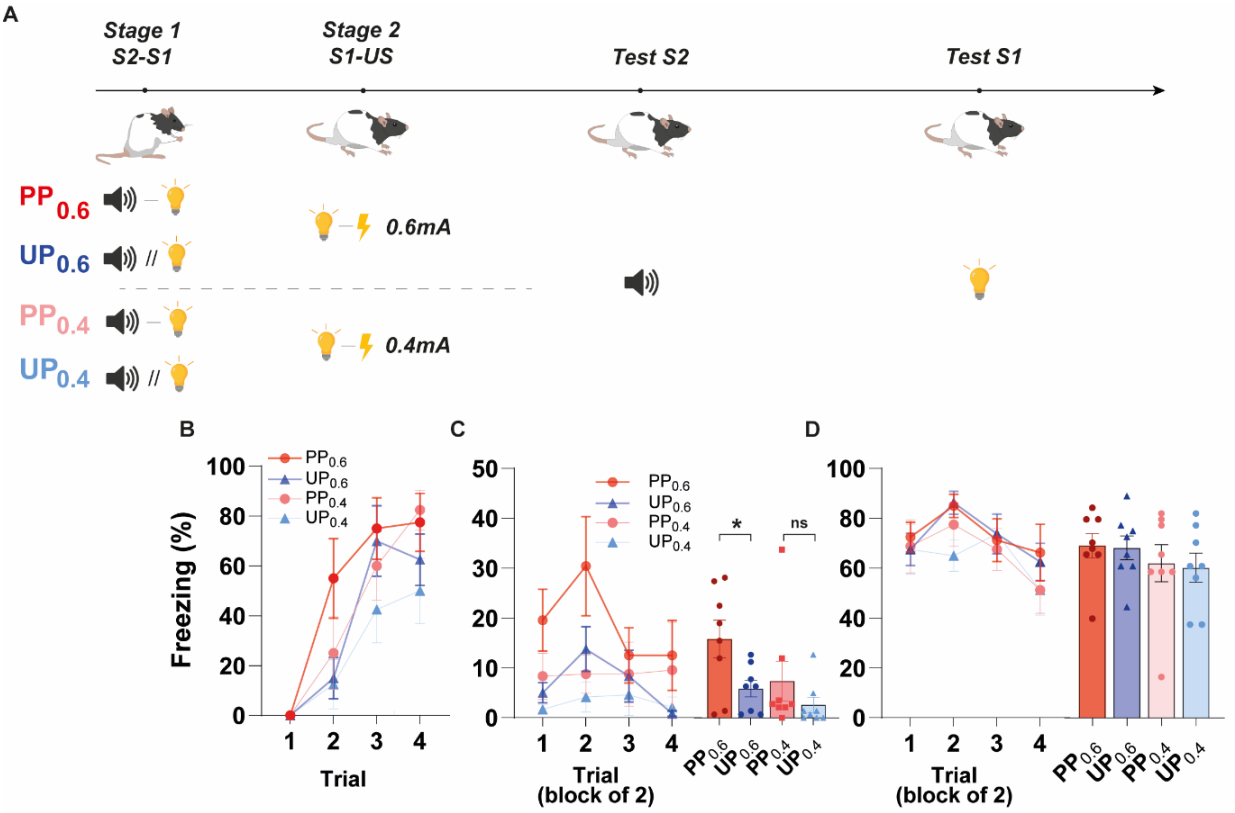


Figure 26. **Lower shock intensity prevents sensory preconditioning but not direct conditioning**

(A) Experimental design. In stage 1, Groups $PP_{0.6}$ and $PP_{0.4}$ received paired presentations of the tone (S2) and light (S1), while Groups $UP_{0.6}$ and $UP_{0.4}$ received interleaved unpaired presentations. In stage 2, all groups received paired presentations of the light and foot shock (US). The US was 0.6 mA for Groups $PP_{0.6}$ and UP0.6, and 0.4 mA for groups $PP_{0.4}$ and $UP_{0.4}$. All groups were subsequently tested for freezing to the tone (Test S2) and light (Test S1). **(B)** Mean (±SEM) freezing during the four S1-US pairings in stage 2. There was a significant effect of pairing ($F[1,28]=5.183$, $p=0.031$), with groups PP freezing more than groups UP, but no effect of intensity nor interaction. **(C, D)** Mean (±SEM) freezing across the 8 test presentations (blocks of 2 trials and averaged over all trials) during Test S2 (tone; C) and Test S1 (light; D). Individual data points are shown in bar graphs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Baseline freezing was measured before the first stimulus presentation at test and was never different between groups. For test to the tone, two planned comparisons were used to compare the mean of Groups $PP_{0.6}$ with UP0.6, and the mean of group $PP_{0.4}$ with $UP_{0.4}$. For stage 2 and testing with the light, we used a two-way ANOVA to test for the effects of pairing, shock intensity and their interaction.

The second, complementary experiment that probed the mechanism of integration involved extinguishing the direct (or first-order) fear of S1 prior to testing for sensory preconditioned fear of the S2. The two mechanisms of integration (mediated learning vs chaining) make opposite predictions for the impact of S1 extinction. If integration occurs through chaining, extinction of S1 should abolish (or substantially attenuate) fear to S2, because S2 fear relies on an intact S1→US association. If, however, integration occurs through

mediated learning, extinction of S1 should leave fear to S2 intact, because the S2 has its own association with the US that is independent of the S1.

To test our hypothesis, we replicated the modified protocol in Groups PP and UP and added two other groups, Group PP_{EXT} and Group UP_{EXT} (**Figure 27A**). After stage 2, Groups PP_{EXT} and UP_{EXT} were exposed to repeated presentations of the light alone while Groups PP and UP remained in their home cage. When the average level of freezing to the light in these groups had decreased to below 20%, extinction was considered successful and all rats were then tested with the tone and the light.

The results are shown in **Figure 27**. Extinction was successful after 4 sessions of 12 light alone presentations, which were administered across 2 days. By the end of the 4^{th} session, the average level of freezing to the light was below 20% (**Figure 27C**), for PP_{EXT} mean freezing was 10.5 +/-1.8%, for UP_{EXT} mean freezing was 11.6 +/- 4.6%). Critically, this extinction had no effect on fear to the tone at test (**Figure 27D**). That is, the analysis of freezing to the tone at test revealed an effect of pairings ($F(1,36)=14.14$, $p=0.001$) but no effect of extinction (largest $F(1,36)=0.428$, $p=0.51$) and no pairings x extinction interaction. By contrast, testing to the light confirmed that Groups PP_{EXT} and UP_{EXT} extinguished successfully (**Figure 27E**) as their levels of freezing to the light were significantly lower than those in Groups PP and UP ($F(1,36)=32.78$, $p<0.001$). There was no effect of pairing nor interaction in this analysis. Thus, these results are an additional replication of sensory preconditioning with the settings of our modified protocol, and show that sensory preconditioned fear to the tone was maintained following extinction of direct fear to the light. Hence, sensory preconditioned fear of the tone and direct fear of the light are independent of each other.

More generally, the pattern of results within and across the two experiments is consistent with integration via mediated learning: the change in shock intensity affected fear to S2 without affecting fear to S1, and the repeated S1 alone exposures reduced fear to this stimulus without affecting fear to S2. These findings indicate that both the original and adapted protocols engage the same mechanism of integration, mediated learning, and thus provide the psychological grounding required for the neural investigations in the following chapters.

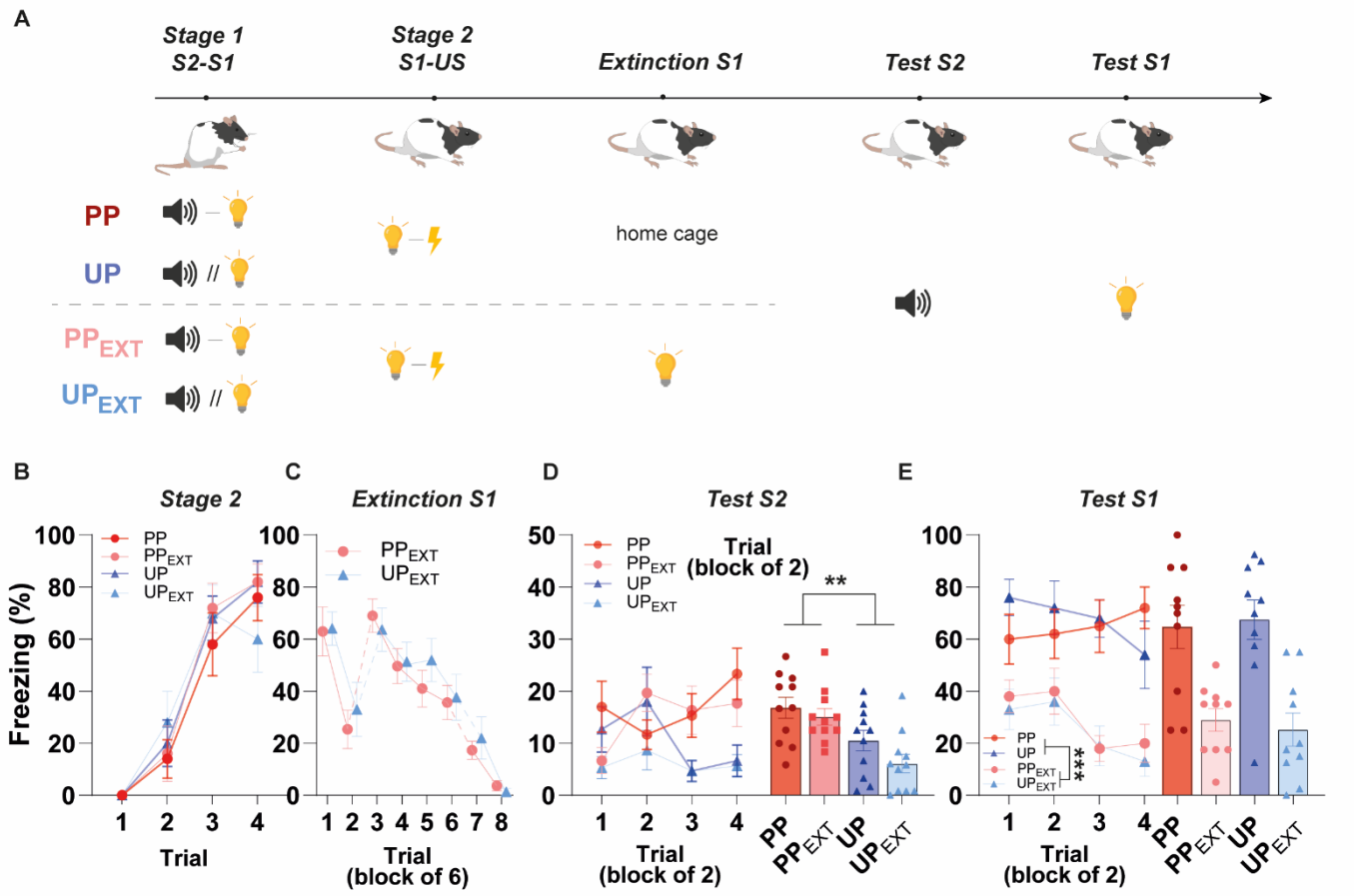


Figure 27. **Extinction of fear to the light spares fear to the tone**

(A) Experimental design. In stage 1, Groups PP and PP_{EXT} received paired presentations of the tone (S2) and light (S1), while Groups UP and UP_{EXT} received interleaved unpaired presentations. In stage 2, all groups received paired presentations of the light and foot shock (US). During extinction, PP_{EXT} and UP_{EXT} received 48 presentations of the light alone across 4 sessions. Groups PP and UP remained in their home cages. All groups were subsequently tested for freezing to the tone (Test S2) and light (Test S1). **(B)** Mean (±SEM) freezing during the four S1-US pairings in stage 2. **(C)** Mean (+/-SEM) freezing during extinction of the light (blocks of 6 trials). Dashed lines indicate 12 hour-delay between sessions. **(D, E)** Mean (±SEM) freezing across the 8 test presentations (blocks of 2 trials and averaged across all trials) during Test S2 (tone; D) and Test S1 (light; E). Individual data points are shown in bar graphs. *p < 0.05, **p < 0.01, ***p < 0.001. Baseline freezing was measured before the first stimulus presentation at test and was never different between groups. For all stages except extinction, 2-way ANOVAs were used to test the effects of pairing, extinction and their interaction. For extinction, one-way ANOVA was used to test for group differences.

Discussion

The experiments reported in this chapter had two purposes: to establish a reliable sensory preconditioning procedure in the two host laboratories for this thesis, and to identify the mechanism of memory integration in that procedure.

Perhaps the most salient outcome of this chapter is that a widely used sensory preconditioning protocol did not transfer without modification from host laboratory 1 (Sydney, Australia) to host laboratory 2 (Bordeaux, France). That is, whereas the procedure produced robust sensory preconditioned fear to S2 in host laboratory 1 where it was well established, the identical protocol failed to yield reliable sensory preconditioning in host laboratory 2. This observation resonates with a broader and increasingly recognised concern in behavioural neuroscience regarding the reproducibility of complex learning paradigms across sites (Crabbe et al., 1999; Kafkafi et al., 2018). Sensory preconditioning is, by its nature, a comparatively fragile phenomenon: fear to S2 depends on the successful integration of two associations, each of which must be neither too weak (such that the constituent memories fail to form) nor too strong (such that, for example, over-conditioning of S1 reduces learning about S2). The effect therefore sits within a relatively narrow parametric window: hence, it is unsurprising that inter-laboratory differences in animals' exact age and weight, apparatus and chamber acoustics, ambient stimulation, handling by non-experimenters should be sufficient to move it outside that window. Nonetheless, the adaptations that we introduced in host laboratory 2 ultimately yielded a robust sensory preconditioning effect in male rats. It was indeed replicated not only for the demonstration but also during the following experiments (reduction of S1 intensity, extinction of S1), strengthening the reliability of our results.

Notably, these adaptations were not sufficient to permit detection of sensory preconditioning in female rats. Several interpretations of this finding are possible. The first is that females did integrate the associations formed in training but that our proxy for this integration, freezing as the index of conditioned fear, was not sensitive to its detection. Freezing is a passive response, and female rodents have been shown to express conditioned fear through active behaviours instead (Gruene et al, 2015), so a freezing-based readout often underestimates their fear. In line with this, Shanazz et al. (2022) found that females froze less than males and yet avoided a shock-associated context just as strongly, indicating that a comparable fear memory is perhaps expressed in a different way; and Chu et al. (2024) reported that females freeze only briefly at cue onset before returning to baseline. The implications of these studies are that freezing as the index of conditioned fear may be sub-optimal for the detection of sensory preconditioning in female rats, particularly as sensory preconditioned fear typically reveals itself in a lower level of responding compared to what is observed following a direct conditioning

protocol. Uncontrolled oestrous cycle variation may also have added noise, as gonadal hormones modulate the circuitry supporting fear learning and its extinction, in ways that interact with stress (Maeng & Milad, 2015). More broadly, sex differences influence the developmental trajectory and neural mechanisms of fear learning: contextual fear develops in opposite directions in males and females, with these differences emerging before puberty (Colon et al., 2018). The second possibility is that females genuinely did not integrate the two memories formed in stages 1 and 2 of the sensory preconditioning protocol. Consistent with this possibility, Pinho and colleagues (2025) showed that female mice exhibit more fear generalisation when tested under the same conditions as males in a light-tone preconditioning protocol (see also Colon et al., 2018). However, it should be noted that the original protocol in host laboratory 1 yields robust sensory preconditioning in mixed-sex cohorts (Wong et al., 2019, 2026), suggesting that the failure of sensory preconditioning in female rats at host laboratory 2 is more likely to result from sex differences in the way that fear is expressed and/or other parametric factors that have not yet been considered.

The second part of the chapter addressed the psychological mechanism of integration in the adapted protocol at host laboratory 2. This was necessary to ensure that the analysis of the neural substrates of memory integration was focused on the correct stage of the protocol (stage 2 for mediated learning, test for chaining); and interpreted correctly with reference to underlying behavioural/psychological processes. Mediated learning and chaining both achieve integration between S2 and the US but operate at different stages or moments of the protocol. Mediated learning occurs at the time of conditioning in stage 2 and builds a memory of a pairing that never physically took place. Chaining, by contrast, forms no such link during conditioning and is instead a retrieval-based process that occurs at test. Importantly, the two mechanisms of integration also differ in their predictions for how manipulations that affect fear to S1 will affect fear to S2 and vice versa. Mediated learning implies an S2-US association that stands somewhat independently of the directly conditioned S1-US association: hence, manipulations which affect fear to S1 will not automatically affect fear to S2, and conversely. By contrast, chaining of the S2-S1 and S1-US associations at test implies that fear to S2 should be reducible in ways that preserve fear to S1; but manipulations that affect fear to S1 will automatically reduce fear to S2.

The first manipulation that we selected for this part of our investigation was a reduction in the shock intensity during conditioning of the S1 (Experiment 1). The reduction in shock intensity reduced sensory preconditioned fear of S2 without affecting directly conditioned fear of S1. This is consistent with integration via mediated learning and has a basis in associative theory. In Wagner's SOP model, the level of activation in A1 scales with the stimulus intensity on a given trial, meaning that a reduced US intensity decreases the pool of US elements in

A1. During stage 2, while S1 and the US are processed in A1, the associatively activated S2 sits in the peripheral state A2. Because S2 and the US are in different states, the excitatory association between them is more sensitive to the US intensity than the association between S1 and the US. Thus, a shock that is sufficient to support learning about a fully activated S1 is insufficient to establish a new association with the activated representation of S2, as was observed in the experiment.

The results seen under the SOP model imply several testable predictions. First, fear to S2 at 0.4 mA should be recoverable by increasing the number of conditioning trials, providing additional opportunities for the activated S2 representation to enter into association with the US. Alternatively, without changing the S1-US pairings, mediated learning could be restored with any manipulation that increases the proportion of S2 sitting in A2 during stage 2. For example, opioid receptor antagonism enhances sensory preconditioning when administered during the preconditioning stage, an effect its authors attributed to associative formation rather than to US processing (Michalscheck et al., 2021). Such a manipulation predicts an increase in fear to S2 at fixed US intensity and could restore the deficit in mediated learning at lower shock intensities. Last, fear to both S1 and S2 should increase with US intensity, but the function for S2 should be shifted towards higher intensities, such that a stronger shock is required to produce any given level of fear to S2 than to S1.

The second manipulation that we selected to investigate the mechanism of integration in the adapted sensory preconditioning protocol involved extinction of fear to S1 prior to testing for fear of the S2 (Experiment 2). In a previous study, Rizley and Rescorla (1972) used a similar design and found that extinguishing fear of S1 dramatically reduced sensory preconditioned fear of S2, in contrast to the present finding that extinguishing fear of S1 completely *spared* sensory preconditioned fear of S2. A possible explanation for these seemingly contradictory results is that, in the Rizley and Rescorla study, rats received a greater number of S2-S1 pairings in stage 1, which may have resulted in chaining of the S2-S1 and S1-US associations at test rather than formation of a mediated S2-US association during conditioning of S1 (see Holmes et al., 2022). That is, while rats in the Rizley and Rescorla study appeared to integrate the two associations formed in training at the time of test in stage 3, rats in our study appeared to integrate the two associations during conditioning in stage 2, consistent with the impact of the shock intensity reduction in the preceding experiment.

Notably, whether manipulations shift from a predominant mediated learning to chaining, or vice versa, could also be reflected in the microstructure of behavioural response at test. That is, not only the averaged freezing percentage is informative, but also the frequency or timing of the freezing episodes during a stimulus presentation. Because associative chaining reconstructs the sequence online, it should leave a temporal signature in behaviour: fear to S2

should develop across the trial and approach its maximum around the moment at which the US would be expected given the sum of the S2–S1 and S1–US intervals, whereas a mediated S2–US memory should generate responding time-locked to S2 onset in the same way as first-order fear. This is the logic underlying the temporal-coding evidence obtained by Miller and colleagues (Barnet et al., 1997; Arcediano et al., 2003), with existing observation in rats' orbitofrontal response to a preconditioned cue reported by Sadacca et al. (2018). In humans, the discrimination between mediated learning and associative chaining can be made by reaction time alone (Nicholas et al., 2025). In our experiments, a within-trial shift of freezing to S2 from onset to delayed responding as stage 1 training is extended would be converging evidence that the parameters influence the integration mechanism.

Taken together, the experiments in this chapter established sensory preconditioned fear in both host laboratories and identified mediated learning as the mechanism of integration in the protocol that was adapted for use in host laboratory 2. That is, fear of the tone in the adapted protocol reflects the retrieval-mediated formation of an integrated tone-shock representation, independent of the light→shock memory, rather than a chaining process operating at test. This identification is essential for what follows: it identifies the critical moment at which integration occurs, which circuit-level interventions should be targeted, and provides a clear framework for interpretation of the subsequent results. The next chapters examine the neural circuits that support this integration. We use the original version of the protocol for behavioural and pharmacological experiments that were conducted in host laboratory 1; and the modified version for the fiber photometry experiments that were conducted in host laboratory 2.

Chapter 3

Roles of the dorsal and ventral hippocampus in auditory-visual sensory preconditioning

Significance statement

Chapter 2 established that the auditory-visual protocol of sensory preconditioning relies on mediated learning, with an integrated S2-shock representation formed during conditioning rather than inferred at test. This chapter uses that framework to ask what the hippocampus contributes to this integration, and whether its dorsal (dHPC) and ventral (vHPC) subregions perform distinct computations, complementary ones, or work together.

What was done. To assess the contributions of dHPC and vHPC to encoding and integration, each subregion was inactivated with muscimol before stage 1 (tone–light pairings) or stage 2 (light–shock pairings), and rats were tested drug-free for fear of the preconditioned tone and directly conditioned light. Calcium activity was then recorded simultaneously in both subregions using fiber photometry across both stages, in rats given paired or unpaired tone–light presentations. Regional stimulus-evoked responses, trial-by-trial co-modulation and within-trial coupling were analyzed, and a composite connectivity index was related to fear at test. Finally, c-Fos expression was quantified after stage 2 in the dHPC, vHPC, perirhinal cortex (Prh) and basolateral amygdala (BLA), and analyzed as an inter-regional correlation structure to identify the network signature of mediated learning.

What was found. The two subregions were doubly dissociated. The dHPC was dispensable during stage 1 but required during stage 2 specifically for mediated fear, whereas the vHPC was required during stage 1 for the tone–light memory and during stage 2 for both mediated and direct fear. The recordings reflected this division of labor. During stage 1, paired rats showed enhanced tone-evoked responses in the vHPC, particularly early in learning, while dHPC and vHPC responses co-varied across trials despite the dHPC being unnecessary. During stage 2, regional responses did not distinguish paired from unpaired rats, but dHPC–vHPC coupling was elevated in paired rats across most of the stimulus window. This coupling predicted fear of the tone in paired rats and fear of the light in unpaired rats. Finally, mediated learning reorganized a broader network: the multivariate c-Fos profile classified rats by group, the overall correlation structure was reconfigured, and the vHPC–Prh correlation was the only individual inter-regional correlation that differed between groups.

Why it matters. Our results provide a stage-resolved **causal dissociation of dorsal and ventral hippocampus** within a single sensory preconditioning protocol, and address two limitations that have constrained the existing literature: use of permanent lesions, which confound all stages of the task, and consideration of the hippocampus as a single structure. It also shifts the question of integration from where and when to how. Because the signature of integration was inter-regional coordination rather than regional activation, **it is coordination along the longitudinal axis, and not the activity of either pole taken alone, that distinguishes whether two memories are combined**. These results identify **dorsoventral coupling as a potential mechanism by which fear spreads beyond experienced contingencies**. Finally, the dissociation maps onto known extrinsic connectivity, positioning the **vHPC–Prh circuit to encode the sensory association and the dHPC as the site where the sensory and emotional associations converge** for mediated learning specifically.

Publication [submitted]

Coordination of dorsal and ventral hippocampal activity during memory integration

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Abstract

The brain often infers relationships between events that have not been experienced together. It does so by integrating experiences with common features in memory. The hippocampus is critical for this process of memory integration. However, at present, it is unknown how its dorsal (dHPC) and ventral (vHPC) subregions contribute to the integration, and/or whether the two regions work together so that the integration is successful. Using stage-specific pharmacological inactivation and dual-site fiber photometry in a sensory preconditioning protocol with rats, we show that the vHPC selectively encodes the details of a distinct sensory memory, and that activity in both hippocampal subregions is required for its subsequent integration with a new emotional memory. Specifically, successful integration was accompanied by enhanced dHPC-vHPC functional connectivity at key moments in the protocol as well as a reorganization of hippocampal-cortical-amygdalar network activity. Hence, the coordination of activity in hippocampal subregions determines whether separate memories are successfully integrated and, thus, the inferences made about relations between events.

Introduction

Animals and humans adapt to their environments by learning to predict motivationally significant events such as the presence of food or danger. These predictions are often based on direct experience of the contingencies between different stimuli and events (e.g., a sound followed by the attack of a predator) but can also be generated in the absence of such experience. For example, by linking the sensory and emotional elements of distinct memories, we can generate novel inferences about stimuli that have only ever been experienced as innocuous: if A and B occur together at time 1 and B signals danger at time 2, we might infer that A also signals danger when it is next encountered. This type of inference has implications for how memories are encoded and stored in the brain; and raises the question of how they are integrated to generate adaptive behavior.

The hippocampus has been shown to be critical for the encoding and integration of memories in a range of species, including humans, rats and mice. The hippocampus is thought

to bind distributed sensory input (including information about space and time) into coherent, event representations (Cohen & Eichenbaum, 1993; Eichenbaum, 2004). It is also important for retrieval-based memory integration, whereby a current experience reactivates a prior memory and enables the formation of a link between otherwise disparate events (in rats, Iordanova et al., 2011; Wheeler et al., 2013; in humans, Molitor et al., 2021; Zeithamova, Schlichting, et al., 2012). The success of this integration has been shown to be predicted by hippocampal connectivity with a network of cortical and subcortical brain regions (Shohamy & Wagner, 2008; Wang, Schoenbaum, et al., 2020; Wimmer & Shohamy, 2012; Zeithamova, Dominick, et al., 2012; Zeithamova & Preston, 2010; Zhu et al., 2022). However, little is known about the role of intra-hippocampal connectivity in the encoding and integration of associative memories. Studies that have causally manipulated activity in the hippocampus and assessed the consequences for these processes have targeted the dorsal hippocampus (dHPC) in isolation or in combination with the ventral hippocampus (vHPC), leaving the specific role of the vHPC in memory unexamined. This is important as the dHPC and vHPC have different extrinsic connectivity profiles, especially with sensory- and emotional-processing brain regions. The dHPC is preferentially connected with the caudolateral entorhinal and retrosplenial cortices, and receives stronger input from postrhinal cortex. By contrast, the vHPC is preferentially connected with the perirhinal cortex (Prh) and rostromedial entorhinal cortex, and exclusively receives direct projections from the basolateral amygdala (BLA) and projects to medial prefrontal cortex. The implication of these different connectivity profiles is that the two hippocampal subregions will be differentially involved and may thus coordinate during the encoding and integration of distinct sensory and emotional events.

To study how dHPC and vHPC differentially contribute to the encoding and integration of sensory and emotional memories, we used a sensory preconditioning task in rats. Sensory preconditioning typically involves three stages: in stage 1, two innocuous stimuli (S2 and S1, such as a tone and a light) are paired together; in stage 2, S1 is conditioned through pairings with an aversive unconditioned stimulus (US; electric foot-shock); at test, S2 elicits fear responses despite never having been directly paired with the shock. These fear responses reflect integration of the sensory (tone-light) memory acquired in stage 1 and the emotional (light-shock) memory acquired in stage 2; specifically, the light activates the memory of the tone in stage 2, which allows for mediated learning about the tone (i.e., mediated tone-shock association) that is retrieved and expressed at test (Holmes, Wong, et al., 2022; Wong et al., 2019, 2025). Using stage-specific pharmacological inhibition, we show that vHPC selectively encodes the tone-light memory in stage 1 but that both subregions are required to encode the mediated tone-shock memory in stage 2. Calcium imaging in both subregions then reveals that successful mediated learning is associated with greater intra-hippocampal functional

connectivity during stimulus presentations, and that this coordination is part of a broader reorganization of network activity. Together, our results provide comprehensive and causal evidence for dissociable roles of the dHPC and vHPC in sensory preconditioning; and demonstrate that it is the coordination of activity in the two subregions rather than their individual activity that tracks whether memories are successfully integrated.

Results

Sensory preconditioning requires the integration of two associations

We first aimed to demonstrate sensory preconditioning using auditory and visual stimuli in rats (Figure 1A, Table S1). Rats in the group of interest were exposed to S2-S1 (tone-light) pairings in stage 1 and S1-US (light-shock) pairings in stage 2. They were expected to show fear responses when presented with the S2 [tone] alone at test. In order to confirm that these fear responses reflect the encoding and integration of associations formed in stages 1 and 2, we used two control groups. The first received the same training except during stage 1, when it was exposed to explicitly unpaired presentations of the S2 and S1 [Group UP]. The second received the same training in stage 1 and testing, but was exposed to explicitly unpaired presentations of the S1 and US in stage 2 [Group PU]. If fear of the S2 in Group PP is associatively mediated, it should be greater in this group than in either of the two controls where the associative mediation is not possible.

Consistent with our predictions, rats in Group PP demonstrated successful sensory preconditioning (Figure 1B, middle panel, Table S1). During the test of the tone, while baseline levels of freezing at test were low (<5% for all groups), Group PP froze significantly more than Groups UP and PU when the tone was presented ($F(1,21) = 10.914$; $p=0.003$; 95% CI: [6.986;30.723]). Groups UP and PU did not differ from each other in their level of freezing ($F=0.001$, $p=0.975$), showing that freezing to the tone is not due to generalization from the light or the context. Indeed, the pattern of freezing across each of the groups during testing with the light was completely different and reflective of their training history; Groups PP and UP exhibited higher levels of freezing than Group PU ($F(1,21) = 79.825$; $p<0.001$; 95% CI: [-72.040;-44.835]), without differing from each other ($F=1.157$, $p=0.294$).

These results show that rats acquire fear of a stimulus (tone) that was never directly paired with danger. This fear of the tone (evident in freezing) reflects integration of associations formed in stages 1 and 2. Specifically, it is dependent on an association between the tone and the light in stage 1, and between the light and the shock in stage 2 rather than generalization of fear from the light or the context.

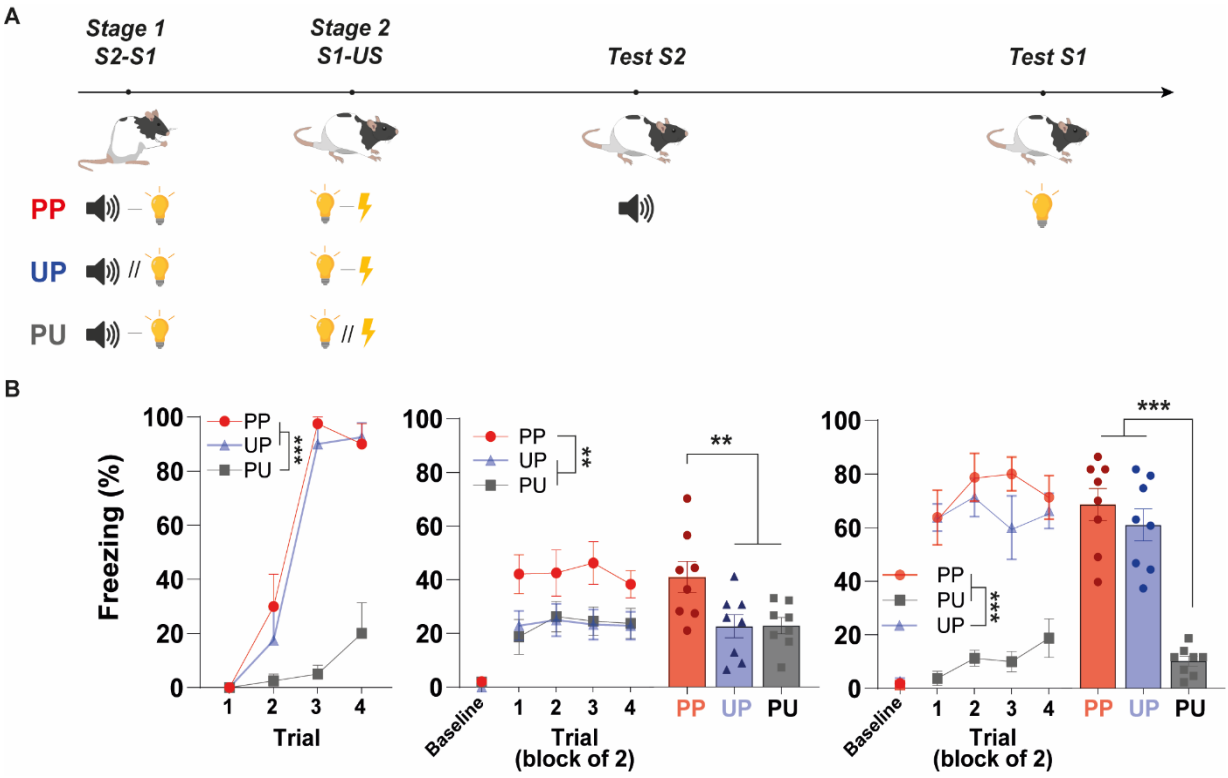


Figure 1. Sensory preconditioned fear of a tone depends on the encoding and integration of tone-light and light-shock associations

(A) Experimental design of the sensory preconditioning task. In Stage 1, Groups PP and PU received paired presentations of the tone (S2) and light (S1), while Group UP received unpaired presentations. In Stage 2, Groups PP and UP received paired presentations of the light (S1) and footshock (US), while Group PU received unpaired presentations. All groups were subsequently tested for freezing to the tone (Test S2) and light (Test S1). **(B) Left**: mean (±SEM) freezing during the four S1 presentations in Stage 2 (*n* = 8 per group). Groups PP and UP exhibited significantly more overall freezing to the light than Group PU ($F(1,21) = 83.056$; $p < 0.0001$; 95% CI: [-55.652; -34.973]) but did not differ from each other ($F=0.581$, $p=0.454$). The presentation x group interaction was significant, indicating that Groups PP and UP learnt at a different rate than Group PU ($F(1,21) = 66.36$; $p < 0.0001$; 95% CI: [-88.26;-52.363]), without differing from each other ($F=0.098$, $p=0.757$). **Middle and right**: mean (±SEM) freezing across the 8 test presentations (blocks of 2 trials and average over all trials) during S2 test (tone; middle) and S1 test (light; right). Individual data points are shown in bar graphs. Error bars represent SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Baseline freezing was measured before the first stimulus presentation at test and was never different between groups (largest $F=2.649$, $p=0.118$). For test levels of freezing to the tone, the first contrast compared Group PP versus the weighted average of Groups PU and UP; and the second contrast compared Group PU versus Group UP. For test levels to the light, the first contrast compared the weighted average of Groups PP and UP vs Group PU, and the second contrast compared Group PP vs UP.

The dorsal hippocampus is selectively required for mediated learning

We next examined the involvement of the dorsal (dHPC) and ventral (vHPC) hippocampus in stages 1 and 2 of the sensory preconditioning protocol. To test whether and when hippocampal activity is causally required for sensory preconditioning, we selectively inhibited dHPC or vHPC using infusions of the GABA agonist muscimol during tone-light pairings in Stage 1 or light-shock pairings in Stage 2. We assessed the consequences of these infusions for expression of sensory preconditioned fear of the tone at test (Figure 2A). Rats in Group MUS/VEH received a muscimol infusion prior to stage 1, rats in Group VEH/MUS received a muscimol infusion prior to stage 2, and rats in Group VEH/VEH received vehicle infusions prior to stages 1 and 2. Cannula placements were verified histologically (Figure 2B, 2F). Final *n*s per group for the dHPC experiment were $n_{VEH/VEH}$=14, $n_{MUS/VEH}$=11 and $n_{VEH/MUS}$=12; and for the vHPC experiment were $n_{VEH/VEH}$=10; $n_{MUS/VEH}$=10 and $n_{VEH/MUS}$=11 .

When infusions took place in the dHPC (Figure 2B-E, Table S1), there was a selective impairment of freezing to the tone only among rats that received muscimol in stage 2 (Group VEH/MUS). Given prior evidence of dHPC involvement in both stages, we contrasted Group VEH/VEH with the weighted average of Groups MUS/VEH and VEH/MUS. Although this contrast was significant ($F(1,34)=6.491$, $p=0.016$, 95%CI [2.40, 21.30]), the effect was entirely driven by Group VEH/MUS. Indeed, follow-up pairwise comparisons with Bonferroni correction showed that freezing of Group VEH/MUS was significantly lower than both VEH/VEH ($F(1,34)=15.226$, $p_{corr}<0.01$) and MUS/VEH ($F(1,34)=10.34$, $p_{corr}=0.009$), which did not differ from each other ($F(1,34)=0.229$, $p=0.635$). This suggests that the dHPC is not required for encoding of the tone-light memory during stage 1 but contributes to mediated learning about the tone during stage 2. Critically, there were no group differences in freezing to the light at test (Figure 2E, largest $F=0.624$, $p=0.435$), indicating that the dHPC is not required for encoding of a direct light-shock association. Importantly, these results were not modality-dependent, as inhibiting dHPC during stage 2 when the preconditioned stimulus was a light and the direct conditioned stimulus a tone produced the same deficit in freezing to the light at test, without affecting freezing to the tone (Figure S1).

Together, these results demonstrate that activity in the dHPC is selectively required for the learning of a mediated tone-shock association at the time of conditioning to the light. It is not required to learn an innocuous tone-light association or a direct light-shock association.

The ventral hippocampus is required for both direct and mediated learning in stages 1 and 2

When infusions took place in the vHPC (Figure 2F-I, Table S1), freezing to the tone was impaired among rats that received muscimol in either stage of training (Figure 2H). That is, rats in Groups MUS/VEH and VEH/MUS froze significantly less to the tone than rats in Group VEH/VEH ($F(1,28)=20.41$; $p<0.001$; CI: [-32.513, -12.228]). Rats in Group VEH/MUS also froze less to the tone than rats in Group MUS/VEH ($F(1,28)=5.63$; $p=0.025$; CI: [-24.889;-1.830]). These results suggest that muscimol infusions in the vHPC impair rats' ability to form a tone-light memory in stage 1 and a mediated tone-shock memory in stage 2.

In this experiment, we anticipated that the vHPC muscimol infusion might disrupt direct learning about the light (Esclassan et al., 2009b; Maren & Holt, 2004). For this reason, we repeated the experiment and tested all rats first with the light instead of the tone. Among these rats (n=9 per group), those in Group VEH/MUS froze significantly less than rats in the two other groups (Figure 2I, $F(1,24)=23.17$; $p<0.001$, $p\eta^2=0.49$; CI: [-53.380;-21.342]), which did not differ from each other ($p=0.92$). This shows that vHPC inhibition during Stage 2 prevented the acquisition of the direct light-shock association. Consistently, this pattern emerges during Stage 2 (Figure 2G, left panel). The rate of acquisition in Group VEH/MUS was significantly lower than that of rats from the two other groups ($F(1,24)=37.83$; $p<0.001$; CI: [-77.906;-38.761]), which did not differ from each other ($F=1.483$, $p=0.235$). The mean (± SEM) levels of freezing to the light on its final pairing with shock were 71.1 ± 13% in Group VEH/VEH, 82.2 ± 9% in Group MUS/VEH, and 6.7 ± 4.8% in Group VEH/MUS, resulting in an effect of group on the overall freezing to the light ($F(1,24)=25.743$; $p<0.001$; CI: [-56.271;-23.729]). As confirmed by the subsequent reduced level of freezing to the light during drug-free test, this impairment reflects that the vHPC is required for the formation of a direct light-shock association, and, possibly, for the expression of fear across presentations.

Taken together, these results show that, unlike the dHPC, activity in the vHPC is required during stage 1 for the formation of a tone-light memory and during stage 2 for both conditioning of the light and formation of a mediated tone-shock memory.

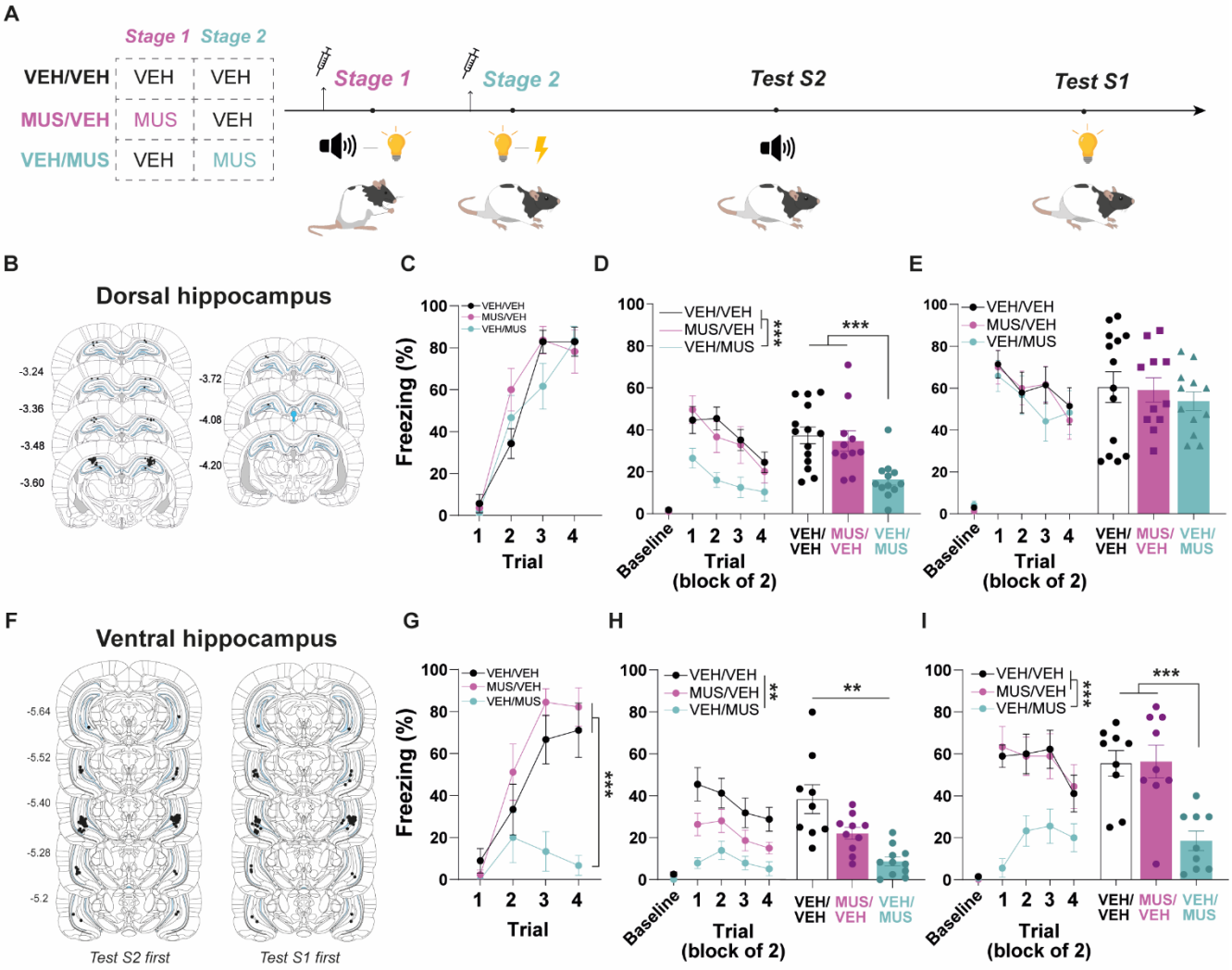


Figure 2. Dorsal and ventral hippocampus have different roles in sensory preconditioning.

(A) Experimental design. In Stage 1, Group MUS/VEH received an infusion of muscimol in the dorsal (dHPC) or ventral (vHPC) hippocampus while Groups VEH/VEH and VEH/MUS received saline. In stage 2, Group VEH/MUS received an infusion of muscimol in the same region while Groups VEH/VEH and MUS/VEH received saline. All groups were tested drug-free to the tone and the light. **(B, F)** Most ventral part of the cannulas in dHPC (**B**) or vHPC (**F**) as verified on Nissl-stained sections. **(C-E)** Results for inhibition of the dHPC. **(G-I) Results for inhibition of the vHPC**. **(C,G)** Mean (±SEM) freezing during the four light presentations of stage 2. In the dHPC experiment, groups did not differ in their rate of acquisition (largest F = 0.711; p = 0.405), or in their overall freezing to the light (largest F= 1.043; p = 0.31). See text for vHPC experiment results. **(D,H)** Mean (±SEM) freezing across the 8 test presentations (blocks of 2 trials and average over all trials) during S2 test. **(E,I)** Mean (±SEM) freezing across the 8 test presentations (blocks of 2 trials and average over all trials) during S1 test. Individual data points are shown in bar graphs. *Baseline freezing was measured before the first stimulus presentation and did not differ between groups across experiments (largest F=3.127, p=0.08). Error bars: SEM. *p < 0.05, **p < 0.01, ***p < 0.001. For test levels of freezing to the tone, the first contrast compared Group VEH/VEH versus the weighted average of Groups VEH/MUS and MUS/VEH; and the second contrast compared Group VEH/MUS versus Group MUS/VEH. For test levels to the light, the first contrast compared the weighted average of Groups VEH/VEH and MUS/VEH vs the average of Group VEH/MUS, and the second compared Group VEH/VEH vs Group MUS/VEH.*

Preferential vHPC recruitment and between-trial dHPC-vHPC coupling during stage 1 are signatures of successful sensory preconditioning

Our previous results establish that the dHPC and vHPC have different roles in sensory preconditioning. While the vHPC alone was required for encoding of the tone-light memory in Stage 1, both subregions were required for encoding of the mediated tone-shock memory in Stage 2. These findings suggest that memory integration may depend on interactions between dHPC and vHPC. We therefore asked whether sensory preconditioning is supported by coordinated activity across the two major hippocampal subregions. To address this, we simultaneously recorded calcium signals from both the dHPC and vHPC throughout stages 1 and 2 using dual-site fiber photometry in rats that received paired (PP) or unpaired (UP) presentations of the tone and light in stage 1 (Figure 3A).

Firstly, we verified sensory preconditioning among rats in Group PP (Figure S2B-C, Table S1). During Stage 2, there was a significant linear increase in freezing across the four light-shock pairings ($F(1,23) = 207.8$; $p < 0.0001$; 95% CI: [60.216; 80.393]). There was no significant effect of group or trial x group interaction, indicating that Groups PP and UP did not differ from each other in their rate of acquisition ($F=2.436$, $p=0.132$) or overall level of freezing to the light ($F=1.44$, $p=0.242$). During testing with the light, levels of freezing in Groups PP and UP remained the same ($F=0.319$, $p=0.578$), indicating successful retention of the direct light-shock conditioning memory. By contrast, during testing with the tone (right panel), rats in Group PP froze significantly more than rats in Group UP ($F(1,22) = 10.242$; $p=0.004$; 95% CI: [-21.516; -4.595]), indicating successful sensory preconditioning of the tone in the former group.

To characterize hippocampal activity across multiple levels of organization, we combined analyses of local and inter-regional dynamics.

We first quantified stimulus-evoked responses in dHPC and vHPC separately to assess regional activity patterns. In Stage 1 (Figure 3B, Table S1), neither tone- nor light-evoked response amplitude (AUC) differed between groups, and no region × group interaction was detected (Figure 3C). Similarly, light-evoked peak amplitudes did not differ between groups. In contrast, tone-evoked peak amplitude revealed significant main effects of region ($F(1,12) = 7.897$, $p = 0.016$ $p_{corr}=0.032$) and group ($F(1,21) = 5.260$, $p = 0.032$, a tendency once correction was applied $p_{corr}=0.064$), with no significant region × group interaction ($F(1,12) = 1.962$, $p= 0.186$). Based on prior evidence that vHPC but not dHPC is required during Stage 1, we performed a priori planned comparisons within each region. Tone-evoked peak amplitude was significantly greater in the vHPC of PP than UP rats (LS mean difference = 0.678, 95% CI [0.124, 1.232], $p=0.018$, $p_{corr}=0.036$), whereas no group difference was observed in dHPC ($p = 0.417$). Complementary comparisons between regions showed that vHPC responses were

significantly greater than dHPC responses in PP rats (p = 0.009, pcorr = 0.019), whereas no regional difference was detected in UP rats (p = 0.354). Thus, vHPC showed selective enhancement of tone-evoked activity in rats for which the tone was predictive of the light. To determine whether this elevated vHPC response reflected active associative encoding, we compared tone-evoked responses during early (1–4) and late (12–16) trials of preconditioning in PP rats (Figure 3D, Table S1). Peak amplitude analysis revealed a significant trial x region interaction (F(1,16)=6.462, p=0.022). Post hoc comparisons with *Bonferroni correction* showed that vHPC responses were greater during early than late trials (LS mean difference = 0.537, 95% CI [0.093, 0.981], p_{corr}=0.041), whereas dHPC responses did not differ across epochs (p_{corr}=0.71). These findings indicate that enhanced vHPC recruitment was most pronounced during the initial phase of tone–light learning, decreasing across presentations.

We then examined inter-regional dynamics and coordination between the two. First, in order to track the dynamics of co-modulation across areas, we assessed trial-by-trial co-variations in response amplitude (noise correlations) to determine whether the two subregions were collectively engaged across learning. Noise correlations, as defined by the trial-by-trial Pearson correlation between dHPC and vHPC response amplitudes (AUC), reflect shared variability between subregions beyond their mean stimulus-evoked activity. Then, in order to investigate the temporal correlation in neural activity, we computed dynamic functional connectivity (DFC) time-locked to stimulus onset to determine whether coupling emerged specifically during the stimulus processing window. DFC, as defined by the sliding-window Pearson correlation between dHPC and vHPC signals within each trial, provides a time-resolved estimate of dHPC–vHPC coupling, allowing coordination to be tracked across the full stimulus window rather than collapsed into a single trial-level value. Together, these analyses allow us to identify how and when the two regions communicate to support sensory preconditioning.

Injection sites and optic cannula placements in the hippocampus were verified histologically (Figures 3A and S2A). For region-specific analyses of dHPC and vHPC activity, the final number of rats per group was n_{dHPC}=12 and n_{vHPC}=8 for PP and n_{dHPC}=9 and n_{vHPC}=8 for UP, respectively. For connectivity analyses (noise correlations, DFC) requiring simultaneous recordings from both hippocampal subregions, only animals with verified placements in both dHPC and vHPC were retained, resulting in final group sizes of n=8 for Group PP and n=6 for Group UP.

In stage 1, trial-by-trial co-modulation of activity (Figure 3E-F, Table S1) was significantly positive during tone presentation in PP rats (Figure 3F, mean r = 0.598 ± 0.097 SEM; t(7) = 5.191, p_{corr} = 0.003), but not in UP rats (mean r = 0.147 ± 0.164 SEM; t(5) = 1.001, p_{corr} =

0.725). Of note, correlations were Fisher-z transformed and tested against zero with a one-sample t-test, with mean r ± SEM reported on the untransformed scale. The groups were significantly different from each other in their trial-by-trial co-modulation of activity ($t(12)= 2.679$, $p_{corr}=0.042$). However, mean DFC during tone and light presentations did not differ between groups, showing no significant within-trial temporal coordination.

Together, these results establish the neural signatures of successful sensory preconditioning in stage 1. They identify a selective elevated vHPC engagement in rats that learn a tone-light association, particularly during the early phase of learning. The signature of associative learning was also found at the trial-by-trial level with co-modulation of dHPC and vHPC amplitude responses.

Mediated learning during stage 2 is associated with higher functional connectivity between dorsal and ventral hippocampus

We applied our analytical framework to the activity measured in the dHPC and vHPC during Stage 2 (Figure 3G, Table S1). Because mediated learning is thought to depend on the retrieval of the tone-light association triggered by the light presentations, we focused our analysis on light-evoked fluorescence responses and compared them between groups. Light-evoked activity revealed a significant main effect of region ($F(1,12) = 46.86$, $p < 0.001$ for AUC and $F(1,12) = 7.089$, $p = 0.02$ for peak amplitude), with vHPC responses exceeding those of dHPC, but no significant effect of group or group x region interaction (Figure 3H, smallest $p = 0.4$). Thus, light-evoked activity in dHPC and vHPC did not differ between PP and UP rats during Stage 2, demonstrating that the signature of mediated learning was not observable in the region-separated signal at our resolution.

However, both measures of inter-regional coordination revealed a pattern specific to PP rats. Noise correlations (Figure 3I, left and middle scatter plot panels, Table S1) during light presentation were significantly positive for PP rats (Figure 3J, right panel, mean $r=0.50 \pm 0.192$ SEM; $t(7) =2.484$, $p=0.042$) but not for UP rats (mean $r =0.126 \pm 0.210$ SEM; $t(5)=0.593$, $p=0.579$), although the between-group comparison did not reach significance ($t=1.585$, $p=0.139$, $\Delta r=0.374$). Given the small sample sizes, this result is presented here as a descriptive measure, rather than as independent statistical evidence of group differences.

To identify the moments during stimulus presentation in which dHPC–vHPC coupling reliably differentiated the two groups, we estimated, at each time window, the Gaussian Copula Mutual Information (GCMI) between individual DFC values and group labels. We did so using an exact permutation test (n=3003) to build a null distribution under label shuffling. Cluster-based correction across the 121 analyzed time windows controlled for multiple comparisons

and allowed detection of contiguous periods of significant group difference. DFC analysis revealed significantly greater dHPC–vHPC temporal synchrony in PP compared to UP rats (Figure 3K). Cluster-based correction identified two significant temporal clusters out of 121 analyzed windows (cluster-corrected $p < 0.05$): an early cluster spanning +0.94 s to +2.94 s after light onset (duration: 2.0 s, 21 windows), reduced to +1.25 s to +2.94 s after accounting for the 2.5 s sliding window, whose leading edge means each DFC value reflects signal from the preceding 2.5 s; and a sustained cluster spanning +3.54 s to +6.14 s (duration: 2.6 s, 27 windows). Together, these clusters indicate that dHPC–vHPC coupling was enhanced in PP rats for 4.29 s of the 7.0 s window over which DFC could be validly estimated (t = 1.25 to 8.25 s, i.e., windows whose full 2.5 s fell within the 9.5 s light), corresponding to 61% of that window.

Taken together, these results indicate that PP and UP rats show equivalent hippocampal response amplitudes to the conditioned stimulus during stage 2. However, PP rats display substantially greater dHPC-vHPC coordination at both the trial level and within individual trials. Thus, functional connectivity between the dHPC and vHPC is a neural signature of memory integration in PP rats, who encode a mediated tone-shock association during conditioning of the light.

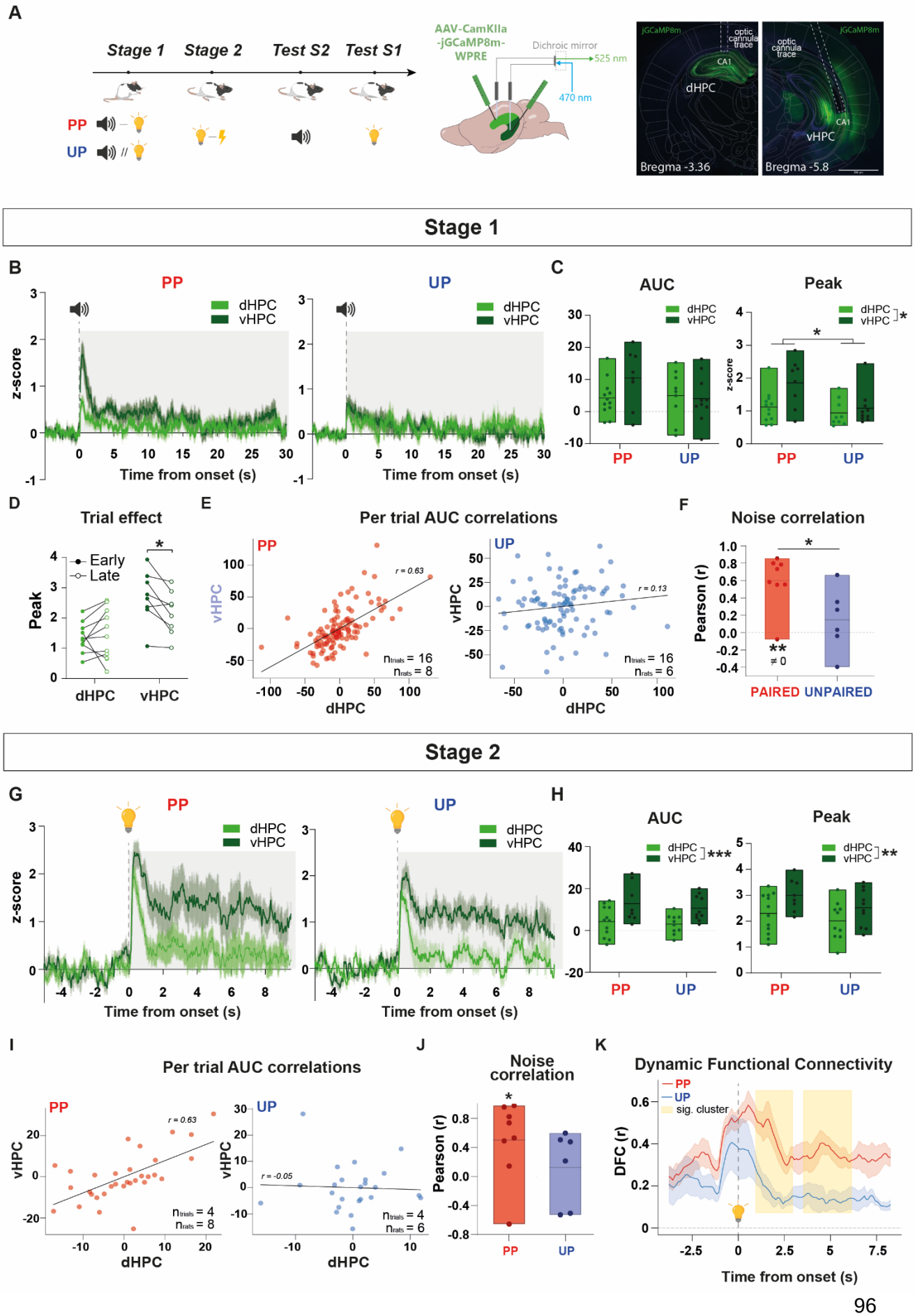

A
Stage 1
Stage 2
Test S2
Test S1
PP
UP
AAV-CamKIIa -jGCaMP8m-WPRE
Dichroic mirror
525 nm
470 nm
jGCaMP8m
optic cannula trace
CA1
dHPC
vHPC
Bregma -3.36
Bregma -5.8
Stage 1
B
PP
UP
dHPC
vHPC
z-score
Time from onset (s)
C
AUC
Peak
D
Trial effect
Early
Late
Peak
E
Per trial AUC correlations
r = 0.63
r = 0.13
n trials = 16
n rats = 8
n rats = 6
F
Noise correlation
Pearson (r)
≠ 0
PAIRED
UNPAIRED
Stage 2
G
H
I
J
K
Dynamic Functional Connectivity
DFC (r)
sig. cluster
r = -0.05
n trials = 4
n rats = 8
n rats = 6

Figure 3. Simultaneous dHPC-vHPC recordings reveal neural signatures of associative and mediated learning.

(A) Experimental design. **Left**: PP and UP groups respectively received paired and unpaired presentations of the tone and light in Stage 1, paired light–shock presentations in Stage 2, and were tested for fear of the tone and the light. **Center**: schematic of the dual-site fiber photometry setup. AAV-CamKIIa-jGCaMP8m-WPRE was contralaterally injected into dHPC and vHPC, and calcium signals were recorded via implanted optic cannulae using 470 nm excitation and 525 nm emission detection. **Right**: representative histological sections showing optic cannula placement in dHPC (Bregma −3.36) and vHPC (Bregma −5.8). **(B)** Tone-evoked calcium responses during Stage 1. Mean (±SEM) z-scored fluorescence time-locked to tone onset for PP (n_{dHPC}=12, n_{vHPC}=8) and UP (n_{dHPC}=9, n_{vHPC}=8) groups, for dHPC (light green) and vHPC (dark green). **(C)** AUC and peak response per group and region. *p < 0.05. **(D)** Trial-dependent modulation of peak amplitude in early versus late presentations during Stage 1 in PP animals. Lines connect individual animals. *p < 0.05. **(E)** Per-trial scatter plots of dHPC versus vHPC responses for PP (r = +0.63, p = 0.001) and UP (r = 0.13, p = 0.149) groups; each dot represents one trial (16 trials per rat). **(F)** Noise correlations (Pearson r of trial-by-trial co-fluctuations from panel E) for PP and UP groups; each dot represents one rat, bars indicate group mean ± SEM with individual data points overlaid. **(G)** Light-evoked calcium responses during Stage 2. Mean (±SEM) z-scored fluorescence time-locked to light onset for PP and UP groups. **(H)** AUC and peak response per group and region. No significant group differences were observed. **(I)** Per-trial scatter plots of dHPC versus vHPC responses for PP (r = +0.63, p < 0.001) and UP (r = −0.05, p = 0.826) groups; each dot represents one trial (4 trials per rat). **(J)** Noise correlations (Pearson r of trial-by-trial co-fluctuations) for PP and UP groups; bars indicate group mean ± SEM with individual data points overlaid. **(K)** DFC during light presentations with cluster-level statistics. Mean (±SEM) dHPC–vHPC DFC time-locked to light onset for PP and UP groups. Yellow shading indicates a significant temporal cluster during which PP animals exhibited greater dHPC–vHPC coupling than UP animals (permutation test, p < 0.05).

Hippocampal connectivity differently predicts indirect and direct fear

Having shown that dHPC-vHPC connectivity during the light in stage 2 was greater in PP than UP rats, we asked whether the DFC measures could predict individual fear expression at test. We therefore computed a composite index of dHPC-vHPC connectivity across both stages, combining the three standardized DFC measures (average DFC during tone in stage 1, average DFC during light in stage 1 and average DFC during light in stage 2). We then examined the relationship between this index and freezing to both the tone and light at test.

For the tone freezing, the model accounted for a significant proportion of variance ($F(3,9) = 10.22$, $p = 0.003$, adjusted $R^2 = 0.70$). Critically, the composite connectivity × group interaction was significant ($F(1,9) = 5.80$, $p = 0.039$), whereas group alone was not ($F(1,9) =$

0.51, p = 0.49): connectivity predicted tone freezing in PP rats (b = 5.55, 95% CI [2.76, 8.35], p = 0.002) but not in UP rats (b = 1.07, n.s.).

For light freezing the model was again significant (F(3,9) = 10.16, p = 0.003, adjusted R^2 = 0.70), with a significant connectivity × group interaction (F(1,9) = 18.13, p = 0.002) and no group difference in overall freezing (F(1,9) = 0.04, p = 0.85). Here, the pattern reversed: connectivity predicted light freezing in UP rats (b = 8.58, 95% CI [4.88, 12.27], p = 0.002) but not in PP rats (b = −0.72, n.s.).

These results show that, among PP rats, functional connectivity was not associated with direct fear of the light but was associated with mediated fear of the tone. By contrast, in UP rats, hippocampal connectivity specifically predicted direct fear of the light rather than mediated fear of the tone. Together, the pattern is consistent with a dissociation whereby sensory preconditioning redirects hippocampal coordination toward mediated learning.

Mediated learning is associated with reorganization of hippocampal–perirhinal–amygdala correlation structure

The selective trial-by-trial co-modulation and within-trial synchrony of dHPC and vHPC in PP rats raises the question of how these regions regulate and are regulated by other components of the circuitry that underlies sensory preconditioning. Both patterns are consistent with the coordinated recruitment of the two subregions by a shared upstream region that is differentially engaged in the presence or absence of mediated learning: slow co-modulation across trials could reflect a common modulatory input, while within-trial coupling points more specifically to a region rapidly activated by the S1 stimulus and capable of simultaneously engaging both hippocampal subregions during the integration window. Auditory-visual sensory preconditioning is known to engage a distributed network including the perirhinal cortex and basolateral amygdala (Holmes et al., 2013, 2018, 2020; Qureshi et al., 2023; Wong et al., 2019), regions with which the vHPC is preferentially connected (Agster & Burwell, 2013; Cenquizca & Swanson, 2006; Fanselow & Dong, 2010). To determine whether integration-dependent hippocampal coordination identified in stage 2 is part of a broader reorganization of this network, we next quantified c-Fos expression across dHPC, vHPC, Prh, and BLA following this stage (Figure 4A-B), allowing us to characterize inter-regional correlation structure across a broader circuit.

We adopted a two-level analytical framework to determine whether mediated learning changed the network organization as reflected by the regional activities. At the regional level, we first tested for group differences in c-Fos expression across all four regions simultaneously using multivariate analysis (MANOVA), and then applied linear discriminant analysis (LDA)

with leave-one-out cross-validation to characterize the multivariate activity pattern that best distinguished PP from UP rats. At the network level, we quantified functional organization by computing inter-regional correlation matrices from c-Fos expression within each group. To assess global differences in network architecture, we compared the Fisher z-transformed correlation matrices using the Frobenius norm, which summarizes the overall magnitude of the difference as a single scalar value. Significant global effects were followed by edge-wise comparisons with false discovery rate correction to identify the connections contributing to network reorganization.

The MANOVA revealed a significant difference in the overall pattern of regional activity between groups (Pillai's trace = 0.644, $F(4,12) = 5.42$, $p = 0.010$), indicating that mediated learning involves a reorganization of activity across the hippocampal–cortical–amygdalar circuit. Consistent with this, LDA correctly classified 82.4% of rats (14/17) based on their regional c-Fos profile alone (Figure 4C). The groups also differed in their inter-regional coordination: the Frobenius norm distance between PP and UP c-Fos correlation matrices (2.95) was greater than all values generated under a 10,000-permutation null distribution (mean = 1.17, $p = 1.0 \times 10^{-4}$; Figure 4D), indicating a robust reorganization of network structure between the groups. Together, these results show that mediated learning reconfigures the functional relationships among hippocampal, cortical, and amygdalar regions, consistent with a network-level integration of associative memories across these systems. To identify the connections driving this effect, we performed edge-wise comparisons across all six pairwise connections. Only the vHPC–Prh connection survived FDR correction, shifting from a strong positive correlation in PP rats to a strong negative correlation in UP rats (PP: $r=0.80$; UP: $r = -0.76$; $z = 3.46$, $p=5.5\times10^{-4}$, FDR-adjusted $p=0.0033$).

Together, these findings indicate that mediated learning is accompanied by a reorganization of hippocampal–cortical network activity, with a selective change of vHPC–Prh coordination, and show that dHPC-vHPC coupling during stage 2 is part of a broader network change in support of this learning.

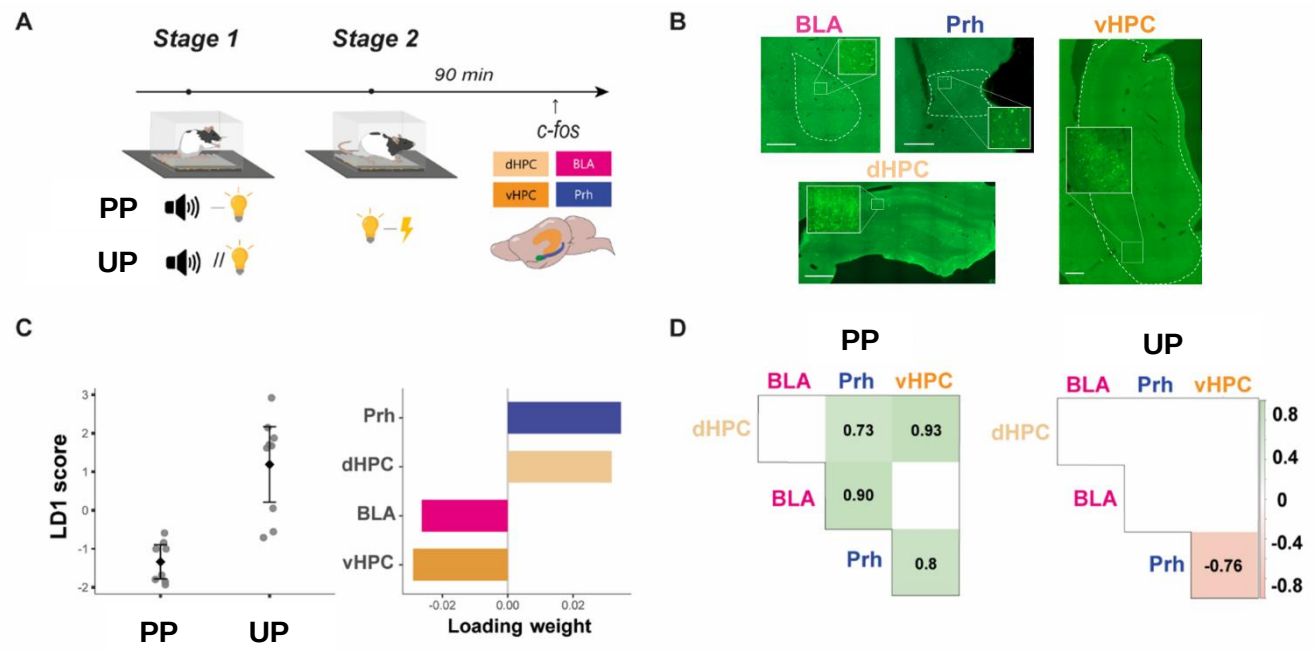


Figure 4. Sensory preconditioning is associated with a reorganization of hippocampal–perirhinal–amygdala network activity during Stage 2

(A) Experimental design. PP and UP groups underwent Stage 1 and Stage 2 training as described in Figure 1. c-Fos expression was quantified in dorsal hippocampus (dHPC), ventral hippocampus (vHPC), perirhinal cortex (Prh), and basolateral amygdala (BLA) following Stage 2. **(B)** Representative histological sections showing c-fos marking (green) in BLA (bregma -2.4), Prh (Bregma –4.56), vHPC (Bregma −5.8) and dHPC (Bregma −3.6). **(C)** Linear discriminant analysis (LDA) of c-Fos counts across the four regions (n = 17 animals total). Left: individual LD1 scores for PP and UP animals; black diamonds indicate group means ± SEM. Right: loading weights of each region on the first linear discriminant (LD1), reflecting their relative contribution to group separation. **(D)** Pairwise Pearson correlation matrices of c-Fos expression across animals, computed separately for PP (left) and UP (right) groups. Numbers indicate correlation coefficients. Color scale reflects correlation strength from −1 (light pink) to +1 (light green). Only significant ($p<0.05$) correlations are displayed.

Discussion

Memory integration allows animals and humans to infer relationships between events that were never experienced together. Our study shows that the encoding and integration of separate events depends on dissociable contributions of hippocampal subregions: the vHPC is selectively required to encode an association between two safe, sensory stimuli; whereas both the dHPC and the vHPC are needed to integrate this association with a new emotional memory. Critically, this integration is accompanied by intra-hippocampal functional connectivity, suggesting that the two subregions coordinate their activity to support integration.

Considered as a whole, the hippocampus was required at every learning stage: inactivating at least one subregion during either stage impaired sensory preconditioned fear.

This aligns with evidence implicating the hippocampus across the successive stages of the protocol: forming incidental associations in stage 1 (Busquets-Garcia et al., 2018; Pinho et al., 2025) and, more extensively, responding to the preconditioned but not the directly conditioned stimulus after stage 2 (Iordanova et al., 2011; Lin et al., 2016; Wheeler et al., 2013; Wimmer & Shohamy, 2012). Recordings further suggest a role at test (Barron et al., 2020; Talk et al., 2002). Consistent with this, we recently showed that hippocampal activity is required both to associate two neutral stimuli in stage 1 and to retrieve the mediated aversion at test, even within a single sensory modality (Talaron et al., 2026).

The hippocampus does not perform this operation alone. Sensory preconditioning engages a distributed circuit that includes the perirhinal cortex (Holmes et al., 2013, 2018; Nicholson & Freeman Jr, 2000; Wong et al., 2019), the basolateral amygdala (Qureshi et al., 2023) and other cortical regions such as the orbitofrontal (Hart et al., 2020; Sadacca et al., 2018) and retrosplenial cortices (Fournier et al., 2020; Robinson et al., 2014). Our c-Fos data show that mediated learning reorganizes this circuit rather than changing any region alone: the multivariate activity pattern across dHPC, vHPC, Prh and BLA distinguished rats that could integrate the two memories from those that could not, and the correlation structure linking these regions was globally reconfigured in the former. The hippocampal contribution is therefore best understood as embedded in a network, with each of its subregions making a distinct contribution to the process of memory integration.

Importantly, we dissociated hippocampal subregions contributions during encoding and integration processes. That fear of the tone was disrupted in a drug-free test following neural manipulation in Stage 2 confirms that the memory integration is performed during the emotional learning, and thus relies on mediated learning, whereby a reactivated memory gets linked to the current experience. This is consistent with studies using the same protocol demonstrating that memory integration at least partly happens in stage 2 (Wong et al., 2019, 2025). Crucially, the absence of effect during Stage 1 might be dependent on the arrangement of the stimuli during learning. Indeed, Pinho et al. (2025) found the reverse — a dHPC Stage-1 requirement in mice, likely because their simultaneous presentations form a compound requiring configural binding, dependent on dHPC's conjunctive representations (O'Reilly & Rudy, 2001; Rudy & Sutherland, 1995), whereas our serial presentations can be supported by predictive associative encoding. dHPC necessity in learning a non-aversive association may thus be conditional on the task's representational demands rather than reflecting a generic associative role. In our version of the task, the dHPC is thus dispensable for learning either association alone, but required when the two must be brought together. The vHPC showed the opposite profile: its inhibition impaired sensory preconditioned fear (in either stage) as well as the direct

light-shock association, extending its role beyond emotional and motivational functions to the encoding of an innocuous auditory-visual association.

Mechanistically, this dissociation maps onto the subregions' extrinsic connectivity. The vHPC is more directly and reciprocally connected with Prh than is dHPC (Agster & Burwell, 2013; Cenquizca & Swanson, 2006), and since Prh is itself necessary for encoding the tone-light association (Holmes et al., 2013; Wong et al., 2019), the vHPC-Prh circuit likely provides the substrate for forming and retrieving this type of learning. Our c-Fos data support this: among the six connections examined, vHPC-Prh was the only one whose coordination distinguished groups, being strongly positive in paired and negative in unpaired rats. The dHPC requirement may instead arise from convergent input from the regions encoding the tone-light (vHPC, Prh) and light-shock (vHPC, BLA) associations. Supporting this, in vitro studies show that co-stimulation but not individual stimulation of Prh and BLA drives activation of the entorhinal cortex, the principal input to dHPC (Kajiwara et al., 2003). The vHPC is thus positioned to encode each association as it forms, whereas the dHPC receives them once both exist.

Our calcium recordings corroborate the dHPC-vHPC dissociation and reveal its dynamics. In Stage 1, the vHPC but not dHPC of paired rats showed an elevated tone-evoked response that decreased throughout the pairings. Because both groups received equivalent tone presentations, this likely reflects the tone's acquired predictive value rather than novelty. Beyond regional activity, trial-by-trial co-fluctuations in dHPC-vHPC amplitude were positive in paired but not unpaired rats, showing joint modulation of both subregions even though dHPC is dispensable for the learning. This co-modulation was restricted to the between-trial level, appearing as a slow trial-to-trial alignment rather than within-trial synchrony – plausibly driven by a shared neuromodulatory or upstream cortical input tracking the tone-light relationship (Hasselmo & McGaughy, 2004). Such co-engagement, even when not strictly necessary, may reflect a network-level encoding state supporting later integration, mirroring human evidence that encoding connectivity predicts later integration (Shohamy & Wagner, 2008; Wang, Schoenbaum, et al., 2020; Wimmer & Shohamy, 2012; Zeithamova, Dominick, et al., 2012; Zeithamova & Preston, 2010; Zhu et al., 2022).

During Stage 2, coordination itself became the signature of mediated learning. Light-evoked activity was comparable across groups in both subregions, so integration was not visible in the region-separated signal; instead, paired rats showed enhanced within-trial dHPC-vHPC coupling and convergent positive noise correlations. Since both paired and unpaired groups received identical light-shock training, the differences in coupling reflect the impact of prior sensory learning on hippocampal processing of the conditioned stimulus, and implicate

the vHPC in integration itself rather than solely direct conditioning. That is, intra-hippocampal communication during presentations of the light appears to support activation of the tone in the paired rats and, thereby, the retrieval-mediated integration of tone-light and light-shock associations. Functional disconnection of dHPC and vHPC would confirm this causal relationship.

Finally, the impact of dHPC-vHPC coordination was traceable in behavior: dorsoventral coupling predicted tone fear in paired but not unpaired rats, and the reverse was true for the directly conditioned light. In line with previous work on dorsoventral integration in direct conditioning (Pompili et al., 2026), we propose that dHPC-vHPC coupling is necessary for fear expression whenever no alternative pathway exists. That is, as direct light fear can be expressed through a vHPC-amygdala route, dHPC-vHPC connectivity is dispensable, whereas the tone, never paired with shock, can only acquire fear through the integrative dorsoventral mechanism, rendering this coupling obligatory. This account situates dorsoventral synchrony as the mechanism through which fear spreads to cues never themselves paired with danger. A limitation of the present work is that it was conducted in males only, as our protocol did not produce reliable preconditioning in females; whether the same dorsoventral division of labor supports integration in females remains an open question.

In summary, our study shows that memory integration depends on activity in both the dHPC and vHPC, with their coordinated activity serving as a signature of the integration. In doing so, it complements work in which hippocampal connectivity at encoding predicts later inference in humans, and suggests that inter-regional coordination is the mechanism of inference. This coordination and inference are adaptive in allowing animals and people to solve novel problems, but can become maladaptive when predictions about the world no longer accord with reality.

STAR★Methods

Key Resource Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-c-Fos (rabbit monoclonal)		
Alexa Fluor 488 donkey anti-rabbit	Thermo Fisher Scientific Australia Pty Ltd	Cat#A21206
Bacterial and virus strains		
AAV9-CaMKIIa-jGCaMP8m-WPRE	Addgene plasmid # 176751	RRID: Addgene_176751
Chemicals, peptides, and recombinant proteins		
Muscimol	In Vitro Technologies Pty Ltd	Cat#RDS028910
Deposited data		
Source data	This paper	Zenodo repository: 10.5281/zenodo.21276654
Experimental models: Organisms/strains		
Rat: Long Evans	Janvier, France	RRID:RGD_151356971
Rat: Long Evans	Biological Resources Centre, University of New South Wales, Sydney	RRID:RGD_151356971
Software and algorithms		
PSY open access statistical program https://www.unsw.edu.au/science/our-schools/psychology/our-research/research-tools/psy-statistical-program	Kevin Bird, Dusan Hadzi-Pavlovic, and Andrew Issac © School of Psychology, University of New South Wales.	N/A
RStudio https://posit.co/	Posit	RRID:SCR_000432
PyCharm https://www.jetbrains.com/fr-fr/pycharm/	JetBrains	RRID:SCR_018221
Custom analysis script	This paper	Zenodo repository: 10.5281/zenodo.21276654
Illustrator https://www.adobe.com/products/illustrator.html	Adobe	RRID:SCR_010279
QuPath https://qupath.github.io/	Open source	RRID:SCR_018257
POLY https://www.imetronic.com/devices/polymodal-system	Imetronic	N/A
OFRS https://www.rwdstco.com	RWD Life Science	N/A
Prism https://www.graphpad.com/	Graphpad	RRID:SCR_002798
Other		
Cannulas Plastics One Plastic dummy Plastics One Internal Cannula Plastics One	BioScientific Australia Pty Ltd	C315G/SPC GUIDE 26GA, 62003 (Cut 5 mm Below Pedestal) + C315DC, 62102 C315I/SP, 62203

Optic fibers	RWD Life Science	Cat# R-FOC-BF400C-50NA

Materials availability

This study did not generate any new reagents.

Data and code availability

All source data is publicly available at Zenodo as of the date of publication. DOI is listed in the key resources table. Original code is available on GitHub (REPO). Any additional information required to reanalyze the data reported in this paper is available from the lead contact on request.

Subjects

For behavioral and pharmacological experiments, subjects were 136 naïve male Long-Evans adult rats (aged from 8 to 10 weeks at surgery) obtained from a colony maintained by the Biological Resources Centre at the University of New South Wales. Rats were housed in plastic cages (40 cm width x 22 cm height x 67 cm length), with between two and four rats per cage. For fiber photometry experiment, subjects were 23 naïve male Long Evans adult rats aged from 9 weeks at surgery obtained from Centre d'Elevage Janvier, France. Rats were housed in pairs in plastic cages in a colony room. Only male rats were used. In pilot work, the sensory preconditioning protocol did not yield reliable preconditioned freezing in females, precluding the fear-based readout used here; we therefore restricted the present study to males.

The facilities were maintained at 21 ± 1°C and kept on a 12hr light/dark cycle (lights on at 7am and off at 7pm), experimentation took place during the light portion of the cycle. All rats received unrestricted access to food (standard laboratory chow) and water for the entire duration of the experiment. Environmental enrichment was provided via tinted polycarbonate tubing. Rats were handled daily for 3 days prior to the beginning of behavioral experiments.

All experimental procedures were conducted in agreement with French (council directive 2013-118, February 1, 2013) and European (Directive 2010-63, September 22, 2010) regulations on animal experimentation and were approved by the French Ministry of Higher Education and Innovation (APAFIS #51028-2024091011369219 v4) and the Australian authorities (iRECS8988, iRECS8991, iRECS11077).

Viral vectors

For fiber photometry recordings, we employed the genetically encoded calcium indicator jGCaMP8m (Zhang et al., 2023) to record a real-time proxy of neuronal activity. The AAV9-CamKIIa-jGCaMP8m-WPRE was a viral construct from Addgene (Cat#176751-AAV9) diluted at a titer of 1.35 x 10^{13} GC/mL.

Drugs

GABAA receptor agonist muscimol (In Vitro Technologies, Australia) was dissolved in nonpyrogenic saline (0.9% w/v) to yield a final concentration of 1 µg/µl. Vehicle solution was made of saline only (0.9% w/v).

Surgeries

Cannulations. Prior to behavioral training and testing, rats were surgically implanted with bilateral cannulas targeting the dorsal hippocampus (dHPC) or ventral hippocampus (vHPC). Rats were first anesthetized with 5% inhalant isoflurane delivered in oxygen at a flow rate of 1 L/min and subsequently maintained on 2.5% inhalant isoflurane delivered in oxygen at 0.6L/min. Rats were then mounted onto a stereotaxis apparatus (David Kopf Instruments) and incisions made over the skull. Two holes were drilled through the skull and 26-gauge guide cannulas (Plastics One) were implanted into the brain, one in each hemisphere. The tips of the cannulas targeted the dHPC at the following coordinates: AP: -3.6 mm; ML:+/- 2.5 mm; DV: -2.8 mm; or the vHPC at the following coordinates: AP: -5.2 mm; ML:+/- 5.4 mm; DV: -6.8 mm to Bregma (Paxinos and Watson, 2013). Guide cannulas were secured in place with four jeweller's screws and dental cement. A dummy cannula was kept in each guide cannula at all times except during drug infusions. Immediately after surgery, rats received an i.p. injection with a prophylactic (0.4 ml) dose of a 300 mg/kg solution of procaine penicillin. Rats were allowed 7 days to recover from surgery, during which they were monitored and weighed daily.

Fiber photometry. Rats were anesthetized with 5% inhalant isoflurane gas with oxygen before being placed in a stereotaxic frame with atraumatic ear bars (Kopf Instruments) in a flat skull position. Anesthesia was maintained at 1.5% isoflurane and complemented with a subcutaneous injection of ropivacaine at the incision site (a bolus of 0.15 mL, 2 mg/mL). All viral injections were performed using a microinjector (UMP3 UltraMicroPump II with Micro4 Controller, World Precision Instruments) connected to a 10µL Hamilton syringe.

Animals received unilateral injections of AAV9-CamKIIa-jGCaMP8m-WPRE in the dHPC, and a contralateral injection in the vHPC; the hemisphere assigned to dHPC versus vHPC was counterbalanced across animals. Each injection consisted of 1 µL infused at a rate of 200 nL/min. Following each injection, the needle was left in place for 5 min to allow viral diffusion

before slow withdrawal. An optic cannula (400 µm, 0.5NA, AquiNeuro) was implanted above each injection site; the cannula targeting the vHPC was implanted at a 10° angle laterally. All cannulae were secured with highly adhesive dental cement (Super-Bond, C&B) and anchored to the skull with 3–4 stainless steel jewelers' screws. The coordinates of dHPC viral injections were as follows (relative to bregma): AP −3.3, ML ±2.2, DV −2.5. The coordinates of vHPC viral injections were as follows (relative to bregma): AP −5.8, ML ±5.2, DV −5.9. The optic cannulas were implanted at the same AP and ML coordinates for both regions, but DV was 0.1 above the injection sites. During surgery, body temperature was maintained at ~37.5°C using a heating pad. Following surgery, animals received a subcutaneous injection of meloxicam (2 mg/kg) and were individually housed in a cage with facilitated access to food and water for 2 h post-surgery. Rats were allowed to recover for 3 weeks before behavioral testing to ensure adequate transgene expression. Injection sites and fiber placements were verified histologically after completion of behavioral experiments.

Intracerebral infusions

All rats were randomly allocated to their groups. Muscimol or vehicle was infused bilaterally into the brain region of interest. For these infusions, infusion cannulas were connected to 25µL Hamilton syringes via polyethylene tubing. The syringes were fixed to an infusion pump (Harvard Apparatus). The infusion procedure began by removing the dummy caps from the guide cannulas on each rat and inserting 26-gauge infusion cannulas with 1 mm protrusion in its place. The pump was programmed to infuse a total of 0.5µL per hemisphere at a rate of 0.25µL/min. The infusion cannulas remained in place for an additional 2 min after the infusion was complete to allow for diffusion of the drug into the dHPC tissue and to avoid reuptake of the drug. After the additional 2 min, the infusion cannulas were removed and replaced with dummy cannulas. Rats were placed back in their home cage for 13 min before the preconditioning or conditioning session. The day prior to the first day of infusions, the dummy cannulas were removed and replaced to familiarize the rats with the procedure and minimize any effect of procedure on the day of infusions.

Behavioral apparatus

Apparatus. Two series of apparatus were used. Behavioral and pharmacological experiments were conducted in 8 identical chambers (30 x 25 x 21 cm) located within the same room. The ceiling, front door, and back wall were clear polycarbonate, and the side walls were aluminum. The floor consisted of 19 stainless-steel rods, each 2 mm in diameter and spaced 13 mm (centre-to-centre), positioned above a stainless-steel waste tray filled with corncob bedding, that could deliver mild foot shocks. At the end of each session, the chambers were

cleaned with 70% ethanol and water. Each chamber was positioned inside a light- and tone-attenuating fibreboard cabinet with a loudspeaker at the top of the back wall. Each chamber was illuminated with an infrared light source and a camera mounted on the back wall of each cabinet was used to record the behavior of each rat. The cameras were connected to a monitor and recording device located in the experimental room. Experimental events were controlled and recorded by a computer equipped with the Med-PC V software.

Fiber photometry experiment was conducted in a conditioning chamber (40 × 35 × 30 cm, Imetronic) made of gray polyvinyl chloride on three sides and transparent Perspex on the opening face (Esclassan et al., 2009a). A grid floor (27 parallel 0.5-cm-diameter stainless-steel bars, 1.5 cm apart) above a sawdust tray could deliver mild foot shocks. A ventilation fan provided a background noise of ∼60dB. The chamber was located inside a tone-attenuating cubicle with a loudspeaker at the top of the back wall. The ceiling contained a 10 cm circular opening to allow cable access for fiber photometry recordings. An infrared camera was placed on the ceiling. Experimental events were controlled and recorded by a computer equipped with the POLY software (Imetronic, France).

Stimuli. For all experiments, the auditory stimulus was a pure tone (1,000 Hz, 70 dB) and the visual stimulus was a pair of flashing lights (3.5 Hz, 57 lux) delivered by two horizontally aligned LEDs placed on a side wall of the chamber. Each LED was separated by at least 15cm. The tone and light were used as S2 and S1, respectively (except Figure S2 where they were reversed). Each S1 presentation was 10 s and each S2 presentation was 30 s.

In the behavioral and pharmacological experiments, the US was a 0.8 mA, 0.5 s foot shock (Michalscheck et al., 2021). In the fiber photometry experiment, based on previous demonstration of sensory preconditioning run in this apparatus, the US was a 0.6 mA, 0.5 s foot-shock.

Behavioral procedures

Context exposure. On Days 1 and 2, rats received two 20-min exposures to the chambers, one in the morning and the other approximately 3hr later in the afternoon. Context exposure aims to familiarize the rats with the training context and eliminate any neophobic reactions.

Sensory preconditioning. On Day 3, all rats received eight presentations of the S2 (tone) and eight of the S1 (light). All rats received the tone as the preconditioned stimulus S2, and the light as the directly conditioned stimulus S1). The first stimulus presentation occurred 5 min after rats were placed into the chambers. For rats that received paired stimuli presentations, the offset of S2 co-occurred with the onset of S1 and the interval between each

paired presentation was fixed at 5 min. For rats that received unpaired presentations, interleaved presentations of S2 and S1 were 2 min and 30 s apart. After the last stimulus presentation, rats remained in the chambers for an additional 2 min. They were then returned to their home cages.

For fiber photometry experiment only, an additional day of sensory preconditioning was performed, where rats received the same 8 presentations of S2 and S1 than on day 3. This modification was based on previous demonstration of sensory preconditioning run in the apparatus.

Direct conditioning. On Day 4 (day 5 for fiber photometry), all rats received four presentations of the S1 and four presentations of the US. The first S1 presentation occurred 5 min after rats were placed in chambers. For rats receiving paired presentations, each S1 presentation lasted for 10 s and co-terminated with foot shock, and the interval between light-shock pairings was 5 min. For rats that received unpaired presentations, blocked presentations of S1 and then the US were 2 min and 30 s apart. Rats remained in the chambers for an additional 1 min after the final stimulus presentation and were then returned to their home cages.

Context extinction. On Day 5 (day 6 for fiber photometry), rats received two 20-min exposures to the chambers, one in the morning and the other approximately 3hr later, in the afternoon. These exposures were intended to extinguish any freezing elicited by the context and thereby provide a measure of the level of freezing to the tone and the light un-confounded by context-elicited freezing. On Day 6 (day 7 for fiber photometry), rats received a further 10 min extinction exposure to the context.

Test. On Day 6 (day 7 for fiber photometry), approximately 2 hr after the context-extinction session, rats were tested for their levels of freezing to the preconditioned tone S2. On Day 7 (day 8 for fiber photometry), rats were tested for their levels of freezing to the conditioned light. For each test, the first stimulus was presented 2 min after rats were placed in the chambers. Each test consisted of eight stimulus alone presentations, with a 3 min interval between each presentation. Each tone presentation was 30 s in duration and each light was 10 s. Rats remained in the chamber for an additional minute after the final stimulus presentation in each test.

Scoring and statistics

Preconditioning, conditioning and testing sessions were recorded. Freezing, defined as the absence of all movements except those required for breathing (Fanselow, 1980), was used as a measure of conditioned fear. Freezing data were collected using a time-sampling procedure

in which each rat was scored as either 'freezing' or 'not freezing' every 2 s by an observer blind to the rat's group allocation. A second blind observer scored a randomly selected 25% of the data. The correlation between the scores were high (Pearson > 0.9). Freezing was scored during the stimulus presentations. The baseline level of freezing was determined as the scoring of freezing during the first 2 minutes of the test session, in the absence of any stimulus delivery. The data were analyzed using a set of planned orthogonal contrasts (Hays, 1963) with the type 1 error rate controlled at $\alpha = 0.05$. The 95% confidence intervals (CIs) were reported for significant results, and Partial eta squared (ηp^2) was reported in Table S1 as a measure of effect size (where 0.01, 0.06, and 0.14 is a small, medium, and large effect size respectively). Non-orthogonal follow-up comparisons were Bonferroni-corrected. The required number of rats per group was determined during the design stage of the study. It was based on our prior experience in running sensory preconditioning studies: experiments of this sort typically require an average of 8 subjects per group to provide sufficient statistical power.

Histology

c-Fos. For c-Fos counts, rats were deeply anesthetized with a lethal dose of sodium pentobarbital ninety minutes following Stage 2 completion, and transcardially perfused with saline followed by 4% paraformaldehyde in phosphate buffer (pH 7.4). Brains were removed and post-fixed in 4% PFA for 24 h, then cryoprotected in 20% 0.1 M phosphate buffer (PB) sucrose for 48h at 4°C until dehydrated. Brains were frozen using liquid nitrogen and stored at -30°C. Coronal sections (40 µm) were cut and stored in cryoprotectant solution containing ethylene glycol and glycerol in phosphate buffer.

Cannulations. For pharmacological experiments, rats were euthanized with a lethal dose of sodium pentobarbital after behavioral training and testing. Their brains were then extracted and sectioned into 40 µm coronal slices. Every second slice was mounted onto a glass microscope slide and stained with cresyl violet. The size of the lesion and placement of the cannula tip was determined under a microscope using the boundaries defined by Paxinos and Watson (Paxinos & Watson, 2013). Rats with extensive damage and/or misplaced cannulas were excluded from the statistical analyses.

Fiber photometry. Rats were euthanized with an overdose of sodium pentobarbital (Exagon Euthasol) and perfused transcardially with 50 mL of saline followed by 250 mL of 3.6% formol in 0.1 M PB. Brains were removed, post-fixed overnight in the same formol 3.6% solution, and subsequently transferred to 0.1 M PB. Coronal sections of 40 µm were cut using a vibratome (VT1200S, Leica Microsystems), and every fourth section was collected to form a series.

c-Fos Immunohistochemistry

Free-floating sections were processed using standard immunofluorescence protocols. Sections were washed 3 × 10 min in phosphate-buffered saline (PBS), then blocked in 5% normal horse serum in PBS containing 0.25% Triton X-100 (PBT) for 1 h at room temperature. Sections were incubated with rabbit anti-c-Fos primary antibody [REF Ab] diluted 1:1000 in 2% normal horse serum in PBT for ~36 h at 4°C under constant agitation. Following 3 × 10 min PBS washes, sections were incubated with Alexa Fluor 488 goat anti-rabbit secondary antibody [REF Ab] diluted 1:500 in 2% normal horse serum in PBT for 4h at room temperature. After final PBS washes (3 × 10 min), sections were mounted on glass slides, air-dried, and coverslipped using DAPI-containing mounting medium (Sigma Fluoroshield F with DAPI).

c-Fos Quantification and statistical analysis

C-Fos positive cells were quantified in the dorsal hippocampus, ventral hippocampus, perirhinal cortex, and basolateral amygdala. The regions were manually outlined based on the DAPI signal following the coordinates from Paxinos and Watson (2013). Images were analyzed using QuPath v0.5.1 (Bankhead et al., 2017) and c-Fos positive cells within the outlined structures were automatically detected with the positive cell detection built-in Qupath function with standardized parameters. At least 4 sections per region, spanning across the anteroposterior axis (for BLA: AP -2.00 to -2.92; for Prh: -3.6 to -5.2; for dHPC: -2.5 to -4.3; for vHPC: -5.2 to -6.2) were analyzed from both hemispheres, yielding to min. 8 counts from 8 outlined areas. C-Fos positive cell density was calculated as the cumulated positive cell counts divided by the cumulated outlined areas (cells/mm²) of a given region to minimize standard deviation within a same animal (for descriptive statistics see Table S2).

All analyses were performed in R (v4.4.1; $\alpha = 0.05$). Group differences in the multivariate c-Fos profile were assessed using a one-way MANOVA (car package), followed by LDA with leave-one-out cross-validation (MASS package); group separation along the primary discriminant function was visualized using ggplot2. Inter-regional correlation matrices were computed and visualized using the corrplot package. Global network differences were assessed using a permutation test (10,000 permutations) on the Frobenius norm of the difference between Fisher z-transformed correlation matrices. Edge-wise comparisons used two-sample z-tests on Fisher z-transformed coefficients with FDR correction across the six pairwise connections.

Fiber photometry set-up and analysis

Fiber photometry recordings were performed using a multichannel fiber photometry system (R811, RWD Life Science). Excitation light sources at 405 nm and 470 nm were used to measure the reference (isosbestic) and jGCaMP8m fluorescence signal, respectively. Optical fiber patch cords (400µm, AquiNeuro) were connected to a rotary joint and photobleached for at least 2 hours before use. All equipment was supplied and installed by AquiNeuro (Bordeaux, France). Light intensity at the tip of the patch cord was measured before each recording day and maintained at 25 µW for 405 nm and 50 µW for 465 nm. Fluorescence signals were digitized at a stable frequency of 50 Hz for all recordings. Each recording lasted the duration of the given behavioral session, plus 1 minute before and 1 minute after the behavioral exercise. Time-To-Live pulses were sent from the operant chamber to the fiber photometry system to mark the beginning of the session and the stimulus presentations (respectively 300 ms-, 200 ms- and 100 ms-duration pulses for the tone, light and shock). These markers were timestamped in the fiber photometry recordings using a synchronization algorithm and saved as CSV files. The CSV files were then processed using two in-house custom-made software programs, which enabled analysis of signal. The fluorescence signal was expressed as a z-scored ΔF/F value, calculated as $z = [(\Delta F/F - \text{mean})/SD]$, with mean and standard deviation being computed over the full trial window. One trial window was defined as a 5 s baseline preceding the stimulus presentation plus the duration of the stimulus of interest. In paired presentations of stimuli, the baseline used for the second stimulus was the same 5 s-window baseline as the one used for the first stimulus.

Stimulus-evoked responses were quantified over a predefined response window following stimulus onset (tone: 30 s; light: 9.5 s). The area under the curve (AUC) was computed as the integral of the z-scored signal over the response window using the trapezoidal rule and served as the primary measure of response magnitude. Peak amplitude, defined as the maximum z-score within a 2 s stimulus-specific search window, was retained as a secondary measure. AUC and peak amplitude were analyzed with a mixed-effects model (REML; GraphPad Prism) with Group (between-subjects) and Region (dHPC vs vHPC, within-subjects) as factors; this model accommodates the unequal number of usable recordings per region (region and interaction terms are estimated only from animals with both dHPC and vHPC recordings). Sphericity was assumed; planned comparisons used Bonferroni correction. All signal processing and connectivity analyses were performed in Python 3 using *NumPy*, *SciPy*, *Pandas*, *Matplotlib*, *Statsmodels*, and the *Frites toolbox* (v0.4; Combrisson et al., 2022).

Trial-by-trial co-modulation of regions activity was quantified using noise correlations. For each stimulus of a given stage, the per-rat Pearson correlation coefficient between dHPC and vHPC AUC vectors across trials was computed using scipy.stats.pearsonr. Fisher's z-transformation on coefficient of correlations was applied prior to group-level inference. Within each group,

one-sample t-tests assessed whether noise correlations differed from zero; between-group differences were assessed using independent-samples t-tests. Both one-sample and between-group tests were Bonferroni-corrected across the stimuli tested within each stage (two in Stage 1, one in Stage 2).

Within-trial temporal synchrony between regions was quantified using dynamic functional connectivity (DFC), computed with the *conn_dfc* function from the *Frites* toolbox. A sliding-window Pearson correlation was applied to single-trial z-scored signals using a window of 150 samples (2.5 s at 50 Hz sampling rate) and a step of 5 sample (100 ms), yielding a DFC time series at 10 Hz resolution per trial. These parameters were chosen to ensure sufficient samples for stable within-window correlation estimates while maintaining sub-second temporal resolution across the shortest stimulus duration analyzed. They are compatible with the fast decay kinetics and transient dynamics of jGCaMP8m, ensuring full capture of individual calcium transients within each window (Zhang et al., 2023). Group differences in DFC over time were assessed using a non-parametric information-theoretic approach. At each time window, the Gaussian Copula Mutual Information (GCMI) between each rat's DFC value and their group label (PP = 0, UP = 1) was estimated using *GCMIEstimator* from *Frites* (mi_type = 'cd', with copula normalization and bias correction). An exact permutation null distribution was constructed by exhaustively enumerating all possible group label assignments given the total sample size (n = 3003 permutations, corresponding to C(14,6), the number of ways to assign 6 labels among 14 rats; random seed = 42). Cluster-based correction for multiple comparisons across the time axis was applied using the *WfStats* function from *Frites*, with fixed-effects inference and a one-sided test (PP > UP). Time windows within clusters with corrected $p < 0.05$ were considered significant.

To examine whether dorsal-ventral hippocampal connectivity predicts fear expression, we computed a composite connectivity index combining the three standardized DFC measures:
$composite = scale(connectivity^{stage1,Tone}) + scale(connectivity^{stage1,Flash}) + scale(connectivity^{stage2,Flash})$

where each connectivity measure was the average DFC during stimulus presentation at the given stage. Tone and light test freezing were each modelled by linear regression with the composite connectivity index, group, and their interaction (`freezing ~ composite * group`). All analyses were conducted on N=13 animals (one animal excluded due to missing data at test). Omnibus term effects were tested with Type II sums of squares (car::Anova), and group-specific (simple) slopes were estimated with emmeans::emtrends.

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Author contributions

Conceptualization, E.C., N.H.; data curation, M.T., formal analysis M.T., A.B; funding acquisition, E.C., N.H., S.K.; investigation, M.T., N.S.; methodology, M.T., A.B., N.H., E.C.; project administration, N.H., E.C.; resources, A.B.; supervision, E.C., N.H., S.K.; visualization, M.T., writing – original draft, M.T.; writing – review & editing, M.T., A.B., E.C., N.H.

Declaration of interests

The authors declare no competing interests.

Supplementary figures

Panel	Effect / comparison	n	Test	Statistic (df)	ES	95% CI	p	p_corr
Figure 1 — Behavioral sensory preconditioning								
1B	Test Tone: PP vs UP+PU	8/grp	contrast	F(1,21)=10.914	ηp²=0.34	[6.986, 30.723]	**0.003**	n/a
1B	Test Tone: UP vs PU	8/grp	contrast	F(1,21)=0.001	—	—	0.975	n/a
1B	Test Light: PP+UP vs PU	8/grp	contrast	F(1,21)=79.825	ηp²=0.79	[−72.040, −44.835]	**<0.001**	n/a
1B	Test Light: PP vs UP	8/grp	contrast	F(1,21)=1.157	—	—	0.294	n/a
1B	Stage 2, PP+UP vs PU, overall	8/grp	contrast	F(1,21)=83.056	ηp²=0.80	[−55.652, −34.973]	**<0.0001**	n/a
1B	Stage 2: rate, PP+UP vs PU	8/grp	contrast	F(1,21)=66.36	ηp²=0.76	[−88.26, −52.363]	**<0.0001**	n/a
Figure 2 — Pharmacology								
2D	dHPC Test tone: VEH/VEH vs (VEH/MUS+MUS/VEH)	14;12;11	contrast	F(1,34)=6.491	ηp²=0.16	[2.40, 21.30]	**0.016**	n/a
2D	dHPC Test tone: VEH/MUS vs VEH/VEH	14;12	contrast	F(1,34)=15.226	ηp²=0.31	[−32.02, −10.09]	**<0.001**	**<0.01** (Bonferroni; 3 comparisons)
2D	dHPC Test tone: VEH/MUS vs MUS/VEH	11;12	contrast	F(1,34)=10.34	ηp²=0.23	[−30.04, −6.77]	**0.003**	**0.009** (Bonferroni; 3 comparisons)
2D	dHPC Test tone: VEH/VEH vs MUS/VEH	14;11	contrast	F(1,34)=0.229	—	—	0.635	>1 (Bonferroni; 3 comparisons)
2E	dHPC Test light (largest effect)	14;12;11	contrast	F(1,34)=0.624	—	—	0.435	n/a
2H	vHPC Test tone: VEH/VEH vs (MUS/VEH+VEH/MUS)	10;10;11	contrast	F(1,28)=20.41	ηp²=0.42	[−32.513, −12.228]	**<0.001**	n/a
2H	vHPC Test tone-first: VEH/MUS vs MUS/VEH	10;11	contrast	F(1,28)=5.63	ηp²=0.16	[−24.889, −1.830]	**0.025**	n/a
2I	vHPC Test light-first: VEH/MUS vs others	9/grp	contrast	F(1,24)=23.17	ηp²=0.49	[−53.380, −21.342]	**<0.001**	n/a
2G	vHPC Stage 2, rate: VEH/MUS vs (VEH/VEH + MUS/VEH)	9/grp	contrast	F(1,24)=37.83	ηp²=0.61	[-77.906; -38.761]	**<0.001**	n/a
2G	vHPC Stage 2, overall light freezing: VEH/MUS vs (VEH/VEH + MUS/VEH)	9/grp	contrast	F(1,24)=25.743	ηp²=0.52	[-56.271; -23.729]	**<0.001**	n/a
Figure S1 — Pharmacology								
S1C	Stage 2, rate of acquisition	10;12	contrast	F(1,22)=2.404	—	—	0.14	n/a
S1E	Test light	10;12	contrast	F(1,22)=3.195	—	—	0.08	n/a
S1D	Test tone	10;12	contrast	F(1,22)=4.773	ηp²=0.30	[−20.981, −0.546]	**0.039**	n/a
Figure S2 — Photometry-cohort behavior								
S2B	Stage 2, rate of acquisition	13;12	contrast	F(1,23)=207.8	ηp²=0.90	[60.216, 80.393]	**<0.0001**	n/a

Panel	Effect / comparison	n	Test	Statistic (df)	ES	95% CI	p	p_corr
S2B	Test light	12;12	contrast	F(1,22)=0.319	—	—	0.578	n/a
S2B	Test tone	12;12	contrast	F(1,22)=10.242	ηp²=0.32	[−21.516, −4.595]	**0.004**	n/a
S2C	Subset — Stage 2, rate of acquisition	8;6	contrast	F(1,12)=79.889	ηp²=0.87	[53.173, 87.452]	**<0.0001**	n/a
S2C	Subset — Test light	7;6	contrast	F(1,11)=0.797	—	—	0.391	n/a
S2C	Subset — Test tone	7;6	contrast	F(1,11)=3.653	ηp²=0.25	[−29.041, 2.046]	***0.082***	n/a
Figure 3 — Evoked responses								
3C	Stage 1 tone AUC: Group	≥1 fiber 12;11	REML	F(1,21)=0.979	—	—	0.334	0.668 (Bonferroni, 2 stimuli)
3C	Stage 1 tone AUC: Region	both fibers: 8;6	REML	F(1,12)=1.658	—	—	0.222	0.444 (Bonferroni, 2 stimuli)
3C	Stage 1 tone AUC: Group×Region	8;6	REML	F(1,12)=3.929	—	—	0.071	0.142, (Bonferroni, 2 stimuli)
3C	Stage 1 light AUC: Group	12;11	REML	F(1,21)=0.375	—	—	0.547	>0.99 (Bonferroni, 2 stimuli)
3C	Stage 1 light AUC: Region	8;6	REML	F(1,12)=22.634	ηp²=0.65	[−11.080, −4.119]	<0.001	**0.001** (Bonferroni, 2 stimuli)
3C	Stage 1 light AUC: Group×Region	8;6	REML	F(1,12)=0.001	—	—	0.976	>0.99 (Bonferroni, 2 stimuli)
3C	Stage 1 tone PEAK: Group	12;11	REML	F(1,21)=5.260	ηp²=0.20	[0.041, 0.833]	**0.032**	**0.064** (Bonferroni, 2 stimuli)
3C	Stage 1 tone PEAK: Region	8;6	REML	F(1,12)=7.897	ηp²=0.40	[−0.861, −0.109]	**0.016**	**0.031** (Bonferroni, 2 stimuli)
3C	Stage 1 tone PEAK: Group×Region	8;6	REML	F(1,12)=1.962	—	—	0.187	0.373 (Bonferroni, 2 stimuli)
3C	Stage 1 light PEAK: Group	12;11	REML	F(1,21)=3.698	—	—	0.068	0.136 (Bonferroni, 2 stimuli)
3C	Stage 1 light PEAK: Region	8;6	REML	F(1,12)=11.552	ηp²=0.49	[−1.182, −0.259]	**0.005**	**0.011** (Bonferroni, 2 stimuli)
3C	Stage 1 light PEAK: Group×Region	8;6	REML	F(1,12)=1.031	—	—	0.330	0.660 (Bonferroni, 2 stimuli)
3C	Stage 2 light AUC: Group	12;12	REML	F(1,22)=0.458	—	—	0.506	n/a
3C	Stage 2 light AUC: Region	8;6	REML	F(1,12)=46.864	ηp²=0.80	[−10.815, −5.593]	<0.001	n/a
3C	Stage 2 light AUC: Group×Region	8/6	REML	F(1,12)=0.001	—	—	0.978	n/a
3C	Stage 2 light PEAK: Group	12;12	REML	F(1,22)=3.661	—	—	0.069	n/a
3C	Stage 2 light PEAK: Region	8/6	REML	F(1,12)=7.089	ηp²=0.37	[−0.923, −0.092]	**0.021**	n/a
3C	Stage 2 light PEAK: Group×Region	8/6	REML	F(1,12)=0.830	—	—	0.380	n/a
Figure 3 — Evoked-response planned comparisons								
3C	Stage 1 Tone PEAK: vHPC PP vs UP	8/8	Planned comparison	t(33)=2.491	Δ=0.678	[0.124, 1.232]	**0.018**	**0.036** (Bonferroni, 2 regions)
3C	Stage 1 Tone PEAK: dHPC PP vs UP	12/9	Planned comparison	t(33)=0.811	—	—	0.423	0.847 (Bonferroni, 2 regions)
3C	Stage 1 Tone PEAK: PP dHPC vs vHPC	8	Planned comparison	t(12)=3.086	Δ=−0.726	[−1.239, −0.214]	**0.009**	**0.019** (Bonferroni, 2 regions)
3C	Stage 1 Tone PEAK: UP dHPC vs vHPC	6	Planned comparison	t(12)=0.964	—	—	0.354	0.708 (Bonferroni, 2 regions)
3D	Stage 1 tone: Trial×Region (PP)	8	REML	F(1,16)=6.462	—	—	**0.022**	n/a

Panel	Effect / comparison	n	Test	Statistic (df)	ES	95% CI	p	p_corr
3D	Epoch tone: vHPC early vs late	8	post-hoc	t(16)=2.566	Δ=0.537	[0.093, 0.981]	**0.021**	**0.041** (Bonferroni, 2 regions)
3D	Epoch tone: dHPC early vs late	8	post-hoc	t(16)=0.935	—	—	0.364	0.727 (Bonferroni, 2 regions)
Figure 3 — Noise correlations								
3F	Stage 1 tone: PP vs 0	8	1-samp t	t(7)=5.191	r=0.598 ± 0.105	—	**0.001**	**0.003** (Bonferroni, 2 stimuli)
3F	Stage 1 tone: UP vs 0	6	1-samp t	t(5)=1.001	—	—	0.363	0.725 (Bonferroni, 2 stimuli)
3F	Stage 1 tone: PP vs UP	8;6	2-samp t	t(12)=2.679	Δr=0.451	—	**0.020**	**0.040** (Bonferroni, 2 stimuli)
3F	Stage 1 light: PP vs 0	8	1-samp t	t(7)=7.340	r=0.498 ± 0.054	—	**<0.001**	**<0.001** (Bonferroni, 2 stimuli)
3F	Stage 1 light: UP vs 0	6	1-samp t	t(5)=2.122	—	—	0.087	0.174 (Bonferroni, 2 stimuli)
3F	Stage 1 light: PP vs UP	8;6	2-samp t	t(12)=0.428	—	—	0.676	>0.99 (Bonferroni, 2 stimuli)
3G	Stage 2 light: PP vs 0	8	1-samp t	t(7)=2.484	r=0.500 ± 0.192	—	**0.042**	n/a
3G	Stage 2 light: UP vs 0	6	1-samp t	t(5)=0.593	—	—	0.579	n/a
3G	Stage 2 light: PP vs UP	8;6	2-samp t	t(12)=1.585	—	—	0.139	n/a
Figure 3 — DFC and connectivity-fear regression								
3H	Stage 2 DFC group diff: cluster 1 (+0.94–2.94 s)	8/6	GCMI perm + cluster	21 windows	—	—	—	**<0.05 (cluster)**
3H	Stage 2 DFC group diff: cluster 2 (+3.54–6.14 s)	8/6	GCMI perm + cluster	27 windows	—	—	—	**<0.05 (cluster)**
3	Composite → test tone: model	13	LM	F(3,9)=10.22	adjR²=0.70	—	**0.003**	n/a
3	Composite → test tone: composite×group		LM	F(1,9)=5.80	—	—	**0.039**	n/a
3	Composite → test tone: PP slope		emtrends	b=5.55	—	[2.76, 8.35]	**0.002**	n/a
3	Composite → test tone: UP slope		emtrends	b=1.07	—	—	n.s.	n/a
3	Composite → test light: model	13	LM	F(3,9)=10.16	adjR²=0.70	—	**0.003**	n/a
3	Composite → test light: composite×group		LM	F(1,9)=18.13	—	—	**0.002**	n/a
3	Composite → test light: UP slope		emtrends	b=8.58	—	[4.88, 12.27]	**0.002**	n/a
3	Composite → test light: PP slope		emtrends	b=−0.72	—	—	n.s.	n/a
Figure 4 — c-Fos network *(edge-wise FDR across 6 connections)*								
4	Multivariate group difference	8/9	MANOVA	Pillai=0.644, F(4,12)=5.42	—	—	0.010	n/a
4C	LDA classification (LOO)	17	LDA	82.4% (14/17)	—	—	—	n/a
4	Global network difference		Frobenius perm (10^4)	dist=2.95 (null 1.17)	—	—	1.0×10^{-4}	n/a

Panel	Effect / comparison	n	Test	Statistic (df)	ES	95% CI	p	p_corr
4D	vHPC–Prh edge		2-samp z	z=3.46	—	—	5.5×10^{-4}	0.0033 (FDR; 6)
4D	Other 5 edges		2-samp z	—	—	—	—	n.s. (FDR; 6)

Corrections. Pharmacology: planned orthogonal contrasts (α = 0.05); non-orthogonal follow-ups Bonferroni-corrected. Evoked responses & noise correlations: Bonferroni across the stimuli analyzed within each stage (×2 in Stage 1, tone and light; ×1 in Stage 2, light). Photometry planned comparisons: Bonferroni across the two regions. AUC and peak treated as primary and secondary metrics, not corrected across each other. Evoked-response omnibus effects are from a REML mixed-effects model (Group between, Region within); Region/interaction df = 12 reflect matched recordings (8 PP + 6 UP); Group df reflects rats with ≥1 usable fiber (Stage 1: N=23; Stage 2: N=24). DFC: GCMI, exact permutation (C(14,6)=3003), cluster correction across time (one-sided). c-Fos: FDR across 6 connections. **Symbols.** —, not reported, or effect not significant; n/a, no correction required; n.s., not significant; Δ, mean difference; Δr, difference in mean correlation; b, unstandardized slope; n given PP/UP or per group.

Table S1. Statistical analyses

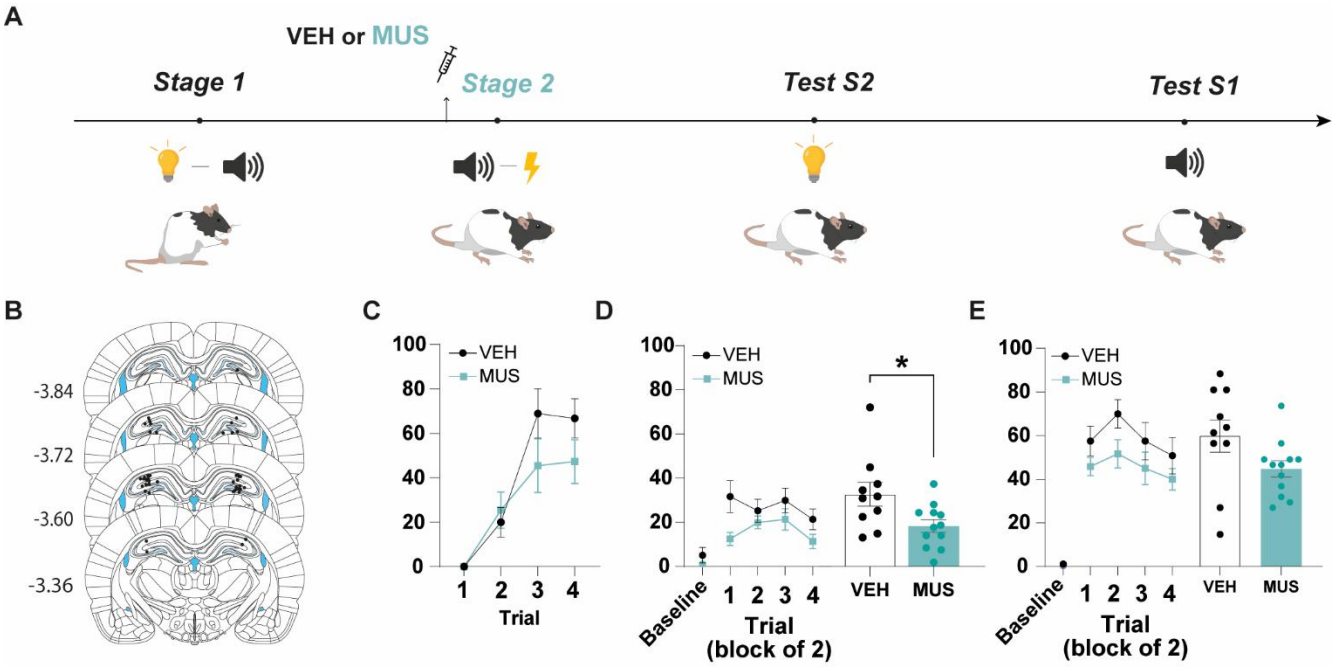


Figure S1. The dorsal hippocampus is necessary to integrate a light-tone memory with a tone-shock memory

(A) Experimental design. In stage 1, all rats received paired presentations of a light (S2) with a tone (S1). In stage 2, Group MUS (n=11) received infusions of muscimol in the dorsal hippocampus while Groups VEH (n=11) received saline. Both groups were tested drug-free to the light and the tone. **(B)** Most ventral part of the cannulas in the dorsal hippocampus as verified on Nissl-stained sections. **(C)** Mean (+/-SEM) freezing across the four tone-shock presentations during stage 2. Groups did not differ in their rate of acquisition (largest F = 2.404; p = 0.14), or in their overall freezing to the light (F(1,22)= 1.155; p = 0.29). **(D)** Mean (±SEM) freezing across the light presentations and average (bar graph) during Test S2. Group MUS froze less than VEH, F(1,22)=4.773, p=0.039, 95%CI[-20.981; -0.546]. **(F)** Mean (±SEM) freezing across the tone presentations and average (bar graph) during Test S1, there was no group difference (F(1,22)=3.195, p=0.08). Individual data points are shown in bar graphs. *Baseline freezing was measured before the first stimulus presentation and did not differ between groups across experiments (largest F=1.656, p=0.21). Error bars: SEM. *p < 0.05, **p < 0.01, ***p < 0.001. For both tests, group difference was assessed with Student t-tests.*

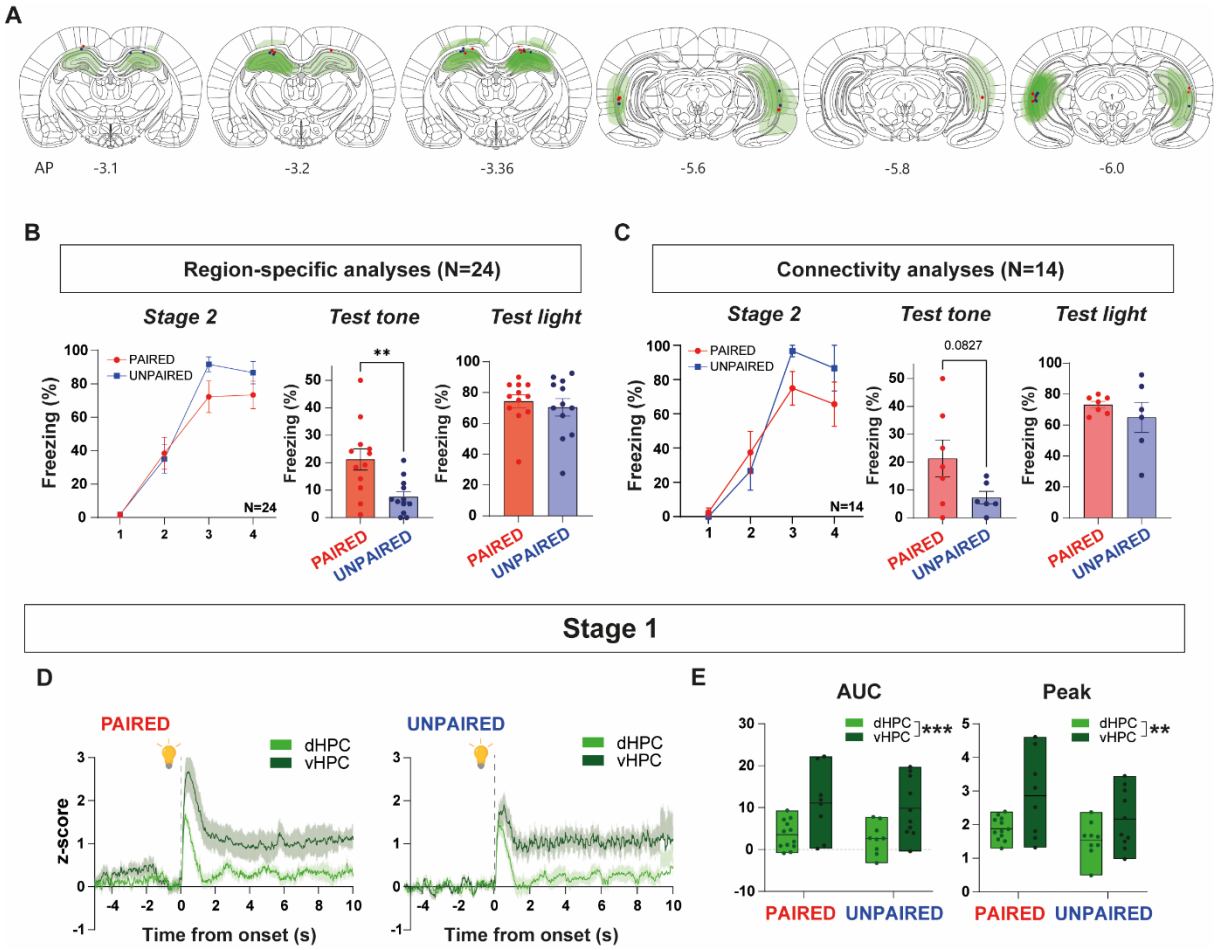


Figure S2. Histological and behavioral characterization of the fiber photometry cohort

(A) Histological verification of viral injection sites and optic cannula placements. Coronal sections show the spread of AAV-CamKIIa-jGCaMP8m-WRPE expression (green shading) in dorsal hippocampus (AP −3.1 to −3.36, left three panels) and ventral hippocampus (AP −5.6 to −6.0, right three panels). Colored dots indicate optic cannula tip locations for PAIRED (red) and UNPAIRED (blue) animals. Only animals with verified placements within the target regions were included in subsequent analyses. **(B-C)** Behavioral performance of the full cohort used for region-specific analyses (panel B, N = 24) and subset with verified dual-site placements used for connectivity analyses (panel C, N = 14). **Left graphs**: Mean freezing (+/-SEM) across the four light-shock presentation in Stage 2. **Middle graphs**: Mean (+/-SEM) freezing to the tone during test. **Right graphs**: Mean (+/-SEM) freezing of the light during test. Individual data points are shown. Error bars represent SEM. **p < 0.01. Baseline was computed over 2 min before the first stimulus presentation and never differed between groups (largest =1.48, p=0.17). **(D)** Light-evoked calcium responses during stage 1. Mean (±SEM) z-scored fluorescence time-locked to light onset for PAIRED (left trace) and UNPAIRED (right trace) rats, for dHPC (dark green) and vHPC (light green). **(E)** AUC (left) and peak amplitude (right) per group and region. There was a region effect for AUC ($F[1,14]=21.33$, $p_{corr}<0.001$) and peak ($F[1,14]=13.04$, $p_{corr}=0.005$) but no group differences.

Group	BLA	n	dHPC	n	vHPC	n	Prh n	
PP	41.12	6	6.28	8	2.77	7	60.46	6
	60.25	5	24.07	9	58.81	8	141.94	6
	89.4	4	13.63	6	41.00	6	146.81	7
	14.90	7	9.67	7	15.78	7	53.17	7
	23.92	4	1.94	6	11.02	7	55.90	6
	40.07	6	4.37	7	14.23	6	72.60	5
	63.66	3	5.27	6	8.50	9	112.49	8
	22.72	5	3.72	6	2.77	10	73.30	4
MEAN	44.51		8.62		19.36		89.58	
UP	58.96	5	8.93	7	23.44	9	143.83	6
	47.27	6	20.93	7	26.05	10	108.53	5
	51.91	5	18.94	6	24.46	7	177.04	6
	60.41	6	1.51	4	21.85	7	128.43	5
	86.10	6	17.47	5	15.82	8	202.97	6
	51.66	6	6.88	5	8.99	8	211.06	6
	64.17	5	4.77	7	9.34	6	200.40	5
	23.65	6	10.11	5	14.71	3	156.30	6
	37.15	6	8.03	6	13.63	7	165.12	6
MEAN	53.48		10.84		17.59		165.96	

Table S2. Individual c-Fos densities per region

Densities are reported as the cumulated c-Fos positive cells count divided by the cumulated defined areas across n number of slices per region per rat.

Chapter 4

Roles of the hippocampus in gustatory preconditioning

Significance statement

Chapter 3 examined the role of the hippocampus in a cross-modal protocol of sensory preconditioning. This chapter asks whether the contribution of hippocampus extends to a protocol in which stimuli are drawn from a single sensory modality.

What was done. A unimodal gustatory preconditioning protocol was used. In the preconditioning stage, rats received presentations of two-taste compounds, AX and BY. Then during conditioning, X, but not Y, was paired with gastric malaise (lithium chloride). The mediated aversion was measured as reduced consumption of A relative to B, and the direct aversion as consumption of X relative to Y. Inhibitory DREADDs (hM4Di) were expressed in the whole hippocampus and CNO was injected either throughout preconditioning or at test, thereby dissociating encoding of the taste–taste association from retrieval of the mediated aversion. Controls received the same procedure, but no virus was injected.

What was found. At test, control rats drank markedly less A than B, demonstrating within-event learning and the expression of a mediated aversion to a taste. In contrast, rats whose hippocampus was inhibited during either preconditioning or test showed no sensory preconditioning, while all groups continued to avoid X relative to Y. Interestingly, this effect was driven by reduction of B consumption in rats that were inhibited during preconditioning, but not for rats inhibited at test. These rats also showed a generalization from X to Y during conditioning, suggesting that the stage 1 deficit may partly reflect impaired differentiation of the two compounds rather than a failure to form the taste–taste associations themselves.

Why it matters. These results provide the evidence that **the hippocampus is necessary for sensory preconditioning within a single sensory modality**, and they resolve the discrepancy with earlier study showing no impact of hippocampal lesions in the same protocol. Specifically, they show that disruption of the hippocampus does not impact general expression or retrieval of fear memory, but rather the retrieval of memory about stimuli never paired with danger. Although the study does not discriminate between an integration mechanism happening in conditioning or at test, they show that **the hippocampal contribution to integration is not restricted to combining information arriving from different sensory streams, as its anatomical position might suggest, but extends to binding elements of the same modality**.

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Chemogenetic disruption of the hippocampus impairs gustatory preconditioning in rats ☆

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ABSTRACT

Adaptive behavior often relies on learning associations between stimuli that were never directly paired with reinforcement. Sensory preconditioning provides a powerful paradigm to investigate such indirect learning: when neutral stimuli A and X are paired during preconditioning, and X is subsequently paired with an unconditioned stimulus (US) during conditioning, stimulus A elicits conditioned response at test despite never being directly paired with the US. While the hippocampus is known to be critical for this process, its involvement in unimodal sensory preconditioning remains unclear. Here, we investigated the role of the hippocampus (dorsal and ventral) during different phases of gustatory preconditioning using chemogenetic approach in rats. We demonstrated that disruption of the hippocampus during either preconditioning or test selectively impaired the mediated aversion to A while leaving the direct conditioned aversion to X intact. These findings provide the first evidence that the hippocampus is necessary for unimodal gustatory preconditioning, extending previous demonstrations of hippocampal involvement in polymodal protocols. Our work therefore clarifies the critical role of the hippocampus in forming and retrieving purely gustatory associations to guide behavior, highlighting its function as a central hub for memory integration.

Introduction

Daily choices and behavioral responses to environmental cues often arise from Pavlovian conditioning. In this form of associative learning, an initially neutral cue is paired with an unconditioned stimulus (US), leading to adaptive responses when the cue is subsequently encountered alone, even in the absence of the US. However, humans and animals do not always acquire knowledge through direct experience with environmental stimuli. Frequently, behavior is guided by indirect learning, involving stimuli that have never been explicitly paired with a positive or negative outcome but rather with other stimuli that are themselves predictive of such an outcome. For example, if one often gets a specific red wine with meat, and they later experience strong hangover after drinking that wine at a party, they might feel bad when eating the meat again, even though it was not the cause of the hangover. Although the term "sensory preconditioning" was introduced later (Brogden, 1939), the conceptual foundations of this type of indirect learning can be traced back to the work of Pavlov. He described that a conditioned stimulus could itself serve as the basis for establishing further conditioned reflexes (Pavlov, 1927). Such higher-order conditioning and related procedures foreshadowed what is now understood as indirect learning through associative integration. Sensory preconditioning paradigms provide a controlled framework for isolating these processes and identifying their neural substrates.

Sensory preconditioning is commonly studied using a three-stages protocol. In stage 1 (preconditioning), rats are exposed to pairing of two neutral stimuli A and X. In stage 2 (conditioning), X is paired with an aversive or appetitive US. Finally in stage 3 (test), A induces an appetitive or aversive response although it was never directly paired with the US (Holmes, Wong, Bouchekioua, Westbrook, et al., 2022; Shohamy, Daw, et al.,

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2015). Sensory preconditioning has been demonstrated in a range of species and protocols using stimuli from all sensory modalities (auditory, visual, gustatory, olfactory) and involves a distributed network of brain regions. So far, the available evidence suggests that the hippocampus is critically involved across all three stages of sensory preconditioning: several studies have demonstrated a role for the hippocampus in linking neutral stimuli during preconditioning (Busquets- Garcia, Oliveira da Cruz, Terral, et al., 2018; Pinho, Ramon-Duaso, Manzanares-Sierra, Busquets-Garcia, et al., 2025), integrating memories during conditioning (Iordanova, Good, Honey, et al., 2011; Wheeler, Chang, Holland, et al., 2013; Wimmer & Shohamy, 2012) and retrieving them at test (Barron, Reeve, Koolschijn, et al., 2020; Talk, Gandhi, Matzel, et al., 2002). However, the choice of stimuli in the protocols has been suggested to impact the neural substrates of sensory preconditioning (Holmes et al., 2022). In particular, the hippocampus is classically viewed as supporting higher-order associative learning by binding arbitrary relations among the elements of experience into flexible memory representations, and is anatomically well positioned to integrate information arriving from multiple sensory processing streams. This raises the possibility that hippocampal contributions to sensory preconditioning could vary as a function of whether the stimuli used in the protocol are selected from the same sensory modality (*unimodal*) or different sensory modalities (*polymodal*). In line with this view, studies cited above demonstrating hippocampal involvement in preconditioning in rodents have predominantly used polymodal protocols. The only studies targeting the hippocampus using unimodal stimuli found mixed results. Talk and colleagues found disruption of responses to preconditioned cues in rats with hippocampal lesions in a protocol involving exclusively auditory cues (noise and tone, Talk et al., 2002). Conversely, a study using unimodal gustatory preconditioning did not find an effect of excitotoxic lesions of the hippocampus on responses to preconditioned tastes (Ward-Robinson et al., 2001). Of note, in all of these studies, the use of permanent lesions prevents assessment of whether and how, the hippocampus is differently engaged in unimodal versus polymodal sensory preconditioning.

Our study aimed at clarifying this gap in the literature by testing the specific role of the hippocampus in different phases of unimodal sensory preconditioning using a gustatory preconditioning protocol (Ward- Robinson et al., 2001). Unsing chemogenetics we temporarily disrupted the hippocampus of rats during preconditioning exposures to two-compounds taste solutions AX and BY or during the tests (mediated response: A versus B and direct response: X vs Y). During conditioning, X but not Y was associated with an aversive US (gastric malaise) without manipulation of the hippocampus. We showed that disruption of the hippocampus during either preconditioning or testing prevented the mediated aversion for taste A but not the direct aversion for taste X.

Our results highlight that chemogenetics disruption of hippocampal activity impairs sensory preconditioning in rats when using a unimodal, gustatory protocol. They demonstrate that the hippocampus is necessary to associate distinct neutral gustatory stimuli, and to retrieve/express the indirect learning about the preconditioned taste but not the direct learning about the conditioned taste. Thus, they help to understand how our brain integrates memories that occurred at different times, providing complementary elements to the current knowledge about associative learning in episodic memory.

Material and methods

1.Subjects

The subjects were 35 males Long Evans rats (Centre d'Elevage Janvier, France) with mean weights of 305 g (range = 267–340 g) before surgery and 315.9 g (range = 279-353 g) at the start of the sensory preconditioning procedure. Rats were randomly allocated to groups HPC-PC, HPC-T and SHAM. Rats were housed in pairs in plastic cages in a colony room illuminated from 08:00 to 20:00 and accustomed to the laboratory facility for two weeks before the beginning of the experiments. The facility was maintained at 21 ± 1 °C and experimentation took place during the light portion of the cycle. Rats were permitted free access to standard Rat Chow. Water was restricted during the sensory preconditioning experiment but was freely available at all other times. The experiments were conducted in agreement with French (council directive 2013–118, February 1, 2013) and international (directive 2010–63, September 22, 2010, European Community) legislations.

2.Surgery

Rats were first anesthetized using a halothane–oxygen mix before placement in a stereotaxic apparatus frame (Kopf). Rats of Group HPC- PC and HPC-T received 4 bilateral injections (2x1µL per hemisphere) of the Designed Receptor Exclusively Activated by Designed Drug (DREADD) AAV8-CamKII-hM4DGi-mCherry virus (Armbruster et al., 2007; Parkes et al., 2018; Zhu et al., 2014) in the following coordinates, relative to bregma and the skull surface: ventral hippocampus (AP: -6.0, ML: +/− 4.6, DV: − 8.2), dorsal hippocampus (AP: -3.8, ML: +/− 2.8, DV: − 3.5). The virus was administered through a needle fixed onto the end of a 10-µL precision syringe held with a precision pump (Word Precision Instrument). The system was left in place for 2 min after the injection allowing diffusion of the solution into the tissue. Because systemic clozapine-N-oxide (CNO, the exogenous DREADD ligand) can produce DREADD-independent effects and may act via conversion to clozapine, we controlled for CNO effects by including SHAM animals that received a similar surgical procedure (the dura was perforated using a standard needle but no viral injection was given) and the same CNO injections at the same relevant stages. Thus, the critical comparison in this study is between hippocampal hM4Di animals and SHAM controls under identical CNO treatment, allowing us to attribute behavioral effects to DREADD-dependent hippocampal disruption rather than to CNO administration per se.

3.Histology

At the end of behavioral task, rats were overdosed with sodium pentobarbitone (Euthatal) and were transcardially perfused, first with 0.9% saline followed by 4% PFA. Brains were removed and postfixed for 18 h before their transfer to phosphate-buffered (0.1 M) 30%-sucrose solution where they remained for 36 h. Coronal brain sections containing dorsal and ventral hippocampus were cut at 40 µm thickness on a vibratome and stored free-floating in phosphate-buffer with saline (PBS) with 0.02% sodium azide at 4 °C. For immunostaining, floating sections were rinsed in 0.1 M PBS with 0.3% Triton X-100 (PBST; 5 × 5 min), and then in 0.5% H_2O_2 in 0.1 M PBST for 30 min. Following further rinsing in PBST, sections were incubated with rabbit anti-RFP (1:1000 in PBST, Clinisciences, PM005) for 24 h at room temperature (RT). Then, the secondary antibody was applied (goat anti-rabbit biotinylated antibody, 1:1000 in PBST containing 1% goat serum)

and sections were incubated for 2 h at RT. Slices were then rinsed in PBST, incubated in avidine- biotinylated complex kit for 2 h at RT, rinsed in PBST and 0.05 M Tris buffer. Staining was revealed using 3,3′-Diaminobenzidine (DAB) solution (10 mg DAB +50 mL 0.05 M Tris +20 µL 30% H_2O_2) for 10 min. Finally, sections were rinsed, mounted and coverslipped with Eukitt (Sigma–Aldrich, St. Louis, MO, USA).

Brightfield whole-slide images of DAB-stained rat brain sections were analyzed using QuPath (version 0.5.1). Hippocampal regions of interest (ROIs) were manually delineated using anatomical landmarks (Paxinos and Watson 7th edition) on at least 10 sections per rat, extending from AP -3.2 to AP -6.24. DAB staining was quantified using a pixel-based classifier. A supervised Random Trees pixel classifier was trained using representative annotations classified as DAB strong (visually strong DAB staining), DAB weak (pale but detectable staining), and DAB negative (absent staining). For each hippocampal ROI, QuPath calculated the percentage of tissue classified as DAB positive or DAB weak. The main outcome was the combined DAB-positive area fraction: *Total DAB staining*% = (DAB positive area + DAB weak area)/(total ROI area).

The saved classifier was applied to all images using QuPath batch scripting, and annotation measurements were exported for analysis (see Table S1). Rat-level of total DAB staining % in the hippocampus were assessed using a 3 standard deviations leave-one-out criterion among all rats that have received the DREADD virus. Outliers were identified and excluded using a leave-one-out approach: for each individual value, the mean and standard deviation (SD) were calculated from the remaining animals, and any value deviating by more than 3 SD from this mean was excluded from further analysis. Additionally, rats with strong off-target expression were also excluded.

4.Apparatus

All stages of the sensory preconditioning procedure occurred in specific cages different from the rats' home cages. The dedicated cages were made of transparent plastic, measured 33 × 21 × 18 cm and contained standard sawdust bedding. For the gustatory sensory preconditioning protocol, deionized water was used to make solutions of 0.33 M sucrose, 0.16 M NaCl, 60.00 µM quinine hydrochloride, and 0.01 M hydrochloric acid (HCl). These solutions were presented in 50-mL centrifuge tubes with ball-bearing tipped spouts, either alone or mixed,

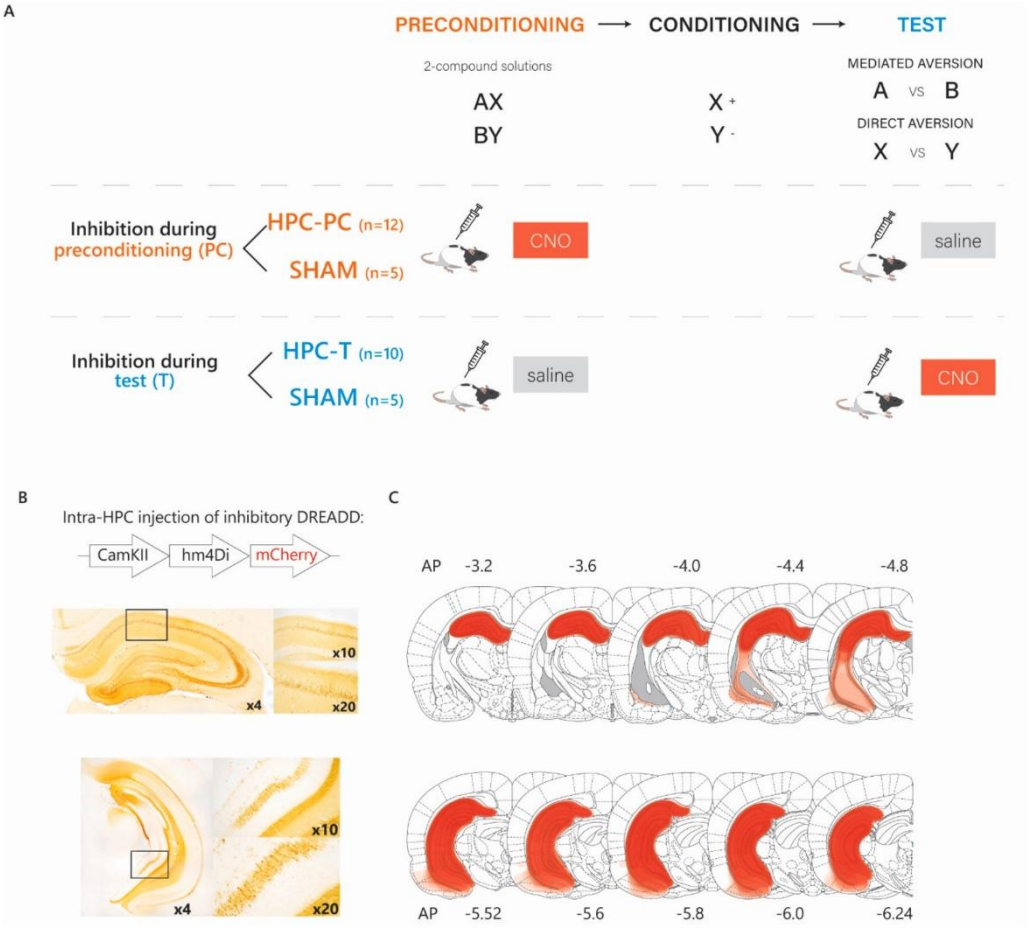


Fig. 1. Experimental procedure and chemogenetic targeting of the hippocampus.
A: Schematic of sensory preconditioning procedure. During preconditioning, rats are presented with two-compound solutions AX and BY. During conditioning, rats receive one-compound solution X and Y, with respectively following injections of lithium chloride 10 mL.kg^{-1} (aversive stimulus) and NaCl 10 mL.kg^{-1}. During test, solutions are presented in choice A vs B (for mediated aversion) or X vs Y (for direct aversion). In preconditioning, group HPC-PC and half of the SHAM receive CNO while groups HPC-T and the remaining SHAM rats receive saline injection. Conversely, during test, Group HPC-PC and the SHAM that have not been injected receive saline while Group HPC-T and the other half of the SHAM receive CNO. **B:** Representative images illustrating the expression of AAV8-CamKII-hM4DGi-mCherry virus in the dorsal hippocampus (top row) and ventral hippocampus (bottom row). Insets represent magnification of the black rectangle area. **C**: Schematics adapted from Paxinos and Watson (2013) showing the viral expression extent in the dorsal and ventral hippocampus for all subjects, where each rat is represented as a unique stacked layer (n = 32).

preserving their molarities. Fluid consumption was measured by the difference of tube weights (nearest 0.1 g) before and after presentations. *2.1.4. Behavioral procedures*

2.1.4.1. Sensory preconditioning protocol. The procedure was based on that used by Ward-Robinson et al. (2001). During the whole procedure, water bottles were removed overnight and access to fluid was restricted to once-daily water for 30 min at 16:00.

Habituation. Rats were habituated to the sensory preconditioning cages for 10 min per day on each of 3 days before the beginning of the preconditioning procedure.

Preconditioning. Fig. 1A shows the procedure of sensory preconditioning. On Day 4, and over 8 days, all rats received 10-g presentations of 2 two-compound solutions: AX and BY. In each group, for half of the rats, HCl was used as X and quinine hydrochloride was used as Y; for the remainder, the flavors assignments were reversed. Within each of these subgroups, half of the rats were given sucrose as A and NaCl as B; the other half was given the same flavors but reversed roles. Rats in each group were represented equally in the four counterbalanced subgroups. One solution a day was presented in the sequence AX, BY, BY,AX, AX, BY, BY, AX for half of the rats and in the sequence BY, AX, AX, BY, BY, AX, AX, BY for the remainder.

Rats from groups HPC-PC and half of the group SHAM received an i.p. injection of CNO, 1 mg/kg, 45 min before each presentation of the solutions. Rats from groups HPC-T and the remaining SHAM rats received an i.p. saline injection (NaCl, 0.9%).

Conditioning. Conditioning began on Day 12 and occurred over the course of 5 days. Half of the rats were presented with 10 g of solutions X or Y in the following sequence over the first 4 days: X,Y,Y,X, the other half received Y,X,X,Y. Presentations of solution X was followed within 20 min by an injection of LiCl 0.30 M, 10 mL/kg, presentation of solution Y was followed with injection of NaCl (0.9%, 10 mL/kg). On day 17, all rats received no flavored stimuli but were offered tap water at 11:00 and 16:00 for 30 min. Water was given in two tubes – the spouts of these tubes were 5 cm apart. This exposure was intended to prepare the rats for the tests in which they would be exposed to two tubes simultaneously.

Testing. Tests began on Day 18. To test the mediated aversion, rats were given 30-min free access to solutions A and B. Two tubes, one containing only solution A and the other containing only solution B, were presented simultaneously. The tubes' spouts were 5 cm apart. Solutions A and B were positioned on the left- and right-hand sides equally often across the subgroups. On Day 19, the test was repeated with the positions of A and B reversed. Direct aversion to X was tested in the same way. On Day 21, rats were presented with 2 separated tubes containing solution X for one and solution Y for the other. The positions of the solutions were counterbalanced and reversed on Day 22. During days of test for mediated and direct aversions, only rats from groups HPC-T and the other half of group SHAM that had not received the drug were injected with CNO (i.p. 1 mg/kg) 45 min before each presentation of the

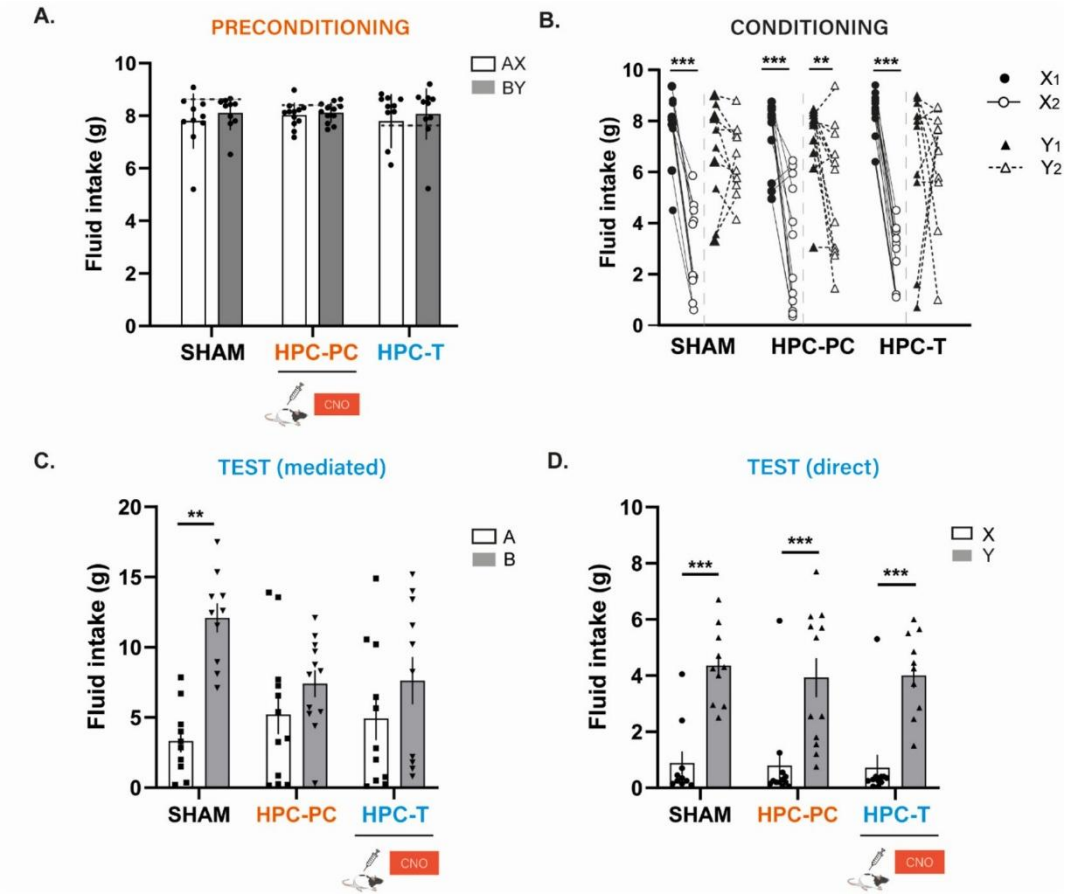


Fig. 2. Role of the hippocampus in the acquisition and expression of sensory preconditioning.
A: Mean consumption (+SEM) solutions AX and BY during preconditioning. Dashed lines: mean water baseline. **B**: Mean consumption (+SEM) of solutions X and Y at each of the two presentations during conditioning. X presentations were followed by i.p. injection of LiCl (10 mL/kg), while Y presentations were followed by i.p. injections of saline. **C:** Mean consumption (+SEM) of solutions A and B during test for mediated aversion. **D**: Mean consumption (+SEM) of solutions X and Y during test of direct aversion. HPC-PC: rats expressing inhibitory DREADD in the hippocampus that received CNO injection during preconditioning. HPC-T: rats expressing inhibitory DREADD in the hippocampus that received CNO injection during tests. SHAM: all control rats.

solutions. Groups HPC-PC and remaining SHAM were injected with NaCl (i.p., 0.9%).

5.Statistical analysis

Data were analyzed using the R software (R.4.4.1 Core Team, 2024). Descriptive statistics are showed in Table S2. To investigate the effects of chemogenetic manipulation of the hippocampus on the intake of the various solutions, we used a linear mixed-effects model analysis to model the solution consumption during each stage of the protocol. The models included a random effect of rat, and the following fixed effects and their interaction terms: solution type (2-level factor) and group (3- level factor). Rats that consumed a total inferior to 2 g of any solution over preconditioning and conditioning were excluded. A total of one rat, from group SHAM, was excluded based on this criterion (total consumption of X = 1.6 g +/− 0.1 g). The final sample sizes are reported the Result section. Rats from group SHAM were pooled after controlling for the absence of difference in solution intake between SHAM rats injected with CNO at preconditioning and SHAM rats injected with CNO at test (Fig. S1). For conditioning, an additional fixed effect of session was included given the expected acquisition of aversion for solution X.

For preconditioning, the statistical significance of the main effects and their interaction terms were assessed using Type III SS Anova performed with the car package (3.1.3). For conditioning and tests, to test our specific, pre-defined hypotheses regarding solution preference, we performed planned contrasts on the fitted models. Using the *emmeans* package (1.11.1), we compared the estimated marginal means for solution intakes between the two solutions specifically within each of the experimental groups (SHAM, HPC-PC, HPC-T). *P*-values for these planned comparisons were reported directly, with statistical significance determined at an alpha level of 0.05.

Results

1.Histology

Fig. 1B and C. illustrates the viral expression in the hippocampus of HPC-PC and HPC-T rats. Two rats were excluded: one due to a low level of viral expression in both regions and one due to strong viral expression in the entorhinal cortex (see Table S1). Ultimately, the sample sizes were 12, 10 and 10 rats in groups HPC-PC, HPC-T and SHAM.

2.Sensory preconditioning

All descriptive statistics for solution consumptions are provided in Table S2. During preconditioning, mean consumption of AX and BY solutions was no lower than water baseline (Fig. 2A), and did not significantly differ from each other ($F(2,58) = 0.39$). There was no group difference ($F(2,87) = 0.77$). This indicated an absence of neophobia and no preference for AX or BY solution during preconditioning.

During conditioning (Figs. 2B and S2), a robust and specific aversion to solution X was acquired across all experimental groups. The intake of solution X was significantly lower on the second day compared to the first for the SHAM (estimate = 4.10, $p < .0001$), HPC-PC (estimate = 4.69, $p < .0001$), and HPC-T (estimate = 5.42, $p < .0001$) groups. Consumption of the control solution Y generally remained stable across sessions as no significant change in intake was observed for the SHAM or HPC-T groups. However, the HPC-PC group showed a small but significant decrease in the consumption of solution Y from the first to the second session (estimate = 2.01, $p = .013$), indicating generalization of the aversion from X to Y (discussed below).

Fig. 2C and D illustrate consumption levels during the test for mediated aversion to A (compared to B) and for direct aversion to X (compared to Y). They suggest that disruption of the hippocampus during stage 1 or stage 3 disrupted the mediated aversion to A without affecting the direct aversion to X. The SHAM group consumed significantly less of solution A compared to solution B (estimate of difference = - 8.76, $p = .0001$). By contrast, there was no statistically significant difference in the intake of solution A versus B in either the HPC-PC group ($p = .22$) or the HPC-T group ($p = .33$). Thus, while SHAM rats were able to acquire and express a robust aversion to solution A, rats with hippocampal disruption during preconditioning or testing could not. Interestingly, all groups showed a robust aversion for X during direct aversion test, indicating that the effects of hippocampal manipulation were indeed specific to testing for the mediated aversion (lowest estimate = -3.13, highest $p < .001$).

Discussion

The current work demonstrates that the hippocampus is essential for both the encoding of taste-taste associations and the later retrieval/ expression of a mediated aversion. The hippocampus was not required for the retrieval/expression of a directly acquired aversion, confirming that the neural basis underlying mediated and direct aversions are different (for a review, see Gewirtz, 2000).

First, we demonstrated that altering the activity in the hippocampus during stage 1 interfered with sensory preconditioned responding at test, contrasting with that of Ward-Robinson et al. (2001), following permanent hippocampal lesions and a similar unimodal protocol. These results suggest that permanent lesions allowed for compensatory mechanisms to support learning and retrieval of taste-taste associations, whereas acute perturbation revealed the necessity of the hippocampus in those processes. Thus, the hippocampus is critically involved in forming purely gustatory associations during sensory preconditioning, extending its role beyond polymodal odor-taste learning (Busquets- Garcia et al., 2018; Wheeler et al., 2013), polymodal auditory-visual learning (Lin, Dumigan, Good, Honey, et al., 2016; Pinho et al., 2025), and more complex learning that incorporates the “what-when-where” components of an event (Iordanova et al., 2011). Hence, there is evidence for a role of the hippocampus in forming associations between the different sensory stimuli that comprise an event, independently of whether they come from the same (as in this study) or different sensory modalities.

The finding that hippocampal disruption in stage 1 interfered with the expression of sensory preconditioned responding at test could be attributed to an impairment in configural learning of the AX and BY associations. The impairment of this configural learning would preclude mediated conditioning of the A-malaise association in stage 2 and/or associative chaining of the A-X and X-malaise associations at test in stage 3. An alternative but related possibility is that hippocampal manipulation disrupted the ability to form distinct representations of the pairs AX and BY. This account implies that, during/after conditioning to X, rats learned an aversion to both A and B. Hence at test, the difference between A and B consumption is reduced mainly because rats reduce their intake of B rather than preserving their intake of A. That is, the hippocampal manipulation in stage 1 impairs gustatory preconditioning not by preventing aversive learning to

preconditioned stimulus A, but instead by increasing generalization of this learning to stimulus B. This account is consistent with the generalization observed during conditioning in the group with hippocampal disruption during preconditioning. In the early days of this stage, rats do not appear to have benefited from previous distinct representations of X and Y tastes, instead attributing the cause of the gastric malaise to both tastes instead of X specifically. The enhanced exposures to X and Y across the second stage (when the hippocampus was functional) may have progressively enabled discrimination between the X and Y tastes, resulting in successful expression of direct conditioning at test.

The finding that hippocampal disruption at test also interfered with the expression of sensory preconditioned responding is consistent with fMRI studies in humans showing activation of the hippocampus at test (Barron et al., 2020; Wang, Schoenbaum, Kahnt, et al., 2020; Yu, Lang, Birbaumer, Kotchoubey, et al., 2014). It is also consistent with studies in mice showing that temporary optogenetic inactivation of the hippocampus impairs responses to a sensory preconditioned tone (Barron et al., 2020). The test effect suggests that hippocampal disruption impaired retrieval of the mediated taste aversion: hence, rats maintained their intakes of both tastes A and B at test. However, it leaves open the question of how/why this impairment occurred. That is, the sensory preconditioning response to A can originate from at least two ways of integration (Holmes, Wong, et al., 2022; Shohamy et al., 2015). During conditioning (in stage 2), presentation of X could reactivate the representation of A thanks to A-X association in preconditioning, allowing this representation to associate with malaise and form an integrated A- malaise memory formed in stage 1, despite A never being directly paired with the US. This process is known as *mediated learning*. Alternatively, the A-X and X-malaise associations remain separate during stage 2 and could be retrieved sequentially at test, with A activating the memory of X, which in turn activates the X-malaise association(i.e., associative chaining, for a review see Holmes, Fam, et al., 2022). Although the current design does not discriminate between these two mechanisms of integration, our results indicate that the hippocampus, when confronted with solution A at test, must either: (1) retrieve an integrated A-malaise representation formed during stage 2 through mediated learning, or (2) reinstate representations of the A-X association and permit its binding with the X-malaise association, enabling a chained retrieval (A → X → malaise). To discriminate between the two mechanisms of integration, one should assess the impact of hippocampal manipulation during taste aversion learning in stage 2. However, the problem with this approach is that taste aversion learning inherently involves trace conditioning of the directly conditioned stimulus (i.e. a temporal gap between taste consumption and the onset of gastric malaise), and the hippocampus has been shown to be critical for exactly this type of leaning. It could be involved because it maintains a representation of the conditioned stimulus (CS) across the delay, effectively bridging the temporal gap to the US (Sellami, Al Abed, Brayda-Bruno, et al., 2017); or because it supports reinstatement of the CS representation at the time of the US (Puhger, Crestani, Diniz, Wiltgen, et al., 2024). Consistent with a role in delay-dependent association rather than taste processing per se, Chinnakkaruppan and colleagues demonstrated that hippocampal NMDA receptor-dependent plasticity in CA1 and dentate gyrus is required for taste aversion learning only when the taste-malaise delay is substantial (Chinnakkaruppan, Wintzer, McHugh, Rosenblum, et al., 2014). Thus, inhibiting the hippocampus during conditioning in gustatory paradigms would likely disrupt taste aversion learning itself, complicating interpretation of stage 2 manipulations.

A growing literature suggests that the hippocampus works with a range of cortical regions to encode sensory associations in stage 1 of the protocols (McKenzie et al., 2014; Schuck, Niv, et al., 2019). These include cortical regions that project to the hippocampus, such as the entorhinal (Ward-Robinson et al., 2005) and perirhinal cortices (Holmes, Parkes, Killcross, Westbrook, et al., 2013; Wong, Westbrook, Holmes, et al., 2019), and regions that receive inputs from the hippocampus, such as the retrosplenial cortex (Fournier, Monasch, Bucci, Todd, et al., 2020; Robinson, Todd, Pasternak, et al., 2014) and orbitofrontal cortex (Jones, Esber, McDannald, et al., 2012; Sadacca et al., 2018; Hart, Sharpe, Gardner, Schoenbaum, et al., 2020). Imaging studies are of particular interest in this respect since they allow us to study the correlates between regions, and, thereby, neural networks that might be involved in sensory-sensory learning. Such studies in humans indicate correlated activations between the hippocampus and the orbitofrontal cortex during preconditioning, suggesting that their connections may be important for processing the generally innocuous associations (Elliott Wimmer, Büchel, et al., 2019; Wang et al., 2020). It is worth noting that most of the studies involving hippocampal manipulations targeted the dorsal but not ventral hippocampus, opening the question about the presence of a functional dissociation between dorsal and ventral hippocampus across the stages as recently suggested (Pinho et al., 2025).

The role of the hippocampus in encoding, storing, and retrieving multiple sensory information suggests that it is a critical hub for flexible, integrative learning. Our work, combined with previous studies, demonstrates a broad hippocampal contribution to sensory preconditioning: it supports both the initial learning of stimulus-stimulus associations and the subsequent use of these associations to generate appropriate predictions and guide behavior. Understanding how memories are integrated to enable flexible behavior has important translational implications, as disrupted memory integration contributes to delusions and cognitive dysfunction –core symptoms of psychopathologies including psychosis and schizophrenia. Future research should address: hippocampal involvement during stage 2 of the gustatory preconditioning protocol using designs that account for conditioning- related confounds, as well as the circuit-level mechanisms by which hippocampal projection neurons facilitate integration of sensory and emotional experiences, particularly when those experiences are separated in time.

CRediT authorship contribution statement

Melie M. Talaron: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Nathan M. Holmes:** Writing – review & editing. **Guillaume Ferreira:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Etienne Coutureau:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.nlm.2026.108187.

Data availability

Data will be made available on request.

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Chapter 5
General discussion

Animals and humans adapt to their environments by learning to predict motivationally significant events. By linking the sensory and emotional elements of distinct memories, an organism can make inferences about stimuli that have only ever been experienced as innocuous. This type of inference has implications for how memories are encoded and stored in the brain, and raises the question of how they are integrated to generate adaptive behaviour. While the hippocampus has been shown to be critical for the encoding and storage of different types of memories, its exact role in the process of memory integration has remained unclear.

The work presented in this thesis investigated how the hippocampus contributes to the encoding and integration of sensory and emotional memories that were acquired at different times. Using a combination of associative learning theory, pharmacology, fiber photometry recordings and chemogenetics, we examined stage-specific involvement of the dorsal and ventral hippocampus in different types of sensory preconditioning tasks. Specifically, we addressed the roles of these regions in the learning of a serial tone-light association and its subsequent integration with a light-shock association, and monitored the real-time neural dynamics of these processes in each subregion. We then compared these results to those obtained in a different study assessing the involvement of the hippocampus in a within-event, gustatory sensory preconditioning task, targeting the learning of flavour-flavour association and the subsequent retrieval of a mediated flavour aversion.

Summary of key findings

Characterization of the integration mechanism in an auditory-visual protocol of sensory preconditioning

The first aim of the study was methodological: to establish an auditory-visual sensory preconditioning protocol in two laboratories (UNSW, Australia and University of Bordeaux, France). We used a well-established protocol in the first laboratory, where two stimuli S2 and S1 are serially paired in stage 1, before S1 is paired with an electric foot shock in stage 2. We found that a direct transfer of this protocol to the second laboratory failed: robust first-order fear to S1 was obtained, but sensory preconditioned fear to S2 was not. Only a combination of adaptations (separating sexes, increasing S2→S1 pairings, adding background white noise, lowering shock intensity, and interleaving unpaired presentations in the control group) produced reliable sensory preconditioning, and this succeeded in males but not females. The second aim was conceptual: to identify the predominant mechanism of integration in the adapted protocol. Using two complementary manipulations, we observed that the conditioned fears to the S2 and S1 stimuli were doubly dissociable. Specifically, lowering the shock intensity during conditioning in stage 2 reduced fear to S2 without affecting fear to S1 as

assessed through freezing, whereas repeated exposures to the already-conditioned S1 extinguished fear to this stimulus while leaving the fear to S2 completely intact. This pattern indicates that integration relies on mediated learning during stage 2 rather than chaining at test, providing the psychological grounding for the neural investigations in subsequent chapters.

Publication 1: Coordination of dorsal and ventral hippocampal activity during memory integration, 2026, First author [submitted]

This study investigated how the dorsal (dHPC) and ventral (vHPC) hippocampus contribute to memory integration using the auditory–visual sensory preconditioning protocol in rats. Combining stage-specific pharmacological inactivation, dual-site fiber photometry, and c-Fos network analysis, we found that the two hippocampal subregions play dissociable roles across the different stages of the protocol. Inactivation showed that the vHPC alone was required to encode the neutral tone-light association in stage 1, whereas both the dHPC and vHPC were necessary to form the mediated tone–shock association during stage 2; and the vHPC was additionally required for direct light–shock conditioning. Photometry revealed that successful preconditioning was marked by preferential vHPC recruitment to the tone in early stage 1 and, critically, by enhanced dHPC–vHPC functional connectivity during light presentations in stage 2. Additionally, we found a double dissociation in how this connectivity predicted fear: among rats engaged in mediated learning, functional connectivity predicted mediated fear but not direct fear, and it was the opposite in control rats. Finally, mediated learning reorganised the broader hippocampal–perirhinal–amygdalar network, with a selective shift in vHPC-perirhinal coordination. Together, these findings demonstrate that it is the coordination of activity across hippocampal subregions, rather than their individual activity, that distinguishes whether separate memories are successfully integrated.

Publication 2: Chemogenetics perturbation of the hippocampus impairs gustatory preconditioning in rats, in *Neurobiology of Learning and Memory*, 2026, First author

In another line of work, we investigated the role of the hippocampus in unimodal gustatory sensory preconditioning, addressing whether hippocampal involvement, previously demonstrated mainly in polymodal protocols, extends to associations between stimuli of a single sensory modality. Using a chemogenetic approach (inhibitory DREADDs), we temporarily disrupted the dorsal and ventral hippocampus during distinct phases of a taste–taste preconditioning protocol. Rats were exposed to two-compound taste solutions (AX and BY) during preconditioning, then X (but not Y) was paired with gastric malaise during conditioning. Mediated (A vs. B) and direct (X vs. Y) aversions were assessed at test. We

found that hippocampal disruption during preconditioning impaired the learning of a mediated aversion (but not direct aversion) by increasing levels of generalisation between the various flavours. We also showed that hippocampal disruption at test prevented the expression of a mediated aversion to A, even though expression of the direct aversion to X was spared. These findings provide evidence that the hippocampus is necessary for unimodal gustatory preconditioning, both for forming purely gustatory stimulus–stimulus associations and for retrieving the resulting mediated aversion, highlighting its function as a central hub for memory integration regardless of sensory modality.

Overall, this work addresses several limitations in the literature on the role of the hippocampus in auditory-visual sensory preconditioning. It refines the distinct contributions of the dorsal and ventral hippocampus to the integration of sensory and emotional memories, showing that their coordinated activity tracks this integration. By extending these investigations to a second, gustatory sensory preconditioning protocol, it further demonstrates that the hippocampus is required for sensory-emotional learning and integration when the task involves unimodal stimuli. The common involvement of the hippocampus in the auditory-visual and gustatory sensory preconditioning protocols shows that this brain region is not engaged by sensory stimuli per se but, instead, is critical for the integration of memories or retrieval of the integrated representations that guide behaviour.

Psychological mechanisms of sensory preconditioning

1. What behaviour tells and does not tell

A fundamental challenge in interpreting behavioural experiments is that the behaviour we observe is an output of an underlying process, not a direct measure of that process. What is experimentally accessible is the animal's response, while the learning, representation, expectation, neural substrates and everything else that give rise to that response must be inferred. Yet, the relationship between a process and its behavioural output is rarely direct, unique and unambiguously reciprocal. One behaviour can be produced by more than one process, and one process can be expressed through more than one behavioural response. That is, pairing a cue with shock does not trigger a single response but engages a system that activates and inhibits a range of behaviours at once, so that scoring any one of them captures only part of what the animal is doing (McDannald, 2023). Both the cue and the situation in which it is delivered shape the form of the conditioned response (Holland, 1977). A choice of measure therefore carries a theoretical commitment, and different choices can support opposite conclusions about the same animals. For example, in an appetitive second-order

conditioning protocol, when the first-order cue is extinguished after the second-order conditioning session, approach responses to S2 survive while rearing to S2 is lost (Holland & Rescorla, 1975a; Setlow et al., 2002). Nothing separates the two readouts except the behaviour that was counted, yet one indicates that the S2–S1 association still controls responding whereas the other indicates that it does not.

This, of course, applies to selection of freezing as a measure of Pavlovian fear conditioning. Freezing is a robust and useful measure of defensive responding, but it is one component of a broader defensive system. The behavioural response recruited by a threat depends on factors including its imminence and the animal's interaction with the threatening event, such that different defensive modes can be engaged under different circumstances (Fanselow, 2026). Thus, even when a cue has acquired aversive significance, the resulting behaviour is not invariant. McDannald (2023) similarly emphasizes that a shock paired cue can engage multiple processes and behavioural outcomes, making it problematic to infer the state of the underlying learning solely from freezing. The critical issue is therefore not whether freezing is a valid measure, but what can legitimately be inferred from its presence or absence. A cue that produces freezing is almost certainly aversive, while a cue that produces no freezing has not thereby been shown to be neutral.

This is the standard against which our failure to obtain sensory preconditioning in females should be read. Male and female rats adopt discrete behavioural strategies and a difference in behavioural output may simply reflect a difference in strategy (Shansky, 2018). That is, our failure to observe sensory preconditioned fear in female rats should not be taken to indicate an absence of memory integration in these animals. Instead, it should be taken to indicate that freezing does not index conditioned fear equally well in both sexes, which is consistent with previous work showing that female rats tend to freeze less than males, expressing learned fear through active responses instead (Gruene et al., 2015). When a task allows fear to be expressed by moving rather than by not moving, the sex difference in freezing persists while avoidance of the shock-associated context is equivalent, indicating comparable memory expressed through a different motor strategy (Shanazz et al., 2022). The same conclusion follows from a measure that does not depend on locomotion at all: males froze more than females during a contextual fear test, yet fear-potentiated startle did not differ between them (Russo & Parsons, 2021). Whether a sex difference is observed therefore depends on which behaviour is scored. Of note, these interpretations are confounded with demonstrated sex differences in aversive learning. The developmental trajectory of contextual fear runs in opposite directions in males and females and is established before puberty (Colon et al., 2018), and gonadal hormones modulate the circuitry supporting fear learning and its extinction in

ways that interact with stress (Maeng & Milad, 2015). More directly relevant, females showed greater generalisation than males in a light–tone preconditioning protocol run under identical conditions (Pinho et al., 2025). Our data cannot separate these accounts: they establish that this protocol, with this readout, does not reveal integration in females, and nothing more. While sex differences in sensory preconditioning are not the core of this thesis, the question of whether females integrate sensory and emotional memories in a sensory preconditioning protocol remains to be addressed.

2. Content of preconditioning

Throughout this thesis, we have indicated that stage 1 of sensory preconditioning involves acquisition of an S2-S1 association. However, such a fixed description does not do justice to the detailed content of what is learned. In Chapter 2, a nominally identical protocol, involving the same stimuli, stages, and behavioural readout, produced sensory preconditioning or failed to do so depending on parameters that left the S2-S1 contingency unchanged. Moreover, the double dissociation observed in the adapted protocol showed that the mechanism through which the two memories were subsequently integrated was at least partially determined by what had been established during stage 1. What is learned during preconditioning is therefore not only fixed by the pairing of S2 and S1. It also depends on the parameters defined by the experimenter and on the animal's experience of those parameters. This content importantly determines what information is subsequently available for integration, but also the psychological mechanism through which integration occurs and the neural circuits by which it is supported.

How the protocol influences representations generated during sensory preconditioning

As reviewed in the Introduction, several parameters influence the nature and content of learning in a sensory preconditioning protocol. The most consequential is the temporal arrangement of S2 and S1. When the cues are presented serially, the animal can learn a directional relation in which S2 predicts S1. When they are presented simultaneously as a compound, the relation instead concerns the sensory characteristics of the two events, producing a within-event or configural representation (Rescorla & Cunningham, 1978; Rescorla & Durlach, 1981). These different representations provide different content for stage 2 integration. The serial tone-light protocol used in Chapters 2 and 3 and the simultaneous taste compounds used in Chapter 4 therefore differ not only in modality, but in the kind of representation established during stage 1.

The amount of preconditioning also changes the content of this representation. Early work on representation-mediated aversion learning showed that an associate can be retrieved after limited training in stage 1, but that this mediated learning disappears with extended exposure (Holland, 1981, 1998). Therefore, the amount of exposure changes what the representation contains, adding temporal and ordinal information that can alter what is retrieved and thereby shift integration from mediated learning towards associative chaining, even though both mechanisms can co-exist (Holmes, Wong, et al., 2022). Temporal structure within a trial is another component of what is learned. A gap between S2 and S1 can abolish sensory preconditioned fear (Kikas et al., 2021), whereas changing the order of the cues does not necessarily prevent that fear (Ward-Robinson & Hall, 1996, 1998). More generally, responding to S2 can reflect the expected temporal relation between S2, S1, and the US rather than simply the strength of first-order conditioning (Arcediano et al., 2003; Matzel et al., 1988; Polack et al., 2013). Temporal information is therefore part of the stage 1 representation and can constrain the form of integration available at test.

Finally, the context in which the cues are experienced also contributes to what is learned, and how. Preconditioning in a context previously associated with shock shifts the neural mechanisms supporting learning towards the BLA. That is, infusion of NMDA receptor antagonist in the perirhinal cortex prior to S2-S1 pairings no longer disrupts learning about S2; however, the same manipulation in the BLA impairs sensory preconditioning (Holmes et al., 2013, 2018; Holmes & Westbrook, 2017; Qureshi et al., 2023). Pharmacological manipulation of arousal can produce related effects and can even rescue preconditioning when the S2 and S1 are separated by a temporal gap (Kikas et al., 2021). The background against which S2 and S1 are experienced, in addition to their temporal and sensory relations, therefore influences integration.

Across these examples, the common principle is that sensory preconditioning is not a fixed computation implemented by a fixed protocol: the representation available for integration depends on what the animal experiences during stage 1, and consequently on what the subsequent stages can retrieve and combine. Thus, apparently inconsistent findings across studies may partly reflect differences in what the protocol allowed the animal to learn.

How the protocol influences the interpretation of the environment

Several of the parameters described above (trace intervals, context shifts, and extended training) may not only influence the content of the encoded representations, but also the animal's inference about how its experience is generated. This view is supported by latent-

cause accounts. They suggest that the animal does not simply record which cues occurred together; it hypothesises about what generated its observations. Each observation is assigned, probabilistically, to a latent cause: an unobservable source with characteristic statistics. Associations are then learned within a cause rather than between raw stimuli, and generalisation from one cue to another follows from the inference that both were generated by the same cause (Courville et al., 2006; Gershman et al., 2010; Gershman & Niv, 2012). The framework was developed to handle phenomena that elemental models do not accommodate straightforwardly, most notably why extinction creates a new memory rather than erasing the old one; if the extinction phase is attributed to a new latent cause, the original cause and its associations survive intact, and renewal and spontaneous recovery follow (Gershman et al., 2010, 2017).

Applied to sensory preconditioning, the model states that stage 1 provides repeated co-occurrence of S2 and S1 with no further structure, and the parsimonious inference is that a single cause generates both stimuli. In stage 2, S1 is paired with shock under circumstances that, if sufficiently similar to those of stage 1, are attributed to that same cause, which thereby acquires aversive significance. Thus, when S2 is presented at test, it signals that the same, danger-associated cause is active, which then leads to the expression of defensive behaviours including freezing. On this description, mediated conditioning is value-tagging of an inferred cause rather than the formation of a new link with an associatively retrieved element.

It is tempting to treat the SOP quantity pA2(S2) as the elemental implementation of this common cause, because both express how strongly an event that is currently-present can activate the memory of an event that is not. But the two should not be treated as equivalent. pA2(S2) is a graded activation state of a single node, whereas a latent cause is a variable that groups events and to which learning is indexed. The first is a property of a representation, the second a hypothesis about the environment. This difference has empirical consequences that provide a way to distinguish it from an elemental account. Because the hypothesis of a latent cause depends on the statistical structure of the events, this view predicts that mediated learning should be reduced by manipulations that make a common cause less likely without necessarily changing V(S1→S2), such as a change of context between stages, a long interval separating them, or variability in the temporal arrangement of stage 1.

Thinking inside the box: the influence of internal states

The external environment also shapes the internal state and actions of the animal which, in turn, influences how the animal learns about the environment. In other words, rats' current

internal or motivational state also has an impact on associative learning processes and has the potential to affect the associative mechanism of integration.

Motivational state differs in the two protocols used in the present thesis. In the auditory–visual protocol, rats are neither food- nor water-deprived, and the experience of the auditory and visual stimuli does not depend on the animal's responses. In the gustatory protocol, rats are water-restricted and, therefore, need to drink the flavoured solutions or compounds that are presented in water. Even in the absence of neophobia, the water-deprivation intrinsically increases motivational engagement. Thus, the two sensory preconditioning protocols used in the thesis do not only differ with respect to the modality of the stimuli and the manner of their presentation; they also differ with respect to the motivational state of the animal at the time of the sensory preconditioning session in stage 1.

In SOP, the impact of internal state on associative learning can be implemented in terms of how effectively a stimulus is processed. In the model, the terms that influence how effectively a stimulus is processed are: 1) p1, which is the proportion of a node's elements driven into A1 upon presentation of the stimulus; and 2) pd1, which is the rate at which elements in A1 decay to A2. Together, these two quantities sit upstream of everything else in determining how strongly the S2–S1 link can grow in stage 1, and, through V(S1→S2), how much of S2 can be primed into A2 when S1 is later paired with the US. Because mediated learning is proportional to pA2(S2)·pA1(US), and pA2(S2) is itself proportional to V(S1→S2)·pA1(S1), the animal's state during preconditioning has consequences through the entire calculation.

Motivational state is a source of variation in that quantity. In the gustatory case, the degree of liquid deprivation modulates the strength of a conditioned taste aversion and the rate at which it extinguishes, and thirst at the time of retrieval in particular weakens the expression of an aversion that has already been acquired (Miranda et al., 2023). More generally, arousal state during encoding influences whether a weakly consequential experience is retained. Indeed, novelty engages locus coeruleus projections to the hippocampus which determine whether an otherwise ordinary event is consolidated (Takeuchi et al., 2016), and in humans, states of curiosity (i.e., how interested in an answer participants were when asked a question) enhance hippocampus-dependent encoding of incidental material (i.e., content unrelated and irrelevant to the question asked) through dopaminergic projections from the midbrain to the hippocampus (Gruber et al., 2014). Because stage 1 of the auditory-visual sensory preconditioning protocol consists of precisely such incidental learning, it is sensitive to the rats' arousal and motivational states. That change in learning is accompanied by changes in the neural substrates through which sensory preconditioning is supported; for example, if stage 1 of sensory preconditioning is administered in a dangerous place, the circuit supporting learning differs: sensory

preconditioned fear becomes entirely supported by the BLA (Holmes et al., 2013, 2018; Holmes & Westbrook, 2017; Wong et al., 2026).

Is sensory preconditioning really incidental?

Stage 1 of sensory preconditioning is often said to involve the learning of an incidental association between two neutral stimuli. Neutral refers to the fact that neither stimulus is motivationally significant, whereas incidental refers to the fact that the association between the two stimuli is acquired in the absence of explicit instruction and has no immediate use for the guiding of behaviour.

Whether stage 1 is truly incidental in both protocols should be examined. In the auditory–visual protocol the tone and light are delivered to the rat, which does not need to make any response and has nothing to gain. In the gustatory protocol the stage 1 compound is consumed, and consumption is an action the animal performs under its motivational state: it is thirsty, and the solution is the object of an ongoing consummatory action rather than an event that happens to them. Therefore, what is encoded in stage 1 may not be a pure stimulus–stimulus association, because the tastes arrive as outcomes of the animal's own licking, and the episode contains an action as well as two events. Of note, encoding is self-paced: the rat determines when and how much it samples, and volitional sampling engages the hippocampus differently from passive exposure (Voss et al., 2011).

This constrains what can be concluded from the comparison between our protocols. At the regional level, the hippocampus was required at stage 1 in both protocols (Chapters 3 and 4), so its recruitment does not depend on whether the animal is a passive recipient of the stimuli or an active sampling agent. However, the common engagement of the hippocampus does not necessarily imply that the same computation is performed in the two cases, since they differ not only in stimulus modality and arrangement but also in the manner by which the stimuli are experienced.

A second question the design leaves open is about the content of “incidental” learning. It is convenient to describe stage 1 as establishing an S2–S1 association and nothing more, but a stimulus reliably followed by no outcome is not a stimulus about which nothing is learned. Since Rescorla (1968), the informational relation rather than the pairing has been the unit of learning, and repeated non-reinforced exposure can produce a loss of associability and, if there is an explicit negative contingency in relation to the US, conditioned inhibition. Stage 1 may therefore establish, alongside the intended sensory–sensory association, something closer to a representation of safety, which should oppose the conditioning that follows. This has an

immediate consequence for how the performance of the control group is interpreted. Unpaired rats receive the same number of tone and light presentations, in the same chamber, according to the same schedule. They are not a group that learns nothing but, rather, a group that learns that the S2 and S1 are unrelated and that neither predicts anything of immediate significance. Any difference between paired and unpaired animals, behavioural or neural, is therefore a difference between two things learned rather than between learning and its absence. This point should be considered when interpreting neural and behavioural data in stage 2 of sensory preconditioning.

Considerations for the present work

The factors reviewed here (protocol parameters, motivational state of the animal, manner through which the stimuli are experienced, interpretation of the environment) determine the representations that the animal forms in stage 1. These representations, in turn, influence the associative basis of memory integration across the subsequent stages of the sensory preconditioning protocol and, thereby, its neural substrates.

To be clear, two studies that both label their first phase preconditioning may be studying a directional prediction in one case and a configural unit in the other, and these may be integrated by different mechanisms and supported by different structures. Indeed, much of the apparent inconsistency in the literature regarding the involvement of the hippocampus in sensory preconditioning (e.g., the hippocampal-independence of sensory preconditioning reported by Ward-Robinson et al. (2001) and the hippocampal-dependence reported by Busquets-Garcia et al. (2018) and Pinho et al. (2025)) may be resolved through consideration of the representations that the animal forms in stage 1 and its implications for how memory integration is achieved across subsequent stages of the protocol.

Read in this light, the finding that the hippocampus played a role across the two experimental lines of this thesis, alongside the literature showing that it also regulates sensory preconditioning in other protocols, is informative precisely because of the differences between them. The hippocampus regulates aspects of associative formation in a serial polymodal fear protocol, in a simultaneous unimodal taste protocol, in an episodic-like ‘what-when-where’ protocol (Iordanova, 2009), as well as in protocols that involve odor-taste learning (Busquets-Garcia et al., 2018). These protocols differ with respect to the stimulus arrangement in stage 1, stimulus modality, how the animal experiences the stimuli (exposed versus earned), and in the motivational state of the animal. Thus, by the arguments above, each protocol should establish representations of very different kinds. A structure whose involvement persists across such a broad range of protocols might make a different functional contribution in each case; or, alternatively, it might support what is common to all of them: e.g., acquiring and

representing an innocuous sensory association, and integrating it with a subsequently formed emotional association. This consideration has implications when interpreting the neural findings, which might reflect a specific computation dependent on the design, or a general computation that acts on a common underlying process.

3. Translational relevance of sensory preconditioning

Because mediated learning involves the formation of an S2-US association that was never actually experienced, it can be framed in terms of “false memory”, illustrating the flexibility of memory: it does not simply record past events but can generate new representations (Bae et al., 2015; Lingawi et al., 2018). In humans, the benefits of this flexibility extend beyond adaptation to the environment. By bringing together elements that have not been experienced, integrated memories support imagination, creativity, decision making, schema development, and the generation of novel knowledge from previously acquired information (for a review, see Schlichting & Preston, 2015). Consistent with its role in this broader set of processes, hippocampal amnesia impairs creative thinking (Duff et al., 2013), and the abstraction of hippocampal representations during higher-order learning predicts subsequent creative output (Zhang et al., 2025). Sensory preconditioning therefore provides a window through which to view the integrative capacities that support these various processes and functions.

However, the same flexibility that supports mediated learning, by definition, produces memories that do not correspond to actual events. In this sense, the capacity to draw inferences from related experiences comes at the expense of memory accuracy. Being able to distinguish internally generated representations of associations from actual experience may then constitute a critical boundary between adaptive behaviour and pathological condition. This boundary is often discussed in the context of “reality testing”, commonly defined in clinical studies as the capacity to distinguish internal fantasy from external reality. Applied to mediated learning and sensory preconditioning, reality testing has been invoked to explain the loss of the sensory preconditioning effect when training in stage 1 is extended (Busquets-Garcia et al., 2017; McDannald et al., 2011; McDannald & Schoenbaum, 2009). One explanation proposes that, with moderate preconditioning, animals form a unified representation of the preconditioned stimuli (S2+S1). With prolonged exposure, however, they acquire more detailed information about the stimuli, allowing them to discriminate their specific sensory features and, consequently, their associated outcomes. This account has motivated the study of when and how animals switch, during stage 1, from a unified or “hallucinatory” representation to a more discriminated representation. Although this approach has proved useful for studying psychotic-like states and positive symptoms of schizophrenia, such as

hallucinations, we argue reality testing is better understood in terms of the content of the representations and their associative relationships.

That is, reality testing in sensory preconditioning may depend less on a change in the nature of the representation formed during stage 1 than on processes occurring subsequently. Specifically, increasing preconditioning does not fundamentally change the nature of the representations of S2 and S1, which are distinguished as two individual identities from very early in training. Instead, it changes their content, including the elements encoded within these representations and their relationship to one another, and consequently the conditions under which one stimulus can evoke the representation of the other. Moreover, the fact that a stimulus evokes the representation of its associate does not imply that the animal mistakes an absent event for a present one. In this view, early in training, the relation between S2 and S1 may be predominantly reciprocal, such that either stimulus can evoke a representation of the other as an event that is known to be absent. This reciprocal association favours mediated learning during stage 2. With further training, however, animals acquire information about the temporal order in which the stimuli occur. Consequently, the first stimulus in the sequence will evoke an expectation of the second stimulus but not vice versa, resulting in a shift in the mechanism of integration from mediated learning in stage 2 to associative chaining at test in stage 3. With extensive training, animals may additionally acquire inhibitory associations and information about the unique elements of the predictor, such that, despite their previous association, the stimuli become increasingly distinguishable and sensory preconditioning is ultimately lost ("reality testing").

Critically, the relevance of these changes lies in how they affect subsequent retrieval. In Wagner's SOP terms, the content acquired during stage 1 may determine the probability that S2 enters the A2 state when S1 is encountered, p(A2). This provides a common mechanism through which different learned content in stage 1 can influence whether and how sensory preconditioning is expressed, with no change in the basic nature of the S1 and S2 representations. If the content is a simple reciprocal association, p(A2)S2 is high and S1 can activate the representation of the absent S2 which supports mediated learning. If the memory contains information about temporal order, p(A2)S2 is low and associative chaining can occur; finally, if S2 undergoes additional inhibitory or safety learning, sensory preconditioning can be abolished. From this perspective, the "hallucinatory" component of sensory preconditioning is not a property of the memory formed during stage 1. It emerges during stage 2 when the representation of the absent S2 is activated by S1. That is, the distinction between discriminated and "hallucinatory" memory is not fixed during stage 1. Instead, reality testing is

something that occurs across subsequent stages of the protocols, perhaps most importantly in stage 2.

Importantly, returning to the original definition, forming a “false” S2–US representation does not necessarily imply a hallucination: representing S2 in its absence is not equivalent to treating S2 as physically present. If it were, the representation of S2 would be functionally indistinguishable from S1 itself. Indeed, Suzuki and Kosaki (2024) reviewed associative-learning accounts of the positive symptoms of schizophrenia, and argued that a core deficit can be described within Wagner’s SOP framework as a failure to suppress focal (A1) processing of two classes of events: stimuli that are physically present but informationally redundant, and stimuli that are physically absent. These processes are relevant to sensory preconditioning but occur at different stages of the procedure. Physically present but irrelevant stimuli occur in stage 1. Reduced processing of a stimulus that has proved uninformative is what latent inhibition and habituation measure, and attenuated latent inhibition has been reported repeatedly in acute schizophrenia and in its pharmacological models, although the literature is not uniform (see Suzuki & Kosaki, 2024, for review). Physically absent stimuli correspond to stage 2, where the absent S2 is retrieved into A2 and enters an association with the US. The capacity to keep that representation in A2 rather than A1 is what distinguishes an inference from an experience. Where the distinction fails, a retrieved representation is treated as though the event had occurred, which is the mechanism proposed for conditioned hallucinations, in which people prone to hallucinatory experience come to report hearing a tone upon presentation of an associated visual cue (Powers et al., 2017) and, more generally, exhibit source-monitoring failures (Simons et al., 2017).

Sensory preconditioning also provides a complementary approach to studying overgeneralisation, as the inferred threat response in aversively motivated protocols is transferred between stimuli through an associative relation and not via perceptual similarity, a distinction that may be particularly relevant to the false and associative memories observed in psychopathology (Lissek et al., 2014; Otgaar et al., 2017).

Taken together, these findings suggest that aberrant inference may represent a core mechanism contributing to psychotic states. As associative representations increasingly guide responding to events that have not been directly experienced, the boundary between inference and experience may become compromised. This could arise through an inappropriate treatment of retrieved representations, excessive retrieval-mediated learning, or a failure to regulate the transition between A2 and A1 processing. Such alterations may contribute to symptoms including hallucinations, overgeneralisation, and false or associative memories.

Importantly, sensory preconditioning does not model any of the clinical conditions described above. Instead, it provides a way to study the processes and computations that underlie certain symptoms of those conditions, and their neural substrates. Interestingly, the processes and computations that are disturbed in clinical conditions are often attributed to hippocampal dysfunction, specifically in its role as a comparator of predicted and experienced episodic events (Gray et al., 1991; Kumaran & Maguire, 2007; Vinogradova, 2001). They also involve broader prefrontal and medial temporal circuits involved in assigning value to inferred outcomes, distinguishing retrieved from perceived information, and regulating emotional responses (Jones et al., 2012; Milad & Quirk, 2012; Sadacca et al., 2018; Simons et al., 2017). Thus, the computations isolated by sensory preconditioning and those proposed to contribute to these clinical symptoms implicate a common distributed network. This convergence makes it important to determine not only which structures support the individual processes that are required for successful sensory preconditioning, but also their relations with each other in a functional circuit.

Neural mechanisms of sensory preconditioning

1. Hippocampal substrates of sensory preconditioning

Hippocampal contributions to preconditioning

The hippocampus appears to be particularly important when information without an immediate behavioural purpose must be preserved for later use. Several studies have now shown the critical role of the hippocampus for this type of learning during stage 1 of sensory preconditioning. For example, blocking CB1 receptors on hippocampal GABAergic interneurons during sensory preconditioning abolished learning about the S2–S1 relationship while leaving first-order conditioning intact (Busquets-Garcia et al., 2018). More generally, in an incidental-encoding paradigm, Zhou and colleagues (2012) showed that temporary CA3 inactivation impaired rats' ability to later retrieve information that had been acquired incidentally, but did not impair their ability to respond when the same information was already available at the time of the test.

As discussed above, the details of a protocol strongly influence what is learned in stage 1 and, consequently, the neural substrates of this learning. One possibility, therefore, is that the hippocampus directly encodes the association between the two neutral stimuli, with the particular hippocampal subregion recruited depending on the preconditioning arrangement. A second, non-exclusive possibility is that hippocampal activity during stage 1 determines which

incidental experiences are subsequently prioritised for offline processing. Awake sharp-wave ripples, for instance, may contribute to selecting particular episodes for later replay during sleep, with ripple characteristics predicting subsequent reactivation and consolidation (Yang et al., 2024).

Offline reactivation may be particularly important to allow later inference using the reactivated memories. Indeed, reactivation during rest can generate predictive relationships between events that were never directly experienced together, producing shortcuts that cannot be explained by strengthening the links that were present during learning (Shearer et al., 2025). Consistent with this possibility, disrupting hippocampal replay prevents the emergence of predictive representations (Liu et al., 2023). Importantly, replay is not allocated uniformly across experience. Huelin Gorriz et al. (2023) showed that replay prioritisation reflects an interaction between novelty and repeated experience. Thus, increasing training may alter hippocampal involvement by changing the subset of experiences selected for replay. These findings raise the possibility that hippocampal processing during stage 1 influences not only how strongly S2 and S1 are initially encoded, but also whether their representations are subsequently preserved, reorganised, and made available for later integration.

A further possibility concerns the organisation of the stimulus representations established during stage 1. For example, in addition to an S2–S1 association, the initial stage of sensory preconditioning may involve hippocampal-dependent encoding of a latent structure that incorporates that association. Consistent with this view, we found preferential ventral hippocampal recruitment to the tone in rats that would later show preconditioned fear, and most prominently during the early tone presentations in stage 1. Thus, aside from a potential role in encoding and/or storing the S2-S1 association, the ventral hippocampus may be involved in the construction of a representation in which the tone and light are treated as arising from a common latent cause. Evidence from other tasks is consistent with this proposal. In mice performing a bandit task with unsignalled reversals, ventral CA1 was necessary for inferring which of two latent contexts was currently in effect (Mishchanchuk et al., 2024). On this account, hippocampal involvement during stage 1 may determine how events are represented (as isolated experiences, as directly associated stimuli, or as elements embedded within a common latent structure) and thereby influence whether and how they can later be integrated to support preconditioned responding.

Hippocampal contributions to memory integration

a. A mechanism for memory integration in the dHPC

Our results demonstrated that the hippocampus is required for mediated learning, a process which may depend particularly on CA1. Anatomically, CA1 is the only hippocampal subfield that receives both Schaffer collateral input from CA3 and direct input from entorhinal layer III, placing internally retrieved and externally driven information on the same dendritic tree. Together with the subiculum, CA1 also provides the principal output of the hippocampus. Functionally, this arrangement is consistent with a division of labour in which the dentate gyrus and CA2/CA3 differentiate related memories, whereas CA1 integrates them (Dimsdale-Zucker et al., 2018; Duncan & Schlichting, 2018; Schapiro et al., 2017). Evidence from human studies supports the link between CA1 and integration of memories: CA1 activity during encoding of events that overlap with prior experience predicts subsequent inference, and the CA1 pattern established during encoding is later reinstated when the inference is made (Schlichting et al., 2014). CA1 is also particularly responsive to information arriving from cortical regions. Perirhinal inactivation, for example, disrupts task-dependent firing in CA1 while leaving CA3 activity intact (Lee & Park, 2013). Given that the perirhinal cortex is itself required for the tone–light association (Holmes et al., 2013; Wong et al., 2019), CA1 provides a plausible site at which the cortical representation of the sensory association could be combined with hippocampally retrieved information to support its integration with the new aversive outcome.

A supplementary experiment to this thesis identified the pharmacological substrate of integration in the dHPC. Infusing the NMDA receptor antagonist D-APV into the dHPC before stage 2 in the auditory-visual sensory preconditioning protocol abolished fear to the tone S2 while leaving fear to the light S1 intact (see **Appendix 2**). This requirement for NMDA receptor-dependent plasticity identifies the dHPC as a candidate site where the integrated memory is formed, though it should also be noted that the exact same pattern of results is obtained following perirhinal NMDA receptor antagonism at the same stage (Wong et al., 2019, 2025). Together, these results suggest that the hippocampus works with or alongside the perirhinal cortex to achieve the integration. However, because the infusion reaches the dentate gyrus, CA3 and CA1 alike, this experiment cannot localise the locus of integration in the hippocampus to a particular subfield.

Nonetheless, there is a close compatibility between mediated learning as the basis of integration and the requirement for NMDA receptor activation in the dHPC. It parallels NMDA receptor-dependent plasticity in the BLA, where associative learning depends on the convergence of CS and US information. In stage 2 of sensory preconditioning, the corresponding convergence would be between the reinstated representation of S2 and

sensory and affective information associated with the current US. The hippocampal circuit provides a plausible substrate for this process: CA3, through its recurrent collateral network, is well suited to reinstate a stored representation from a partial cue and could, therefore, provide the reinstated S2 representation to CA1. CA1, in turn, receives both CA3 output and direct entorhinal projections carrying information about the current event, allowing these representations to converge and potentially become bound through NMDA receptor-dependent plasticity (Duncan & Schlichting, 2018). Consistent with this possibility, both CA3–CA1 and entorhinal–CA1 inputs undergo potentiation during associative learning (Wang et al., 2025). Thus, integration may depend on complementary contributions from CA3 and CA1, with CA3 supporting reinstatement of the absent stimulus and CA1 providing a site at which this representation can be associated with the current event. Importantly, this is a hypothesis about the division of labour among hippocampal subfields rather than about the longitudinal axis, along which the organisation of the transverse circuit is broadly conserved.

b. Examining the ventral hippocampus in memory integration

Inactivation of vHPC during stage 2 impaired both direct and mediated fear, meaning that we cannot distinguish a role for the vHPC in memory integration from a role in first-order conditioning. One way to resolve this ambiguity is to change the class of manipulation performed. The amygdala provides a useful template for this approach: muscimol infusion before test abolishes fear to both the directly conditioned and the preconditioned stimulus (like infusions in vHPC), whereas manipulations targeting plasticity impact only one of the two fears. The additional experiment conducted for this thesis shows that mediated learning in the dHPC is NMDA-receptor dependent, as is the corresponding process in the perirhinal cortex. By contrast, Zhang et al. (2001) found that NMDA receptor antagonism in the vHPC spares direct fear conditioning to a tone. Thus, replacing intra-vHPC muscimol by an NMDA receptor antagonist administered before stage 2 could potentially distinguish between the roles of the vHPC in memory integration and first-order conditioning: if vHPC NMDA-dependent plasticity is required for integration, the manipulation should selectively affect the mediated fear to S2 while sparing the directly conditioned fear to S1.

A second approach would be to target pathway specificity. Ventral CA1 output is functionally parcellated, with projections to the amygdala implicated in context-dependent fear, and projections to prefrontal cortex supporting other functions such as instrumental actions (Bakoyiannis et al., 2023; Dong & Clem, 2026; Li et al., 2024). If the vHPC contributes primarily to fear expression, silencing the vCA1–amygdala projection during stage 2 should impair both direct and mediated fear. If, instead, the vHPC contributes to the integration of the two representations, a cortically directed projection might be expected to affect mediated fear

selectively. Such a dissociation would help determine whether the contribution of the vHPC reflects a general influence on fear expression or a more specific role in the formation of the mediated association.

Future studies of memory integration in the hippocampus will benefit from a focus on the neuronal populations involved rather than just regional activity. For example, in the dorsomedial prefrontal cortex, Gu and Johansen (2025) combined neuronal recordings across learning stages with projection-specific manipulations. They found that multisensory-responsive neurons emerged after paired presentations and remained stable across stages, and that silencing the amygdala-projecting population specifically disrupted the inferred association in sensory preconditioning. A similar strategy could be applied to the hippocampus by imaging dorsal and ventral CA1 across the stages of sensory preconditioning. In paired animals, this should reveal whether the population of cells that responds to the light during stage 2 overlaps with the population that represented the tone during stage 1. Such cross-stage stability would provide more direct evidence that the hippocampus contains a neuronal population capable of linking the two stimulus representations.

c. Chaining integration and the hippocampus

The conditions of preconditioning influence what is learned and, thus, how integration occurs across the subsequent stages of the protocol. Conditions that favour mediated learning trigger the animal to form a never-experienced association involving an absent stimulus, whereas conditions that favour chaining involve the successive retrieval of experienced memories. These are two different processes and, as such, potentially supported by different neural substrates. Hence, the role of the hippocampus in mediated learning cannot necessarily be extended to chaining.

The extended training version of the sensory preconditioning protocol, which shifts integration from mediated learning to chaining (Wong et al., 2019), would be particularly informative for determining how the hippocampus contributes to a different type of memory integration. In this extended version, dHPC inactivation during stage 2 should no longer disrupt sensory preconditioned fear, because fear to S2 relies on processes occurring at test. By contrast, because the vHPC is required for first-order conditioning of S1, its inactivation during stage 2 should prevent S2 from eliciting fear at test, as the second link in the S2-S1-US associative chain will have been broken.

The dorsal hippocampus should, however, continue to play an important role during stages 1 and 3 of the extended sensory preconditioning protocol. Importantly, sequential information

encoding and retrieval have been shown to depend on the hippocampus. Hippocampal lesions leave recognition of individual odours intact while disrupting memory for the order in which they were presented (Fortin et al., 2002); and impair the rapid acquisition of higher-order sequential associations while sparing their slower, extrahippocampal acquisition (Ergorul & Eichenbaum, 2006). They also disrupt incidental memory for the order of trial-unique events (Allen et al., 2020), which closely resembles the incidental learning in stage 1 of sensory preconditioning. Beyond order, the dHPC is also required for interval information: lesions shift the peak of conditioned responding earlier than the time of reinforcement, degrade its precision (Tam et al., 2015; Tam & Bonardi, 2012), and impair the maintenance of temporal information across gaps when the cue itself is absent (Tam et al., 2013). Hence, if rats solve the preconditioning task by chaining, the dHPC should be required during stage 1 to encode the order of the stimuli, and again at test, when the sequence has to be retrieved and run forward to generate defensive, fear responses.

d. Hippocampal representations for inference

The hippocampus has a role in mediated learning and is predicted to play a role in associative chaining. However, the methods used in this thesis do not allow us to determine exactly which functions the hippocampus supports in mediated learning, which might include retrieving the S2 representation, associating the retrieved representation with danger, facilitating plasticity in downstream regions, or storing the resultant S2-US association. A useful way to approach this determination is therefore to consider the broader set of integrative functions attributed to the hippocampus.

As reviewed in the Introduction, the hippocampus is not typically considered to be the site of a particular association. Instead, it is taken to be a structure that organizes relationships between events and integrates distributed representations into memory. From this perspective, the question is not whether the hippocampus is required for mediated learning or chaining but, rather, how it systematises and integrates representations to support the process of inference.

Three representational structures have been proposed for how the hippocampus may organise relational information across overlapping experiences (Zeithamova, Schlichting, et al., 2012). They differ primarily in the extent to which they preserve the original episodes and in whether the inferred relationship is established during encoding or only at retrieval. In the first structure, the overlapping element (S1) triggers pattern completion of the earlier S2–S1 episode when the S1–US episode is encountered. The reactivated and current experiences are then integrated into a single composite representation, directly linking S2 and the US. The

cost of this integration is that the distinction between the two original episodes may be reduced or lost. This is broadly consistent with the idea of a cognitive map: overlapping experiences are incorporated into a unified representation that is updated as new information is encountered (O'Keefe & Nadel, 1978; Tolman, 1948). In the second structure, the S2–S1 and S1–US episodes remain distinct, with their shared element providing the route between them. This preserves episodic detail, but the S2–US relationship is not stored as an integrated relation and must instead be inferred by retrieving and combining the two trained episodes at test (McClelland et al., 1995). The third structure combines these possibilities. The original S2–S1 and S1–US representations remain distinguishable, but reactivation of the first during encoding of the second also establishes an integrated relationship across them. Inference can therefore draw both on preserved episode-specific information and on a relational network that links the two experiences. This hybrid account is compatible with indexing theory, in which hippocampal representations act as pointers to distributed cortical representations, allowing individual experiences to remain distinct while also supporting their joint retrieval and relational integration (Rudy, 2024; Teyler & Rudy, 2007). Such a representational architecture predicts a role for the hippocampus at both encoding, when overlapping experiences are linked, and retrieval, when the resulting network is accessed to support inference.

Reconsidering the dorsoventral division of hippocampal function

The dHPC and vHPC are dissociably involved in encoding of the three associations acquired during sensory preconditioning (S2-S1; S1-US and S2-US). The formation of both the S2–S1 and S1–US associations required the vHPC, but not the dHPC. The dissociation observed for first-order conditioning is consistent with previous evidence for distinct contributions of the two hippocampal poles to associative learning (Esclassan et al., 2009b; Maren & Holt, 2004), and is also consistent with their anatomical connectivity. In particular, the requirement for the vHPC during S1–US conditioning is expected given its reciprocal connections with the basolateral amygdala (Bast et al., 2001; Dong & Clem, 2026).

However, our finding that the vHPC was required to encode a tone-light association that predicts no biological significance suggests that its contributions extend beyond associations containing direct affective value, and challenges the historical view of the vHPC being engaged for processing of emotional and affective information (Moser & Moser, 1998b). Because the tone–light association was never paired with a US, its acquisition may have endowed the cues with a non-threatening or "safe" meaning, in the sense that it does not signal danger. In this respect, our finding may be relevant to work showing that vHPC representations are engaged by explicitly learned safety, suggesting that vHPC involvement in associative encoding does

not necessarily depend on biologically aversive or rewarding outcomes. Consistent with this, Nguyen et al. (2023) showed that when a conditioned stimulus acquires *safety* properties during extinction, vHPC activity to that stimulus is reactivated during sleep and strengthened at retrieval, and that these representations depend on glutamatergic amygdalar input during learning. More broadly, the involvement of vHPC output pathways in object memory, social memory, and instrumental action supports the claim that their functional contributions extend beyond Pavlovian and affective processes (Bakoyiannis et al., 2023).

The broader contribution of the vHPC to associative learning could imply that its functions overlap more extensively with those of the dHPC than a strict functional dichotomy would predict. Yet, the dissociable roles of the dHPC and vHPC across stages of sensory preconditioning suggest that some form of longitudinal specialisation is preserved. A way to reconcile these results is to consider that, rather than implementing fundamentally different computations, the two subregions may be dissociated by the information to which each has access. McNaughton and Bannerman (2024) argue that the transverse hippocampal circuit operates in essentially the same manner along the longitudinal axis, with apparent functional differences arising from the distinct situational and motivational information entering, and being transmitted by, different portions of the circuit. From this perspective, the dHPC–vHPC dissociation observed here may reflect differences in the inputs available to each subregion, and, consequently, in the information that can be incorporated into the same underlying computation. The requirement of both subregions for mediated learning could be a reflection of such a view. The anatomical organisation of the human hippocampal long axis, characterised by both gradual connectivity gradients and more circumscribed zones of dense connectivity, likewise argues against a simple division into two discrete functional structures (Dalton et al., 2022).

Dorsoventral coordination in mediated learning

The fact that dHPC and vHPC perform different computations, or have access to different information, implies that integrating two previously acquired associations will require their coordination. In our study, dorsoventral coupling during stage 2 was higher in paired rats during light presentation, and coupling strength predicted subsequent freezing to the tone in paired rats and to the light in unpaired rats. These measures were correlational, however, and therefore cannot establish whether dorsoventral communication is necessary for mediated learning. If dorsoventral communication is necessary for integrating the retrieved tone representation with the current event, preventing this communication while leaving local processing within each subregion intact should impair mediated fear. This could be tested

using a crossed disconnection, in which the dHPC and vHPC are inhibited contralaterally, provided that commissural fibers do not compensate with inter-hemispheric connectivity. For this disconnection, a chemogenetic approach may be preferable to local pharmacological inhibition, given that unilateral hippocampal muscimol infusions can themselves produce behavioural impairments (Cimadevilla et al., 2005; Gilmartin et al., 2012).

An alternative interpretation of the thesis findings is that dorsoventral coordination does not mediate integration itself but, instead, reflects the updating of previously acquired representations when they are involved in new learning. The paired and unpaired groups differ in the amount of stimulus representations that are updated during stage 2: whereas unpaired rats only learn about the light that is explicitly paired with shock, paired rats must also incorporate the reinstated tone representation into a new association. On this account, the relationship between coupling and subsequent freezing at test reflects which of the representations present during the light–shock event is preferentially associated with the shock. Notably in paired rats, the retrieved tone representation should compete with the light for the association with shock. Greater representational updating would therefore predict stronger tone–shock learning and greater subsequent freezing to the tone, at the expense of learning about the light. In unpaired rats, by contrast, only the light gets conditioned, explaining why coupling predicts subsequent freezing to the light but not the tone. The capacity of the tone to undergo such updating may itself differ between groups: because the tone was previously presented alone and without reinforcement in unpaired rats, it is subject to latent inhibition, whereas its prior relationship with the light in paired rats may attenuate this effect. The greater plasticity of the tone representation in paired animals could therefore be reflected in greater dorsoventral coordination during stage 2.

Several neural mechanisms could support this coordination. One possibility is direct longitudinal communication within the hippocampus. Schaffer collateral projections extend along the longitudinal axis, and recent optogenetic mapping indicates that excitatory transmission can propagate along this axis while feedforward inhibition remains relatively local (Okamoto et al., 2025). This provides a potential substrate for direct communication between dorsal and ventral hippocampus. However, longitudinal transmission has been characterised most clearly in the dorsal-to-ventral direction, whereas the requirement of vHPC in stage 1 suggests a ventral-to-dorsal flow of information during stage 2. Ventral-to-dorsal communication could instead occur indirectly through ventral CA1 and the subiculum, with projections through entorhinal and perirhinal cortices providing a route back to dorsal hippocampal processing. Notably, this pathway also provides a potential link to the extrahippocampal network identified by our c-Fos data.

A second possibility is that coordination is mediated by hippocampal oscillations. Theta activity propagates along the longitudinal axis as a travelling wave, generating systematic phase differences between dorsal and ventral hippocampus (Lubenov & Siapas, 2009; Patel et al., 2012). A stable dorsoventral phase relationship could therefore coordinate processing across the two poles, predicting that the coupling observed here should manifest as phase-locked interactions. More generally, functional coupling can provide information not captured by regional activity alone. For example, vHPC–prefrontal theta synchrony predicts anxiety-related behaviour even when theta power in either region does not (Adhikari et al., 2010).

The present findings raise the possibility that the direction of dorsoventral communication changes with computational demand. Ventral-to-dorsal interactions could allow representations established during stage 1 to influence subsequent dorsal processing, whereas dorsal-to-ventral interactions could support the incorporation of information about the current event into an already retrieved representation. However, the nature and direction of this coordination cannot be resolved with the present recordings. Fibre photometry measures bulk calcium fluorescence from CaMKIIα-expressing neurons, providing an indirect and temporally slow measure of population activity. Our analysis therefore captures co-fluctuations over hundreds of milliseconds to seconds, but not the oscillatory dynamics, including theta phase locking, gamma coherence, or cross-frequency coupling, through which such communication could occur. Thus, the present results show that the two poles are jointly recruited during integration, but do not establish that they are synchronised in an oscillatory sense or reveal the direction of their interaction. Electrophysiological recordings would provide complementary information, with local field potentials indexing afferent input and network state and calcium signals reflecting local excitatory output. Simultaneous dHPC and vHPC recordings could therefore test whether the 1 to 6 s epochs identified here coincide with increased theta coherence at light onset and whether this coordination has a preferred direction. Recent work has begun to examine longitudinal hippocampal interactions during first-order fear conditioning and identified cell assemblies from the dHPC and vHPC whose activity might reflect the integration of the acquisition and expression of fear (Pompili et al., 2026).

Finally, dorsoventral coordination may be expressed through coordinated ensemble reactivation. Morici et al. (2026) simultaneously recorded dHPC and vHPC activity during reward- and aversion-motivated exploration and subsequent sleep, identifying dorsal, ventral, and joint dorsoventral neuronal assemblies. Dorsoventral coordination was associated with coordinated sharp-wave ripples and ripple bursts, and joint assemblies formed during aversive experience were preferentially reactivated during subsequent non-REM sleep. This reactivation occurred during coordinated ripples and was related to the strength of assembly

activation during the task, leading the authors to propose that dorsoventral communication contributes to integrating contextual and valence-related information. In the present context, such coordinated assemblies provide a plausible physiological substrate through which stimulus representations and aversive information could become integrated, although whether this coordination is required for integration or instead reflects the updating of representations remains to be determined.

2. A neural circuit for sensory preconditioning

Taken together, the broader literature on sensory preconditioning and the findings presented here point to a circuit organisation that is difficult to describe in terms of a one-to-one mapping between regions and functions. Sensory preconditioning depends on a distributed network in which individual regions can contribute to more than one function, as illustrated by the involvement of the vHPC and perirhinal cortex in distinct aspects of learning, while a single function (such as mediated learning) can depend on multiple regions. Moreover, a region that does not differ between groups in its overall activity in the task can nevertheless differ in its functional communication: the vHPC, for example, shows different patterns of coupling with dHPC between sensory preconditioned animals and controls despite being engaged similarly on its own. It shows that higher-order learning emerges from interactions between different regions which themselves contribute to different functions. Indeed, across protocols that differ methodologically, the variable that often predicts successful integration is coordination between regions at the time the overlapping event is encoded: activation changes in a functionally coupled hippocampal–ventromedial prefrontal circuit track the formation of integrated memories and predict inferential success (Zeithamova, Dominick, et al., 2012) and hippocampal–orbitofrontal functional connectivity supports inference-based decisions (Li et al., 2025; Wang, Schoenbaum, et al., 2020).

Overall, these observations suggest that the regions involved in sensory preconditioning should not be assigned fixed functions independently of one another. Instead, their contributions are shared and interact through anatomical and functional connections to produce adaptive behaviour. This view motivates a shift from mapping functions onto regions, or regions onto functions, towards a circuit-level account in which partially overlapping systems are defined by the regions and anatomical pathways that support a common process. On this basis, we suggest below a comprehensive view of the sensory preconditioning circuit as organised into three interacting systems, which provide a neural implementation of the processes formalised by the mSOP model of learning.

Sensory preconditioning as a product of three processing systems

a. Description of the model

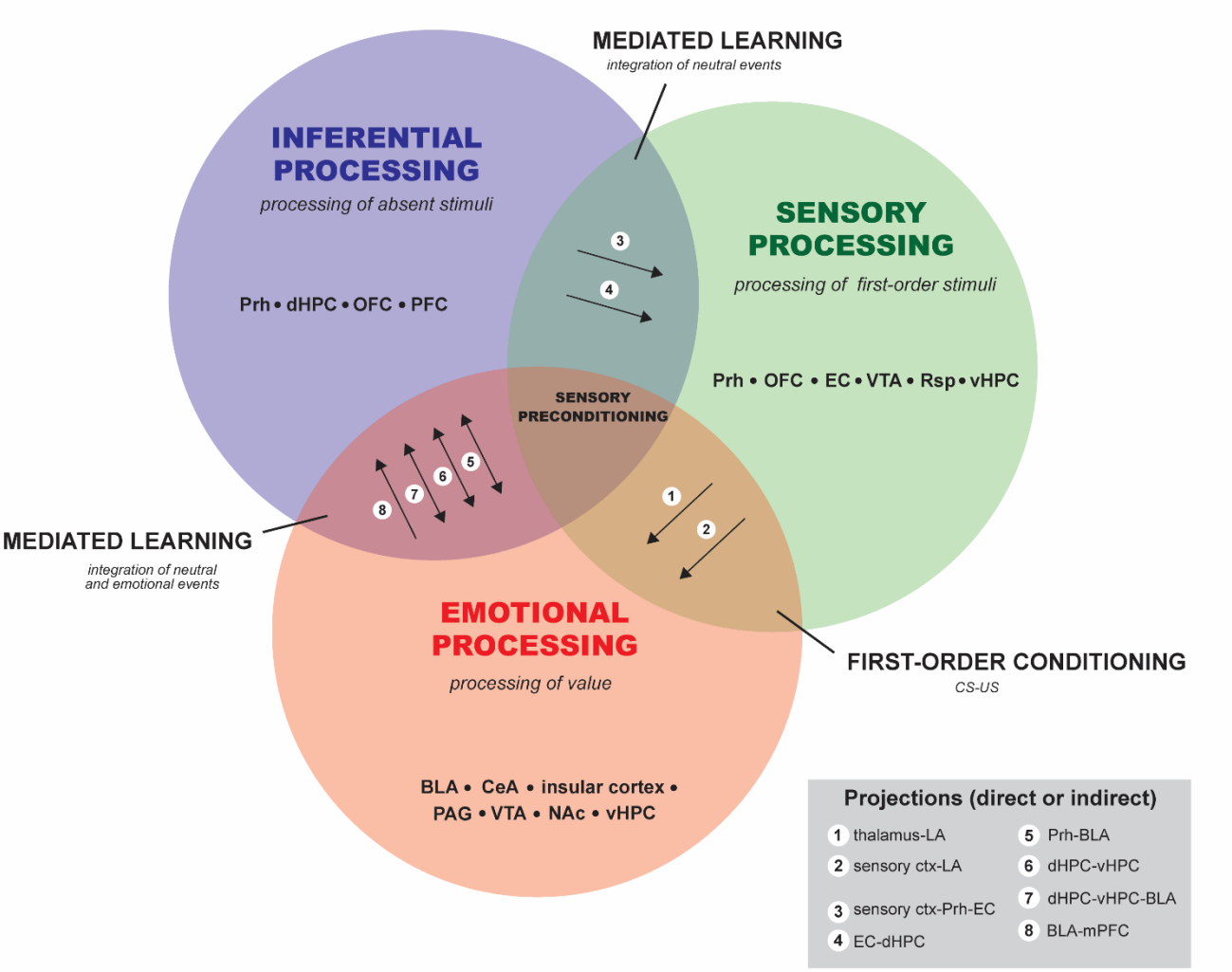


Figure 28. The neural circuit of sensory preconditioning viewed as an interaction between three systems.

Essentially, the three systems (***Figure 29***) fall into the three types of associations learnt in sensory preconditioning: a sensory association, an emotional association, and an inferential association.

The “sensory processing” system operates on the perception of stimulus properties that are physically present. The system of significance, “emotional processing” operates on how much an event matters and generates the biological response to this event. It is not specific to aversive value, and reads current significance irrespective of how that significance was acquired. Finally, the “inferential processing” system operates on relations between events that are no longer physically present. Each system is supported by a set of brain regions.

So conceived, each pairwise interaction of the systems corresponds to a phenomenon of learning. The sensory and significance systems together support first-order conditioning, which requires both a CS and US to be physically present. The sensory and inferential systems together support the integration of neutral events (one absent and one present). Lastly, the significance and inferential systems together support the integration of a present emotional event and an absent neutral event, such as mediated learning described in sensory preconditioning, but also generalisation. In sensory preconditioning, responding to the stimulus never paired with the US is then the phenomenon that requires all three systems. Learning the S2-S1 association relies on the sensory system, the S1-US association on the sensory and emotional systems, and the S2-US association on the emotional and inferential system. The conclusion towards which both parts of this discussion have been building is that each system partially depends on the others, and no single structure participates in all three systems.

b. Critical component of mediated learning: the inferential system

During sensory preconditioning, the inferential system allows the representation of the absent S2 to be incorporated into the S2–US association supported by the emotional system. This account explains why the signature of integration in our data was coordination rather than activation, and why inactivating a single region can selectively affect an association learned through sensory preconditioning. If the inferential system maintains representations of absent events, manipulations confined to this system should spare first-order conditioning and selectively disrupt the inferred association. This pattern is observed following manipulations of the perirhinal cortex (Holmes et al., 2013; Wong et al., 2019), the dorsomedial prefrontal projection to the amygdala (Gu & Johansen, 2025), the lateral orbitofrontal network (Wang, Howard, et al., 2020), the retrosplenial cortex (Fournier et al., 2020), and the dHPC during conditioning. The vHPC, whose inactivation impaired both direct and mediated learning, occupies a less clearly defined position and may lie at the interface between the inferential and emotional systems, pending a more precise account of its contribution.

Notably, the regions grouped within the inferential system also share a broader involvement in relational processing beyond sensory preconditioning. Their functions include constructing predictive and cognitive maps, controlling whether memories are integrated, and using stored relationships to infer the significance of events. The hippocampal contribution to these processes has been discussed earlier. The remaining members of the system include the perirhinal cortex, prefrontal cortex, and orbitofrontal cortex, whose respective contributions further define the operations supported by the system.

The perirhinal cortex is required at every stage of the auditory–visual protocol, for encoding the tone–light association (Holmes et al., 2013; Nicholson & Freeman Jr, 2000), for integrating

it with the light-shock association during conditioning, and for retrieving the mediated tone-shock association at test (Wong et al., 2019). This is the pattern that one would expect of a structure positioned at the interface between systems and engaged whenever information passes from one to another. The perirhinal cortex has been proposed to learn a predictive map of a task environment, i.e. which stimulus features predict which outcome (Lee et al., 2024), and, on that account, to evaluate an internal model held via the hippocampus against the sensory information currently arriving from neocortex. The relationship is therefore a loop: the hippocampus supplies a model of what the situation is predicted to contain, and the perirhinal cortex supplies a comparison at the level of stimulus features, with damage to either disrupting integration (Zhang & Zhang, 2026).

In sensory preconditioning, dorsomedial prefrontal neurons encode an internal model that links stimuli to aversive events whether those relationships were acquired directly or inferred. Within this population, the neurons projecting to the amygdala represent the inferred association and are required for its expression (Gu & Johansen, 2025). The dorsomedial prefrontal cortex therefore provides a point at which relational information converges with behavioural significance, in a role that parallels the interface function proposed for the perirhinal cortex. Beyond sensory preconditioning, the prefrontal cortex is suggested to have a control function: it determines under which conditions the representations are incorporated into subsequent learning. Prefrontal lesions impair the critical inference judgments required in transitive inference and acquired equivalence while leaving the directly trained discriminations intact (DeVito et al., 2010; Iordanova et al., 2007). The ventromedial prefrontal cortex also regulates whether memories are integrated in the hippocampus according to the contextual similarity between experiences (de Sousa et al., 2026). This division of labour suggests that hippocampal circuitry supplies the mechanism through which representations can be integrated, while prefrontal circuitry regulates the conditions under which that integration occurs. It also provides a possible account of what the unpaired animals learn. A system that controls integration according to the relation between experiences could implement the decision that two events should remain separate.

Finally, the orbitofrontal cortex is concerned with the use of these relational representations to guide responding and subsequent learning. It is required for responding to preconditioned cues in appetitive sensory preconditioning, both during cue-cue learning and at test (Hart et al., 2020; Jones et al., 2012); and its neurons signal the sensory associations that support model based inference (Sadacca et al., 2018). In humans, network targeted stimulation impairs performance specifically when a sensory preconditioning task requires outcome expectations to be inferred mentally, while leaving behaviour based on directly experienced associations

intact (Wang, Howard, et al., 2020). The relationship between the orbitofrontal cortex and hippocampus has also been examined at the level of task representations. Suppressing ventral subicular output degrades the integrated representation of task structure in the orbitofrontal cortex (Wikenheiser et al., 2017), suggesting that hippocampal output contributes to the construction of these representations. However, inactivation of the same output can leave orbitofrontal schema correlates intact (Zong et al., 2025). Recordings from mice learning serial odour-outcome sequences help account for these apparently divergent findings. Hippocampal activity was governed by the particular combination of past and current cues, whereas orbitofrontal activity generalised across sequences that shared a common structure and expected outcome (Lin & Zhou, 2024). The two regions therefore represent different levels of the same associative structure. The hippocampus carries information about the particular episode, while the orbitofrontal cortex extracts its general structure and expected consequence.

c. Critical component of mediated learning: the emotional system

The emotional system includes the basolateral amygdala and its central and midbrain outputs, together with the ventral tegmental area, nucleus accumbens, and insular cortex. The role of BLA in mediated learning might depend on bidirectional communication with the relational system so that the retrieved representation gains value (Wong et al., 2019). Anatomically, amygdalar input permits excitatory activity to spread from the perirhinal cortex into the entorhinal hippocampal circuit (Kajiwara et al., 2003), and long-term potentiation induced at one node of the perirhinal-hippocampal-amygdalar network potentiates synapses at the other nodes when the network remains connected (Supcun et al., 2012). This provides a candidate substrate for exchange of information with the inferential system. Consistently, amygdala-modulated hippocampal–neocortical coupling during emotional learning drives the retroactive integration of neutral associations acquired beforehand (Zhu et al., 2022).

Notably, the emotional system is not specific to fear. Appetitive sensory preconditioning recruits the BLA, the ventral tegmental area and nucleus accumbens (Sadacca et al., 2018; Sharpe, Chang, et al., 2017), and intrinsic connectivity in humans links the hippocampus, nucleus accumbens, and midbrain through a circuit centred on the ventral hippocampal body (Kahn & Shohamy, 2013). In appetitive sensory preconditioning, coupled hippocampal and midbrain activity during encoding predicts subsequent generalisation (Shohamy & Wagner, 2008). The common property is therefore behavioural significance, whose valence depends on the current outcome and motivational context. The significance system can provide the information that makes a retrieved representation worth updating, while the inferential system determines what those representations contain and how a current event can be incorporated.

A reinstatement in terms of SOP model and future directions

Importantly, the three systems just described can be mapped to different components of the SOP model:

(1) **Systems map to states**. Sensory and emotional processing systems operate in the A1 state, while the inferential system operates in A2. Of note, the regions suggested to support these systems support learning in the corresponding states but need not be thought of as constituting the states themselves.

(2) **The overlaps between systems map to rules or equations for associations**. Sensory and emotional systems together realise the A1–A1 case, which is first-order conditioning. Sensory and inferential systems together build the $V_{(S2\text{-}S1)}$ (i.e., A2-A1 association) in stage 1, whose strength later determines how much of S2 can be driven into A2 (i.e., $p_{(A2)}S2$). In stage 2, emotional and inferential systems together realise the case of a stimulus in A2 meeting a US in A1, which results in mediated learning (***Figure 30***). Because the inferential system influences the proportion p(A2)S2, it influences the interaction with US nodes in A1 processed by the emotional system. How much of S2 can be linked to the US is determined by cooperation between regions belonging to the two systems. For example, the dorsoventral coupling we measured is an index of that interaction being available.

(3) Finally, **neural processes shown to regulate associative learning map to key parameters in the model**. For example, noradrenaline spreads associations of hippocampal cognitive maps (Koolschijn et al., 2026), which is an increase in pA2(S2), the proportion of a node's elements recruited into A2.

The scheme is built on aversive, cross-modal sensory preconditioning in rats, and extending it to appetitive paradigms or to human sensory preconditioning remains an assumption. It also specifies which systems are required for the different processes, but not the order in which those systems interact.

The scheme also brings together two literatures that are usually connected mainly at the level of interpretation, which open a future possible route of investigation. Indeed, formal models specify the rules by which learning should proceed, while circuit neuroscience identifies the structures and interactions through which those rules might be implemented. Yet the terms of the former rarely have a directly measurable counterpart in the latter. In the mSOP model, for example, p1 and pA2 carry much of the explanatory weight, but no study has directly measured a neural quantity that can be identified with either variable. Establishing a correspondence between these model quantities and measurable patterns of neural activity is therefore an

important unresolved question. The framework proposed here is intended as a first step towards making the computational and circuit levels commensurable, by asking not simply where sensory preconditioning occurs, but how the representations and interactions specified by the model could be instantiated in the brain.

What this comprehensive view adds, therefore, is a change in the level at which the question of the behavioural and neural substrates of sensory preconditioning is posed. Rather than asking whether a structure is required at a particular stage, it asks which computation is being performed, which representation is in the relevant activation state, and at which interface information from different systems is brought together. Sensory preconditioning consequently becomes more than a procedure for studying higher-order conditioning: it provides a framework for studying how representations acquired in the learning systems are retrieved, integrated, and ultimately imbued with behavioural significance.

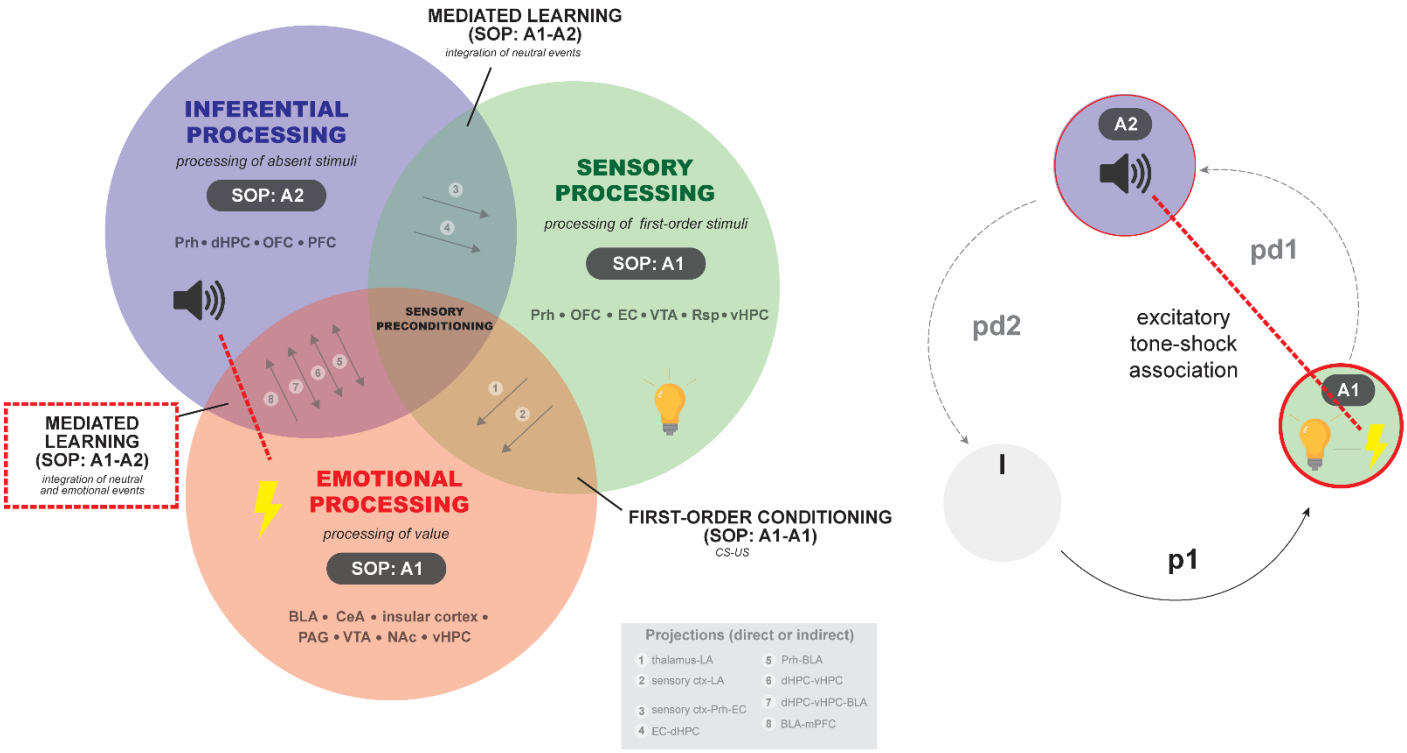


Figure 29. The circuit of sensory preconditioning maps on the SOP model (example of stage 2). *Sensory and emotional systems map on A1 state, inferential system maps on A2. Overlaps between systems correspond to associative learning driven by the occurrence of stimulus representations in the different states. For example, during stage 2 of sensory preconditioning, the light and the shock physically delivered are processed by the sensory and emotional systems, supporting first-order conditioning, while the tone representation is reinstated by the inferential system which supports mediated learning via its interaction with the emotional system. Mediated learning thus maps the A2-A1 excitatory association of mSOP model.*

Appendix

Appendix 1 – Studies of the hippocampus in sensory preconditioning

Study	Model	Motivational system	Stage-1 arrangement	Modality	Technique	Hippocampal target	Stage(s) targeted	Key finding
Talk et al., 2002	Rat (male)	Aversive	Serial	**Unimodal** auditory	Lesions; CA1 evoked-potential recording	Whole (lesions); dorsal CA1 (recording)	No specific (lesions)	Lesions abolished suppression to the preconditioned noise but spared direct conditioning to the tone. No evoked-potential change during stage 1; the signal emerged only after stage 2, dissociating storage from expression.
Ward-Robinson et al., 2001	Rat (male)	Aversive	Simultaneous	**Unimodal** gustatory	Lesions	Whole hippocampus	No specific (lesions)	Preconditioning was INTACT: lesioned rats avoided A as much as shams. The principal null in this literature, and confounded with lesion permanence.
Lin et al., 2016	Rat (male)	Aversive	Simultaneous	**Polymodal** auditory–visual	Lesions	Whole hippocampus	No specific (lesions)	Double dissociation: shams showed more fear to AX than BX (configural), lesioned rats more fear to A than B (elemental). Pattern completion is not one process.
Iordanova et al., 2009	Rat (male)	Aversive	Simultaneous	**Polymodal** auditory–context–temporal	Lesions; muscimol inactivation	Whole (lesions); dorsal (muscimol).	Stage 3 (and no specific for lesions)	Hippocampus required for retrieval of configural memories but not of those encodable elementally;
Iordanova et al., 2011	Rat (male)	Aversive	Simultaneous	**Polymodal** auditory–context–temporal	muscimol and AP5 infusions	Dorsal hippocampus (CA1)	Stage 2 and stage 3	AP5 before stage 2 abolished the effect, AP5 after did not, AP5 at test had no effect, and neither drug affected learning about single components. Plasticity is required at the moment of integration, and only for conjunctions.
Wheeler et al., 2013	Rat (male)	Aversive	Simultaneous	**Polymodal** olfactory–gustatory	Muscimol inactivation (dHPC)	Dorsal hippocampus	Stage 2	dHPC inactivation impaired mediated learning while sparing direct learning
Busquets-Garcia et al., 2018	Mouse (male)	Aversive	Simultaneous	**Polymodal** olfactory–gustatory + auditory–visual	Global and conditional CB1R knockout; pharmacology; in vivo electrophysiology	Dorsal hippocampus (GABAergic CB1R)	Stage 1	Inactivating hippocampal, specifically GABAergic, CB1 receptors before stage 1 disrupted preconditioning while sparing direct conditioning.

Study	Model	Motivational system	Stage-1 arrangement	Modality	Technique	Hippocampal target	Stage(s) targeted	Key finding
Barron et al., 2020	Mouse + human	Appetitive	Serial	**Polymodal** auditory–visual	In vivo electrophysiology + optogenetic inactivation (mice)	Dorsal hippocampus (CA1)	Stage 3	Optogenetic inactivation of dCA1 at test impaired reward-seeking to the preconditioned cue but not to the directly conditioned one; at test, the preconditioned cue evoked higher representational similarity with its paired associate.
Pinho et al., 2025	Mouse (male)	Aversive	Simultaneous	**Polymodal** auditory–visual	Fibre photometry + chemogenetic	**Dorsal + ventral, targeted separately**	Stage 1 (primary); also 2 and 3	Chemogenetic inhibition of dorsal CaMKII+ neurons during preconditioning abolished mediated learning while sparing direct learning; ventral inhibition had no effect.
Wimmer & Shohamy, 2012	Human	Appetitive	Simultaneous	**Unimodal** visual	fMRI	Hippocampus (whole); HPC–striatal coupling	Stage 2	Hippocampal activity and hippocampal–striatal coupling during the reward phase predicted the magnitude of the later decision bias toward the preconditioned stimulus.
Wang, Schoenbaum, et al., 2020	Human	Appetitive	Serial	**Unimodal** visual	fMRI	Hippocampus (whole)	Stages 1 and 3	Orbitofrontal but not hippocampal activity reinstated stage-1 representations at test, placing the hippocampal contribution earlier than the inference itself.
Zhu et al., 2022	Human	Aversive	Simultaneous	**Polymodal** auditory–visual	fMRI + skin conductance	Hippocampus (whole) + amygdala + sensory cortices	Stages 1 and 2	Trial-specific hippocampal reactivation during stage 2, followed by increased hippocampal–amygdala and then hippocampal–neocortical coupling; retroactively promoted integration.
Zhu et al., 2023	Human	Aversive	Simultaneous	**Unimodal** visual	fMRI	Hippocampus (whole) + amygdala + cortices	Stages 2 and 3	Emotion induced in stage 2 reactivated hippocampal representations of the stage-1 pair; reactivation enhanced or interfered with integration depending on valence.

Appendix 2 – Inhibition of NMDA receptors in the dHPC during stage 2

Aim

Chapter 3 showed that preconditioned fear, but not directly conditioned fear, required activity of the dHPC during stage 2 of sensory preconditioning. Based on previous work on the role of NMDA receptor-dependent plasticity in the perirhinal cortex in the same protocol, we hypothesized that mediated learning was similarly dependent on NMDA receptor activity in the dHPC.

To test this hypothesis, rats were randomly allocated to groups MUS (n=10), D-APV (n=9) and VEH (n=8). Muscimol, D-APV or vehicle was infused bilaterally into the dHPC prior to stage 2 (Figure S1A).

Methods

All methods were the same as pharmacological experiments in Chapter 3 (refer to STAR★Methods for details). For this experiment, two drugs were used: GABAA receptor agonist muscimol (Sigma, Australia) was dissolved in nonpyrogenic saline (0.9% w/v) to yield a final concentration of 1 μg/μl. The NMDA receptor antagonist D-2-amino-5-phosphopentanoic acid, D-APV (Sigma, Australia) was dissolved in ACSF to yield a final concentration of 10 μg/μl. Vehicle solution was made of saline only (0.9% w/v).

Results

Histology.

Figure S1B shows the most ventral location of cannula tips in the dHPC.

Behaviour.

Consistent with our previous findings, the three groups showed equivalent levels of freezing to the light (Figure S1E, largest $F=1.257$, $p=0.273$), indicating that NMDA receptors or general activity in the dHPC are not required for first-order conditioning to the light. By contrast, when tested with S2 (Figure S1D), rats that were infused with muscimol or D-APV in the dHPC prior to stage 2 showed significantly lower levels of freezing compared to rats infused with vehicle ($F(1,24)=13.1$, $p<0.01$, $\eta p^2=0.35$, 95%CI =[−23.410;-6.407]), and did not differ from each other ($F=0.003$, $p=0.958$). These results suggest that NMDAr activity in the dHPC is necessary for the encoding of a mediated tone-shock association at the time of conditioning to the light.

Consistent with test results, fear conditioning to the light during stage 2 was successful (Figure S1C). The mean (± SEM) levels of freezing to the light on its final pairing with shock were 95

± 5 % in group VEH, 68 ± 12.3 % in group MUS, and 80 ± 8.1% in group D-APV. There was a significant linear increase in freezing across the light-shock pairings ($F(1,24) = 134.32$; $p < 0.0001$; 95% CI: [55.955, 80.202]), as well as a significant presentation x group interaction, indicating a different rate of learning between group MUS vs D-APV ($F(1,24) = 5.792$; $p = 0.024$, $\eta p^2 = 0.19$, 95%CI=[4.787;62.435]), with group MUS learning at a faster rate than group D-APV. Thus, this interaction did not reflect an inability to learn the fear for S1. Additionally, the overall freezing to S1 did not differ between the groups (largest $F= 0.344$; $p = 0.516$), indicating that the muscimol or D-APV infusions did not disrupt the expression of the association between the S1 and the US.

Conclusion

Together, these results show that mediated learning in the dHPC not only depends on general activity in this region, but can be specifically attributed to NMDA receptor-dependent plasticity. They provide a mechanism and a site for memory integration in our auditory-visual protocol of sensory preconditioning, discussed in section ***A mechanism for memory integration in the dHPC*** of Chapter 5 (p.147).

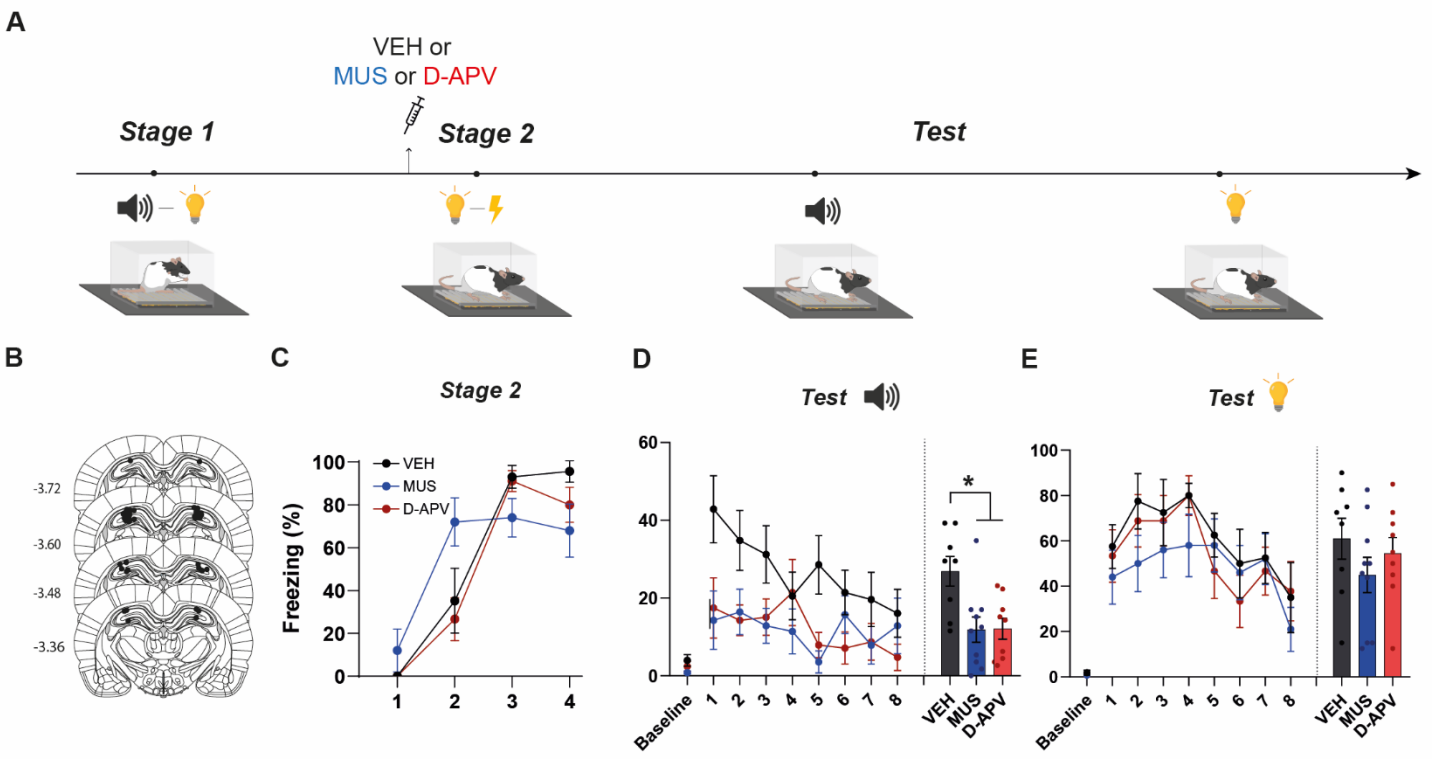


Figure 30. Blocking of NMDA receptors in the dorsal hippocampus specifically disrupts mediated learning.

(A) Experimental design. In stage 2, Group MUS and D-APV respectively received an infusion of muscimol and D-APV in the dHPC while Groups VEH received saline. All groups were then tested drug-free to the tone and the light. **(B)** Most ventral part of the cannulas in dHPC as verified on Nissl-stained sections. **(C)** Mean (±SEM) freezing during the four light presentations of stage 2. Changes in freezing across presentations were assessed by a within-subject linear trend analysis using planned contrasts. **(D)** Mean (±SEM) freezing across the 8 test presentations of the tone and averaged across all presentations (bar graph) during testing of the tone. **(E)** Mean (±SEM) freezing across the 8 test presentations and averaged across all presentations (bar graph) during testing of the light. Individual data points are shown in bar graphs. *Baseline freezing was measured before the first stimulus presentation and did not differ between groups across experiments (largest F[1,29]=1.703, p=0.20). Error bars: SEM. *p < 0.05, **p < 0.01, ***p < 0.001. For each test levels of freezing, the first contrast compared Group VEH versus the weighted average of Groups MUS and D-APV; and the second contrast compared Group MUS versus Group D-APV.*

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