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**Plasticity of instinct: Interactions of the memory
engram with innate behaviour**

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DECLARATION

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A handwritten signature in black ink, appearing to read 'Paul Conway', is written over a horizontal line.

Paul Conway

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Go raibh maith agaibh!

Abstract

The brain has access to two sources of information to guide behaviour – instinct and memory. While research into these two phenomena is advancing rapidly, much less is known about how they interact with one another. This thesis investigates the interplay between memory and instinct in the brain, using two behavioural models: the innate defensive responses of *Mus musculus* to visual looming stimuli, and the olfactory responses of *Mus musculus* and *Mus caroli* to the odorant trimethylamine (TMA).

I demonstrate that innate fear responses to visual looming stimuli are subject to experience-dependent plasticity. This plasticity results in reduced activation of circuitry responsible for driving the behaviour, specifically the superior colliculus (SC) and periaqueductal gray (PAG). Using engram labelling technology, I identify a novel role for the hippocampus in mediating adaptive behavioural responses to visual looming stimuli.

Additionally, I show that the TMA response of *Mus musculus* is influenced by both innate and learned factors, with early life sensory exposure crucial to the development of an appropriate behavioural response. I identify the anterior olfactory nucleus (AON) as a key region involved in processing TMA responses in *Mus musculus*. Comparative c-Fos-based mapping in *Mus caroli* reveals divergent neural circuits underlying their instinctive aversion, suggesting a shift in olfactory circuitry accompanying the evolution of attraction in *Mus musculus*.

Together, these findings provide insight into how memory can modify instinctive behaviours and how learning contributes to the development and evolution of instinct. This work advances our understanding of the neural circuits underlying instinct-memory interactions, demonstrating that these two systems are not isolated but are constantly shaping one another to drive adaptive behaviour. Understanding this relationship will advance our understanding of how the brain controls behaviour, the role for learning in this process, and how information is stored in the brain.

Publications

Plasticity of visual looming response reveals a dissociation of innate and learned components.

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Abbreviations

AAV	Adeno-associated virus
AON	Anterior olfactory nucleus
BLA	Basolateral amygdala
BNZ	Benzaldehyde
CCF	Common coordinate framework
CFC	Contextual fear conditioning
CNO	Clozapine-N-oxide
CoA	Cortical amygdala
CRHN	Corticotropin releasing hormone neurons
CS	Conditioned Stimulus
DG	Dentate Gyrus
DREADD	Designer Receptors Exclusively Activated by Designer Drugs
EPSP	Excitatory post-synaptic potential
FMO3	Flavin-containing monooxygenase 3
LGN	Lateral geniculate nucleus
LPTN	Lateral posterior thalamic nucleus
LTP	Long term potentiation
MH	Methyl hexanoate
MOB	Main olfactory bulb
MOE	Main olfactory epithelium
NMR	Nuclear magnetic resonance
OR	Olfactory receptor
PAA	Peri-amygdalar area
PAG	Periaqueductal gray
PBG	Parabigeminal nucleus
PBS	Phosphate-buffered saline
PBT	Phosphate-buffered saline with Tween
PFA	Paraformaldehyde
PIR	Piriform cortex
PO	Paraffin oil
PTSD	Post traumatic stress disorder
PV	Parvalbumin
PVN	Paraventricular nucleus
RGC	Retinal ganglion cell
SC	Superior colliculus
TAAR	Trace amine associated receptor
TMA	Trimethylamine
TMAO	Trimethylamine-N-oxide
TMT	Trimethylthiazole
US	Unconditioned stimulus
VMH	Ventromedial hypothalamus

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1. Introduction

1.1 Information in the brain

1.1.1 How does the brain know what to do?

The brain's job is to process incoming information from sensory organs and execute an appropriate response. In the context of behavioural neuroscience this can mean the processing of visual, auditory, olfactory, gustatory and tactile cues for the execution of behavioural responses such as fleeing, freezing, mating, hunting and feeding. Of course, this list is non-exhaustive, as the range of potential inputs and outputs to and from the brain is incredibly vast.

The brain can handle this challenge due to its ability to store information about certain sensory stimuli. Such information may be innate to the animal – the animal will execute an appropriate behavioural response to the stimulus upon its first encounter with the stimulus. This is known as an instinct. Examples of this include defensive responses to threatening stimuli, such as predator odours (Kondoh et al., 2016, Pérez-Gómez et al., 2015, Rosen et al., 2015, Root et al., 2014), or threatening visual looming stimuli (Yilmaz and Meister, 2013, Shang et al., 2018, Evans et al., 2018). Innate behaviour emerges over the process of evolution to stimuli which are reliably present in the environment, allowing the animal to engage in behavioural responses which are adaptive to particular stimuli which are important for survival or reproduction (Tinbergen, 1951, Lorenz, 1935). However, the environment constantly changes through seasons, the appearance of new food sources, or shifts in social dynamics to name a few. How, then, does the brain adapt to novel stimuli?

The brain can adapt to new stimuli via learning - the process of storing new information about the world. This stored information is known as memory. Learning allows an animal to change its behaviour based on experience. For example, a mouse may learn to associate a stimulus such as a tone with a mild shock (Schafe and LeDoux, 2000, Kim and Fanselow, 1992) or press a lever to receive a sucrose reward (Mishima et al., 1986). These learned associations enable animals to navigate their ever-changing environments, adjusting their behaviour in ways that enhance survival and reproduction.

Instinct and memory differ from one another in how they form. Learning is hypothesised to be driven by synaptic plasticity, which alters the strength and number of

connections between different neurons, leading to a new learned behavioural response upon re-exposure to the stimulus (Hebb, 1949, Cajal, 1894, Bliss and Lømo, 1973, Han et al., 2009, Castellucci et al., 1970, Engert and Bonhoeffer, 1999, Ryan et al., 2015). Meanwhile, instinct is genetically encoded, with particular neuronal connections established during development to drive specific behaviours (Tinbergen, 1951, Felix et al., 2023, Alaux et al., 2009, Wang et al., 2018). Despite these differences in formation, both instinct and memory are ultimately represented through patterns of neuronal connectivity and activity (Root et al., 2014, Evans et al., 2018, Wu et al., 2023, Agrabawi and Kim, 2018, Aso et al., 2014, Frankland et al., 2019, Kitamura et al., 2017, Liu et al., 2012a, Ortega-de San Luis et al., 2023). As such, instinct and memory can interact with one another.

Until recently, neuroscience has often treated instinct and memory as separate areas of study. This approach has been helpful in elucidating many features of the underlying circuitry for these two kinds of behaviour. However, it does not address how these systems might interact or even rely on each other. Understanding the interplay between instinct and memory could have significant implications for our understanding of information in the brain, the role of experience in behaviour (Badyaev, 2009, Ito and Feldheim, 2018, Kveim et al., 2023, Alaux et al., 2009, Baier, 2020, Choi et al., 2005, Toma et al., 2002), the management of phobias and post-traumatic stress disorder (PTSD) (Silva et al., 2016, Fadok et al., 2017, Kondoh et al., 2016, Pérez-Gómez et al., 2015, Rosen, 2004, Rosen et al., 2015) and the development of uniquely human faculties such as language (Hauser et al., 2014, Heyes et al., 2020, Velichkovsky and Rumbaugh, 2013). However, before we consider how instinct and memory interact with and depend on one another, let us first examine more deeply what is known about instinct and memory separately. By understanding their separate mechanisms and roles in the brain, we can better appreciate how they converge and influence behaviour.

1.1.2 The memory engram

As previously mentioned, memories are the brain's means of storing new information. In the early 20th century, Richard Semon coined the term *engram* to describe the physical or biochemical changes in the brain that store and recall memories (Semon, 1921). Not long after Richard Semon's theory, Donald Hebb proposed the cell assembly theory and Hebb rule of synaptic plasticity, whereby networks of interconnected cells increase their connectivity when

undergoing concurrent activity (Hebb, 1949). This theory further posited that the activation of a subset of this cell assembly would be sufficient to induce activity of the entire assembly, and hence may serve as a mechanism for memory recall (Hebb, 1949).

While the theoretical underpinnings of memory were developing, experimental neuroscience struggled to localise the engram within the brain. Karl Lashley was one researcher who championed this campaign, hypothesising that the engram of his maze learning paradigm for rats may be situated in certain parts of the cerebral cortex. Lashley systematically performed lesions throughout different areas of the cortex, finding that while cortical lesion size correlated with memory performance, lesion location did not. As a result, Lashley concluded that memories were distributed throughout the cortex, rather than in any particular region (Lashley, 1931a, Lashley, 1963, Lashley, 1931b). However this view was later challenged by findings in both humans and animal models.

One example of this is the famous case of patient H.M. Patient H.M. underwent lesions of the medial temporal lobes as a treatment for debilitating, treatment-resistant epilepsy. These lesions included the destruction of the hippocampus. While this intervention helped relieve these symptoms, it resulted in the unintended side effect that the patient lost the ability to form new episodic memories (Scoville and Milner, 1957), but not procedural memories (Milner, 1965). This prompted widespread interest in the role of the hippocampus in memory, as well as supporting the idea that specific types of memory may require the activity of particular brain regions. Meanwhile, other lesion studies found roles of other brain regions in particular components of memory, such as the amygdala in emotional memories (Weiskrantz, 1956).

Landmark electrophysiological studies later uncovered the processes of long-term potentiation (LTP) and synaptic plasticity (Kandel and Tauc, 1965, Bliss and Lømo, 1973, Brown et al., 1988, Chapman et al., 1990). These phenomena were proposed as mechanisms by which changes in strengths of synaptic connections could occur to facilitate memory formation (Bliss and Collingridge, 1993). This work has been complemented by molecular studies indicating the importance of certain genes in memory formation – especially a host of genes collectively referred to as immediate-early genes (IEGs) such as *c-fos*, *arc* and *npas4* (Greenberg and Ziff, 1984, Sheng and Greenberg, 1990, Herschman, 1991). IEGs show an increase in transcription following increased activity of the neuron. Many IEGs are transcription factors which activate secondary genes that are believed to be involved in various processes central to synaptic

plasticity (Morgan and Curran, 1989, Sheng and Greenberg, 1990, Tonegawa et al., 2015). This demonstrated that memory formation does indeed result in changes in synaptic wiring of specific cells, suggesting a potential mechanism of memory encoding. However, the functional role of these cells in memory storage and recall remained to be demonstrated.

In the past two decades, the development of engram labelling technology has utilised these genes alongside new genetic tools to revolutionise memory neuroscience. This technology hi-jacks the expression of IEGs, such as *c-fos*, to express fluorescent markers and effector proteins in neurons in an activity-dependent manner (Reijmers et al., 2007, Liu et al., 2012a, Pignatelli et al., 2019, Ramirez et al., 2013, Ryan et al., 2015, Guenther et al., 2013).

Using these strategies, experimenters have learned that neurons which are active during memory acquisition are also active during memory recall (Sweis et al., 2021). Furthermore, their activity is necessary for memory retrieval (Tanaka et al., 2014, Denny et al., 2014), and that their artificial re-activation is sufficient to drive learned behaviour (Liu et al., 2012a, Ramirez et al., 2013). Additionally, the information in these ensembles has been shown to relate to specific memories. For example, when cells tagged in a neutral context are artificially stimulated during application of a mild electric shock, mice will fear this context upon their next exposure to it, despite the animal having never experienced fearful stimuli in the context (Ramirez et al., 2013). Together, these studies provide strong evidence that for a given memory there is a specific ensemble of cells, which is active during natural memory formation and retrieval, and whose activity is both necessary and sufficient for memory retrieval. This model is reminiscent of the original engram and cell assembly theories put forward by Richard Semon and Donald Hebb (Semon, 1921, Hebb, 1949).

Much of this work has focused on individual brain areas, especially the hippocampus. However, engram cells have been identified in other brain regions too, including the amygdala and throughout the neocortex (Agrabawi and Kim, 2020, Zaki et al., 2022, Milczarek et al., 2018, Reijmers et al., 2007, Frankland et al., 2004). Indeed there is emerging evidence that many of these engram ensembles are part of a larger engram circuit, or *engrime*, throughout the brain (Tayler et al., 2013, Wheeler et al., 2013, Lecoq et al., 2014, Tonegawa et al., 2015), and that the hippocampal engram can orchestrate reactivation of amygdalar and cortical engrams during memory recall (Tanaka et al., 2014, Nyberg et al., 2000) (illustrated in figure 1.1).

1.1.3 The instinct *ingram*?

An instinct is information the brain possesses about appropriate behavioural responses to a given stimulus, without having prior learning experience to inform it (Lorenz, 1935, Tinbergen, 1951, Piaget, 1978). However, it may require some degree of developmental sensory exposure or “practice” to mature into a full-fledged instinct (Alessandro et al., 1989, Fehér et al., 2009, Han et al., 2017, Inoue et al., 2021, Galvin et al., 2021). Moreover, unlike sensorimotor reflexes, the specific behavioural response during instinctual recall can vary depending on context, but will be within a predictable class of behaviours for a given species (Campagner et al., 2023, Bedford et al., 2022, Yilmaz and Meister, 2013, Hebb, 1949). Examples of instinct include innate fear of predator odours in rodents (Buron et al., 2007, Rosen et al., 2015, Saito et al., 2017), defensive responses to threatening visual stimuli (Yilmaz and Meister, 2013, Deng et al., 2016, Evans et al., 2018), pheromone-mediated sexual behaviours (Demir et al., 2020, Ishii et al., 2017) and vocalisation attempts in infant human and non-human primates (Gultekin and Hage, 2017, Lameira et al., 2022, Heyes et al., 2020, Velichkovsky and Rumbaugh, 2013, Hauser et al., 2014)

While memory forms via learning, instinct is developed innately by genetically encoded, developmentally constructed pathways (Tinbergen, 1951, Felix et al., 2023, Alaux et al., 2009, Wang et al., 2018, Lorenz, 1935, Lorenz, 1973, Piaget, 1978). While the *engram* is defined as “whatever biochemical or biophysical substrate that underlies a memory”, we may define the instinctual *ingram* as “whatever biochemical or biophysical substrate that underlies an instinct” (Ryan, 2020, Ryan et al., 2021). As discussed previously, the search for a memory engram is often guided by the observation of synaptic plasticity and IEG expression, during and following learning. These processes that correlate with learning can then be identified and investigated as candidate engram formation mechanisms. With the study of innate behaviour on the other hand, we do not get to see the “before” version of the brain, and hence are not privileged to such guidance. This has been one of several challenges relating to studying the neuroscience of instinct over the years. As such, our general understanding of how an instinct forms and is represented as individual units of information, comparable to the engram, remains underdeveloped. However, modern advances in the technology and tools available to neuroscientists allow us to overcome this to begin dissecting the components of innate behaviour, from gene to circuit to behaviour (Deng et al., 2016, Dolan et al., 2019, Evans

et al., 2018, Gore et al., 2015, Kim et al., 2013, Kobayakawa et al., 2007, Pérez-Gómez et al., 2015, Root et al., 2014, Shang et al., 2018, Wang et al., 2018, Wei et al., 2015, Wu et al., 2023).

What is currently known about how instincts form and persist in the brain? Instincts are encoded by the interaction between genes over the course of development. In some cases, a single gene can be very influential in the development of a particular behaviour or set of behaviours (Choi et al., 2005, Shaw et al., 2019, Wang et al., 2018, Harringmeyer et al., 2023), although in most cases many genes will each make small contributions to the formation of any given innate behaviour (Mulligan and Williams, 2015, Civelek and Lusi, 2014, Flint and Mackay, 2009, Zwarts et al., 2011). These genes interact not only with each other, but also the developmental environment (Power et al., 2022, Bateson, 2007, Varki et al., 2008). The transition from gene and environment interactions to neuronal circuitry is a vastly complicated process and unsurprisingly remains largely a mystery. However, it is understood that certain genes which are involved in axon guidance during development are important in this process. For example, the *taar* chemosensory receptors which are important for detection of volatile amines in vertebrates, also participate in axon guidance from sensory neuron to olfactory glomeruli (Hashiguchi and Nishida, 2007, Johnson et al., 2012, Liberles, 2015, Pacifico et al., 2012). As such, these genes serve not only as sensory receptors for particular ethologically relevant stimuli, but to guide the axons of these sensory neurons to particular glomeruli in a labelled-line manner. Other examples include the repulsive interactions of *Nrp1/Sema3a* for topographical organisation of the olfactory bulb (Kawakami et al., 1996, He and Tessier-Lavigne, 1997), or the *Ephrin-A/EphA* repulsive interaction in topographical mapping in the superior colliculus (SC; Drescher et al., 1997, Flanagan and Vanderhaeghen, 1998, O'Leary et al., 1999).

In the developed brain, most instincts rely on a specialised mechanism for the detection of the stimulus, such as a dedicated olfactory receptor (Dekker et al., 2006, Hashiguchi and Nishida, 2007, Shaw et al., 2019, Li et al., 2013b, Wang et al., 2018), or specialised circuitry for feature detection in the case of visual stimuli (Lee et al., 2020, Wu et al., 2023). This ensures that certain salient stimuli may be readily detected and easily identified by the brain.

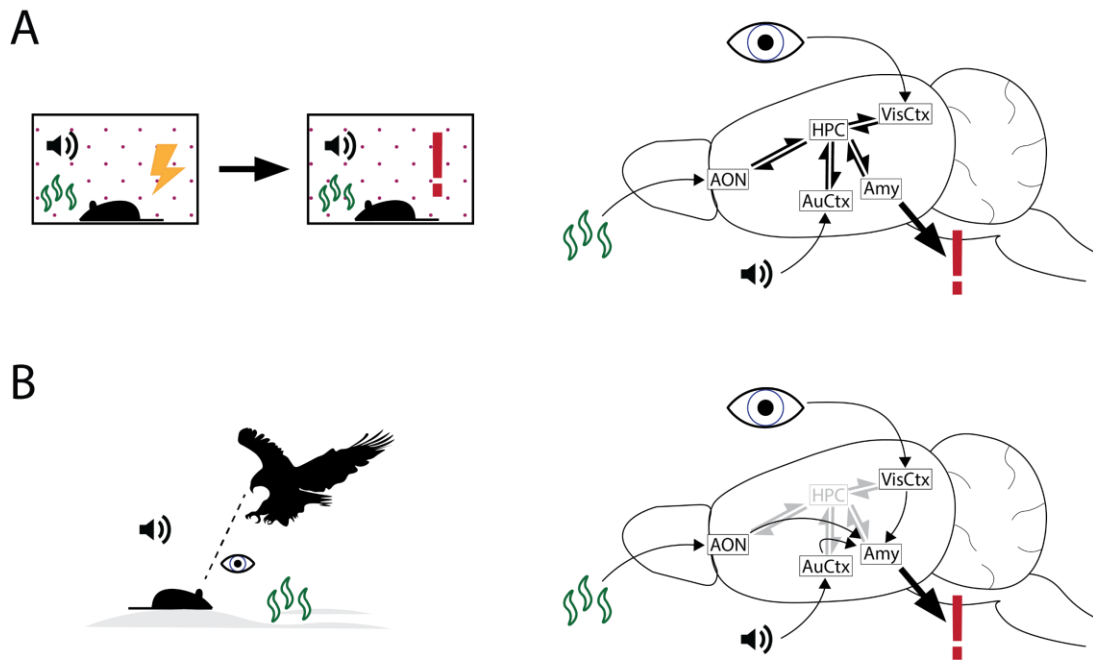


Figure 1.1 Example circuitry of innate and learned behaviour. Multiple sensory cues can trigger learned or innate behavioural responses. (A) Sensory stimuli related to a given memory will be processed by their respective brain regions, eventually leading to activation of a hippocampal engram. (B) Certain innately salient stimuli may activate direct projections from sensory areas to downstream targets, in addition to hippocampal or para-hippocampal projections.

The precise next steps on the circuit are sparsely studied, and vary for different kinds of instinct, but commonly involves relaying stimulus information onto another area or circuit where some value or meaning is implicated, such as odour representation in the anterior olfactory nucleus (Aqrabawi and Kim, 2020, Aqrabawi and Kim, 2018, Brunjes et al., 2005) or threat recognition in the superior colliculus (Evans et al., 2018, Lee et al., 2020). This will then activate downstream areas which can orchestrate the execution of appropriate behavioural responses such as freezing or fleeing (Evans et al., 2018, Deng et al., 2016, Deichler et al., 2020, Fadok et al., 2017). Additionally, brain regions involved in emotional regulation such as the amygdala may be activated (Gore et al., 2015, Ishii et al., 2017, Kim et al., 2013, Root et al., 2014). This model of innate circuitry is similar to that of the memory engram - sensory stimuli activate specific ensembles which coordinate the activation of downstream regions to produce appropriate behavioural and emotional responses (figure 1.1).

1.2 Instinct-memory interactions

1.2.1 Instinct and memory as a spectrum

While it is sometimes helpful to draw a distinction between memory and instinct, the two phenomena may not be as dichotomous as often assumed. Indeed, the definition of instinct presented earlier included the caveat whereby an instinct “*may require some degree of developmental sensory exposure or “practice” to mature into a full-fledged instinct*”. This experience-dependent component of many innate behaviours has been recognised since the early days of modern ethology (Lorenz, 1935, Lorenz, 1973, Tinbergen, 1951, Piaget, 1978). For example, geese chicks will innately imprint onto parental figures, but the recognition of the parental figure is itself achieved by memory. In fact, this interaction of instinct and memory is so strong, that geese chicks have even been demonstrated to imprint onto their human caregiver (Lorenz, 1935).

Another example relates to the innate “waggle dance” of the honeybee, whereby the honeybees communicate the location of food sources to their fellow honeybees via a series of stereotyped movements. This behaviour, while innate, requires memory of the location of the food (von Frisch, 1967). Furthermore, other honeybees from the same hive which recall a danger being present at that food source, such as a predator, will innately perform a “headbutt” on the naïve dancer to prevent them from continuing their danger-inviting dance (Nieh, 1993). In mammals, rodents retain a memory of shelter location to which they rapidly flee while performing innate flight responses to threatening visual stimuli (Campagner et al., 2023), and can learn to avoid areas where innately threatening stimuli were previously presented (Evans et al., 2018, Mackenzie et al., 2010).

These examples clearly demonstrate that there is cross-talk between instinct and memory. However, their interconnectedness may go much deeper, and rather than considering instinct and memory as distinct systems available to the brain for information, it may be more accurate to consider instinct and memory as a spectrum, with some behaviours being largely learned, others largely innate, and many somewhere in between (figure 1.2).

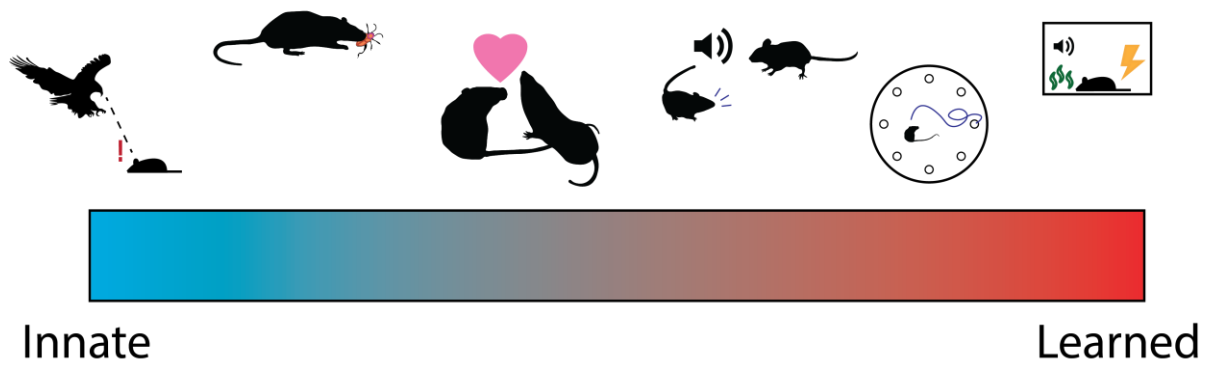


Figure 1.2 Animal behaviours as a spectrum of innate and learned behaviour. Some behaviours, such as fleeing from predators, are very innate and require little learning. Others such as hunting, mating and communication, may be innately driven but require varying amounts of learning to fully develop. Learned behaviours such as maze learning or fear conditioning may even have innate components, such as associating neutral stimuli with innately rewarding or punishing unconditioned stimuli.

One case study which illustrates this clearly is that of the zebra finch birdsong. Zebra finch males sing to attract mates, and young male zebra finches learn their songs from their fathers (Jones et al., 1996). These songs show species-specific properties, but when young males are raised in an environment isolated from other male birds, they develop abnormal songs which are distinct from the wild-type song of laboratory and natural colonies (Williams et al., 1993). However, when these very same isolated males are then allowed to establish new colonies, with successive generations the song properties trend back towards the wild-type song despite not a single bird in the colony having heard the original wild-type song (Fehér et al., 2009). This case study demonstrates how even learned behaviours such as birdsong may be directed and constrained by innate preferences. In fact, this interaction of cultural learning and innate direction of behaviour has been observed in humans too. A particularly well known case of this pertains to a group of deaf children in Managua, Nicaragua, who spontaneously developed sign language without having been taught to do so (Kegl et al., 2002). These examples highlight an interaction of instinct and memory not just within the lifetime of the individual but spanning several generations.

1.2.2 Impact of learning on the evolution of instincts

Given that innate behaviour is sculpted over the process of evolution, and that innate behaviour interacts with learning, could it be possible that learning impacts the evolution of instinct? This is a topic of great discourse in recent years, with theoretical implications that are complex, multi-faceted and context-dependent (Mery and Burns, 2010, Heyes et al., 2020, Axelrod et al., 2023, Lande, 2009, Levis and Pfennig, 2016, Morgan et al., 2020, Paenke et al., 2007, Schlichting and Wund, 2014, Hinton and Nowlan, 1996, Tierney, 1986).

Firstly, can the evolution of innate behaviour be guided by learning? This relates to a concept known as the Baldwin effect (Baldwin, 1896, Simpson, 1953). The Baldwin effect proposes that innate properties of a species may have first appeared in a population as an adaptive response to their environment via phenotypic plasticity. An example of this may include an animal seeking a new food source when it enters a new environment, as likely occurred in the ancestors of *Drosophila sechellia* when they first colonised the Seychelles island which is home to the pungent noni fruit – aversive to other species of *Drosophila* (Dekker et al., 2006, Hungate et al., 2013, Lachaise et al., 1986, Shaw et al., 2019). Genetic variation within the population will result in variation in the neural architecture of different brains. Some individuals may be better able to adapt their behaviour, such as by overcoming aversion towards volatiles of the noni fruit, hence providing them a selective advantage. In the absence of behavioural plasticity, these changes may not have provided a selective advantage to the flies, so in this sense, learning reveals the advantage of certain phenotypes and guides the process of evolution. This can manifest in the descendent population in different manners, depending on how costly learning is to the animal, the selection pressure to adapt the behaviour, and how many generations have passed. For example, it has been hypothesised that interactions of learning and natural selection have produced the phenomenon of “prepared learning”, where an animal does not possess a complete instinct but can learn a particular behaviour much more rapidly than its sister species (Dunlap and Stephens, 2014, Mery and Burns, 2010, Mery and Kawecki, 2002). While the Baldwin effect is by no means a new concept, there appears to have been a resurgence in interest in the concept within the scientific community within recent years (Badyaev, 2009, Heyes et al., 2020, Morgan et al., 2020, Axelrod et al., 2023). The Baldwin effect has been proposed as a possible mechanism for the rapid expansion of various animals into new environments, such

as the house finch in North America (Badyaev, 2009). It is backed up by many computational studies (Lande, 2009, Paenke et al., 2007, Hinton and Nowlan, 1996) and experimentally during artificial selection experiments (Mery and Burns, 2010, Dunlap and Stephens, 2014, Morgan et al., 2020).

However, this is not the only way learning may interact with evolution. In fact, where the learning of a certain behaviour occurs readily and with little cost to the individual, the canalisation of an innate behaviour can be delayed. This theoretical phenomenon is sometimes referred to as the “buffering effect” (Axelrod et al., 2023), where behavioural plasticity can buffer a selection pressure for a certain behavioural response. As such, learning may alter the trajectory of evolution in various ways (figure 1.3), and paying attention to this may lead to novel insights on the evolution of behaviour.

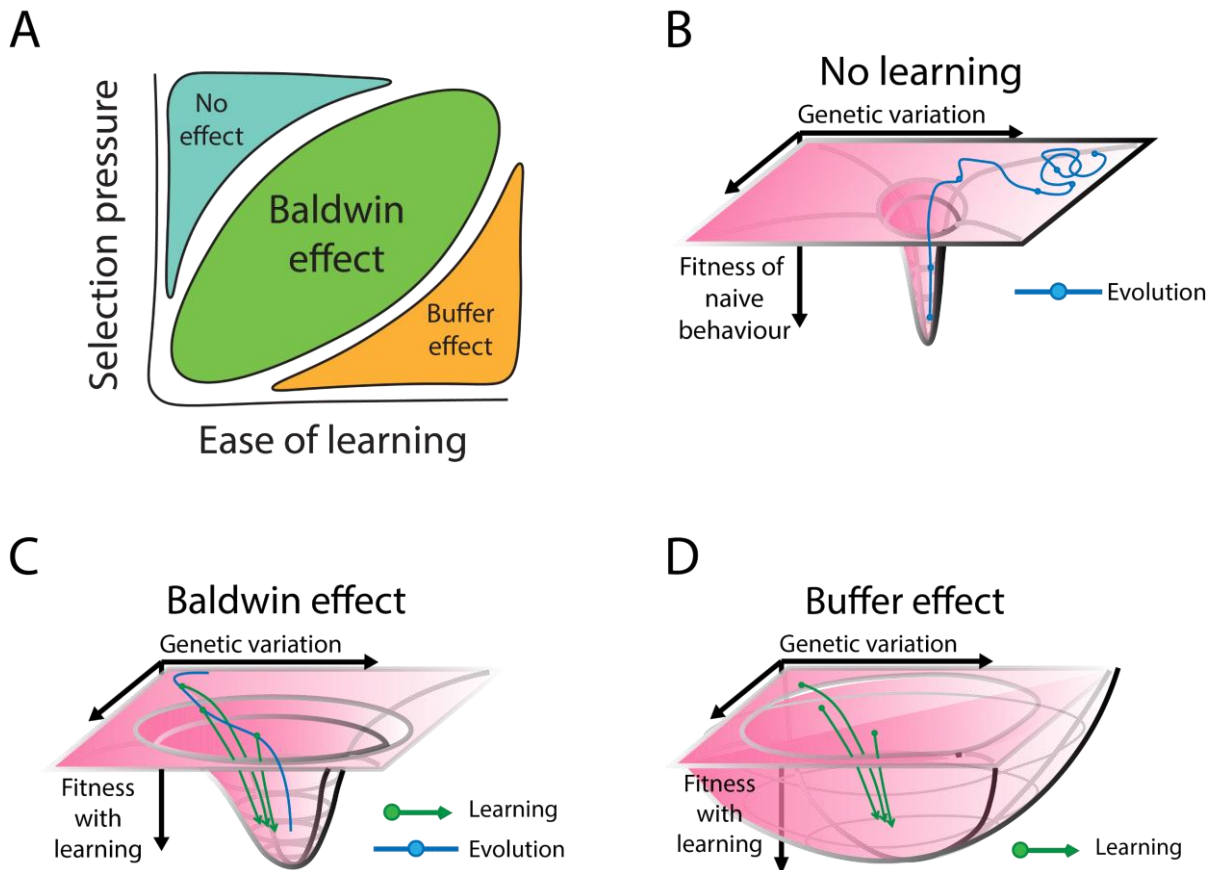


Figure 1.3 The interaction of instinct and memory across evolution. (A) Interactions of selection pressure and learning may hypothetically lead to different outcomes for evolution, including the Baldwin effect and the Buffer effect. (B) Natural genetic variability in a population predisposes some individuals with increased fitness in a given environment, but the range of genetic combinations which lead to an innately advantage behaviour may be narrow. (C) Learning can reveal the advantage of certain genetic combinations which are closer to the “ideal” behaviour by leading to faster learning of the appropriate behaviour, even if it does not improve the innate fitness of the individual. However, learning is costly so there is still a selective advantage in canalising the behaviour. In this scenario, learning may “guide” evolution (“Baldwin effect”). (D) In some situations, a behaviour may be so readily learned, that genetic combinations which make the behaviour more innate provide little or no selective advantage. This is known as the “buffering effect”.

1.2.3 How to study instinct-memory interactions?

In order to advance our understanding of the *ingram* as a unit of information, we may draw inspiration from the memory engram field. In just over the past decade, advances in memory engram labelling technology (Josselyn and Tonegawa, 2020, Liu et al., 2012a, Ramirez et al., 2013, Poo et al., 2016, Ryan et al., 2015, Power et al., 2022) (figure 1.4) have made it possible to label and manipulate the cells responsible for a given memory. This has given

researchers unprecedented control, allowing them to identify, monitor and perform functional manipulations on engram cells. This technology has now been shown to be effective in labelling the neurons coding for innate behaviours (*ingrams*) as well (Root et al., 2014). In combination with other tools such as *in vivo* fiber photometry (Silva et al., 2021) and optogenetics (Liu et al., 2012b), we may identify, monitor and manipulate an instinct.

This thesis will present two models of innate behaviour which will implement these techniques to understand instinct and memory interactions. First, the defensive responses of *Mus musculus* to innately threatening visual looming stimuli (Shang et al., 2018, Yilmaz and Meister, 2013, Evans et al., 2018, Vale et al., 2020). Second, the olfactory responses of the *Mus* clade to trimethylamine (TMA), a component of *Mus musculus* urine (Li et al., 2013a, Lee et al., 2021b). These two models will serve to provide unique perspectives on the interaction of innate behaviour and memory. Additionally, a computational model, inspired by the olfactory behaviour of *Drosophila melanogaster*, will be used to understand how varying parameters such as selection pressure, plasticity and cost of learning may interact with one another over the course of evolution.

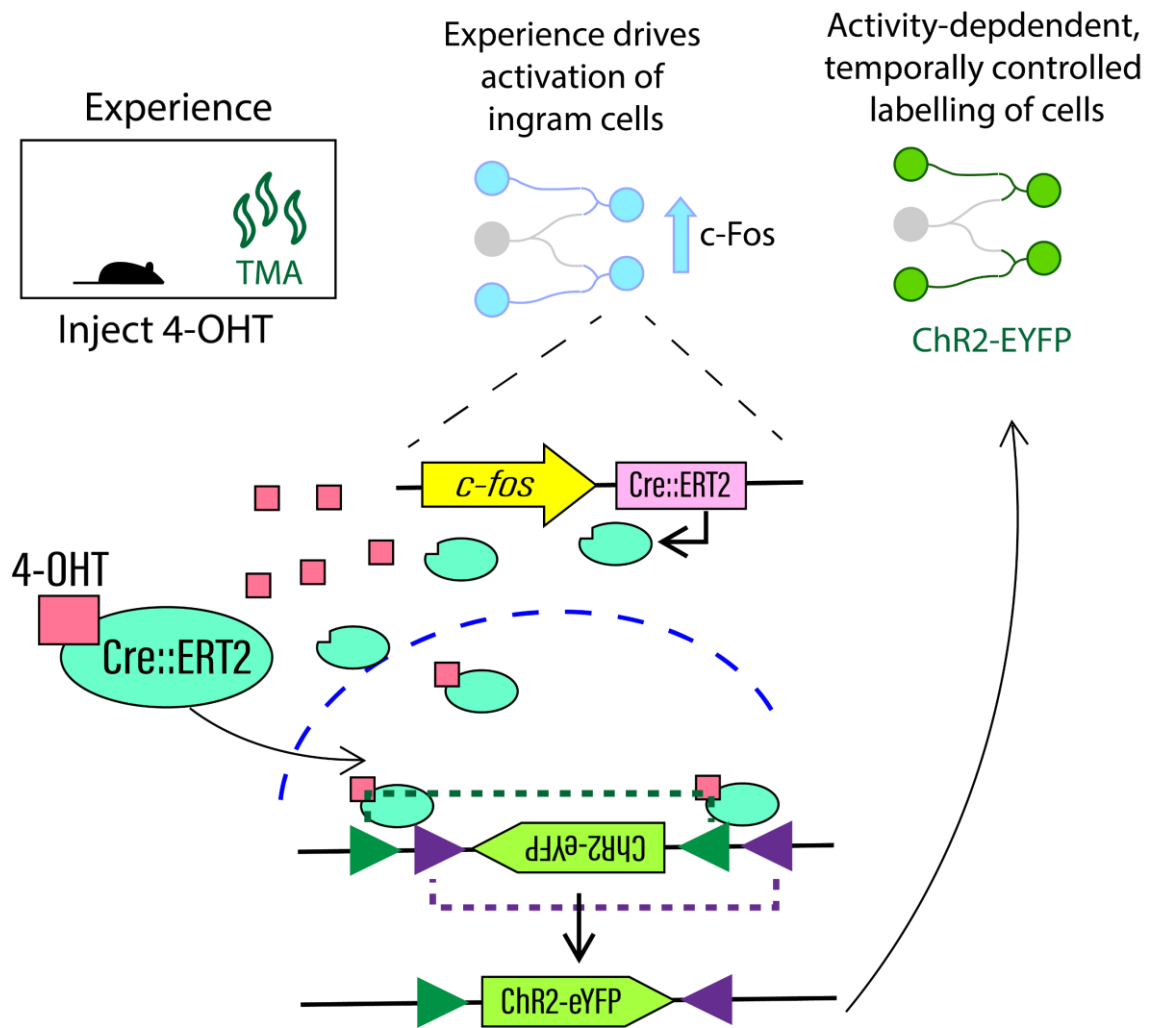


Figure 1.4 Engram labelling technology. Neurons increase *c-Fos* expression when their activity increases. Engram labelling technology hijacks this process to tag neurons which are active during a given experience. Transgenic TRAP2 mice express *Cre:ERT2* under the control of the *c-Fos* promoter, leading to activity dependent expression. This *Cre:ERT2* cannot cross the nuclear membrane except in the presence of 4-hydroxytamoxifen (4-OHT). Injecting 4-OHT allows the *Cre* to transiently cross the nuclear membrane, recombining the gene of an effector protein such as Channelrhodopsin, such that the cell now permanently expresses the transgene. This system result in the ability to induce expression of specific transgenes in neurons in an activity-dependent and temporally controlled manner.

1.3 Defensive behaviour to visual looming stimuli

1.3.1 Behavioural responses to visual looming stimuli

The presentation of an overhead expanding black disk, resembling a rapidly approaching object (such as an attacking predator), can be used to trigger innate defensive responses of rodents such as freezing or fleeing towards shelter (Evans et al., 2018, Vale et al., 2020, Yilmaz and Meister, 2013) (figure 1.5). This stimulus, known as the visual looming stimulus, has been shown to induce defensive behaviours in various species of rodent, fish, reptiles, amphibians, birds, non-human primates, infant humans and invertebrates (Yilmaz and Meister, 2013, Gallagher and Northmore, 2006, Carlile et al., 2006, Ingle and Karin, 1990, Wang et al., 1993, Schiff et al., 1962, Ball and Tronick, 1971, de Vries and Clandinin, 2012, Oliva et al., 2007). The ability to engage in defensive behaviour in response to visual looming stimuli is so highly conserved across species due to its importance in survival – a rapidly approaching object, be it a rock or a predator, is dangerous. This important role in survival is also a likely explanation for why this behaviour evolved to be innate rather than learned – there is no opportunity to get it wrong.

The precise defensive behaviour carried out may vary across differing contexts. For example, when a shelter is present, mice will rapidly flee to said shelter upon detection of the stimulus (Yilmaz and Meister, 2013, Evans et al., 2018, Campagner et al., 2023). However, if the shelter is distant, or the stimulus is less intense, mice may opt for other defensive approaches such as freezing (Dean et al., 1989, Yilmaz and Meister, 2013). The bias towards one response or the other can also vary across species. For example, *Peromyscus maniculatus*, who naturally reside in dense shrubby environments, have a strong bias towards fleeing responses when tested in the lab. Meanwhile *Peromyscus polionotus*, whose light coloured fur blends into the sandy plains of their natural habitat, strongly bias towards freezing even when a shelter is available (Baier et al., 2023). Further to this, there is some precedent for experience-dependent changes in behaviour. For example, mice can avoid areas where looming stimuli were previously presented (Evans et al., 2018, Burnett et al., 2024). Developmental experience matters too, as mice who spent 7 days in a dark box starting at 21 days old show reduced escape probability, although freeze probability is less impacted (Narushima et al., 2022). Additionally, mice with genetic models of neurodevelopmental disorders show impairments in escape responsiveness and place avoidance to looming stimuli

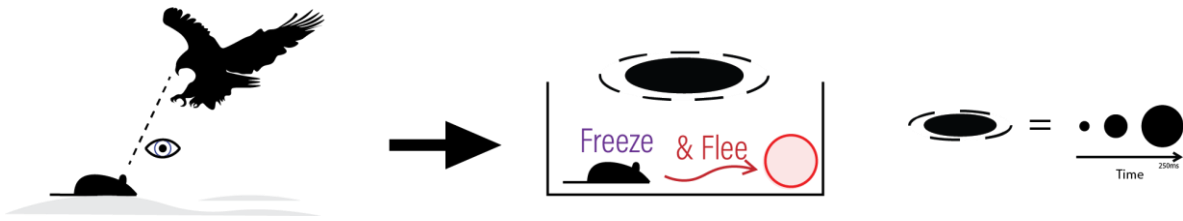


Figure 1.5 Defensive behaviour to visual looming stimuli. Mice innately fear rapidly approaching objects, such as a predator. This may be replicated in the laboratory environment via an expanding black disk which takes up an increasing portion of the visual field over time. Mice will engage in defensive behaviours in response to this stimuli, such as freezing in place or fleeing to a nearby shelter.

(Burnett et al., 2024). Furthermore, the behavioural response can be prevented by initial presentation of a very low contrast stimulus and gradually increasing contrast (Lenzi et al., 2022, Mederos et al., 2024).

1.3.2 Circuitry underlying looming stimulus responses

Despite being recently introduced as an experimental model to the field of behavioural neuroscience (Yilmaz and Meister, 2013), the circuitry underlying the innate visual looming response is quite well studied (summarised in figure 1.6). The visual looming response has been studied at the level of sensory detection (Ito and Feldheim, 2018, Lee et al., 2020, Wu et al., 2023), stimulus identification and threat assessment (Lee et al., 2020, Shang et al., 2018, Wei et al., 2015, Wu et al., 2023, Evans et al., 2018) and behavioural response (Campagner et al., 2023, Deng et al., 2016, Evans et al., 2018, Fadok et al., 2017, Salay et al., 2018, Shang et al., 2018, Tovote et al., 2016, Wei et al., 2015).

The retina of the eye, where all visual information is first detected, is retinotopically mapped onto the superficial layer of the superior colliculus (SC) (Ito and Feldheim, 2018). The first step in the detection of looming stimuli specifically occurs via the action of OFF-transient alpha-retinal ganglion cells (OFF-RGCs) (Wang et al., 2021). These neurons, as their name suggests, transiently respond to a decrease in light within their receptive field. The visual looming stimulus causes an expansion of a dark area in the visual field, triggering the activation of more OFF-RGCs which expands spatially and temporally in the retina. The OFF-RGC neurons project to parvalbumin-positive (PV+) neurons in the superficial SC (Wu et al.,

2023, Shang et al., 2015), where their ability to induce excitatory post-synaptic potentials (EPSPs) is bounded by a band-pass filter (Wu et al., 2023). If PV+ neurons in the SC then receive successive inputs from the OFF-RGCs within a certain bandwidth (not too fast, not too slow), it will result in the production of an action potential. This provides a mechanism by which an expanding area of darkness, within a certain range of speed, will result in the activation of PV+ neurons in the superficial SC. This is the detection of the visual looming stimulus.

Meanwhile, the deeper layers of the SC filter out and respond to behaviourally relevant cues, such as the visual looming stimulus (Lee et al., 2020). This activity builds with successive cues as a measure of threat accumulation (Evans et al., 2018). Above a certain threshold, this accumulated threat measure will activate the dorsal periaqueductal gray (dPAG) via weak synaptic connections, leading to the generation of an escape response (Evans et al., 2018). This is the canonical pathway of the visual looming response, but there are other pathways involved too. For example, PV+ neurons of the superficial SC also project to the parabrachial nucleus (PBG), the lateral posterior thalamic nucleus (LPTN) (Shang et al., 2018, Shang et al., 2015) and the lateral geniculate nucleus (LGN) (Li et al., 2023). Stimulation of SC to PBG projections leads to the generation of flight responses (Shang et al., 2018), while the SC to LPTN and LGN projections generate freezing response (Shang et al., 2018, Li et al., 2023)s. Additionally, inactivation of these nuclei results in a bias towards the opposite behavioural response (Shang et al., 2018, Li et al., 2023), providing a mechanism by which the brain can generate varying defensive responses. The ventral midline thalamus (vMT) is another region which plays a role in response selectivity, with subregions of the vMT also promoting specific freezing and fleeing responses via projections to the basolateral amygdala (BLA) and prefrontal cortex respectively (Salay et al., 2018). Indeed it is likely that in natural looming-evoked defensive responses, all of these regions contribute to the generation of a behavioural response.

In terms of deciding which response and associated circuitry to activate, the exact mechanism remains inconclusive. However, it is known that the superficial SC receives innervation from many brain regions which may provide additional information, including the visual cortex (Mederos et al., 2024, Salay et al., 2018, Zhao et al., 2014), retrosplenial cortex (Campagner et al., 2023) and prefrontal cortex (Ritter et al., 2024). It is likely that these projections contain information including shelter location, general threat and anxiety level,

and memories of threat history. How this information is ultimately compiled is currently unknown.

1.3.3 Investigating plasticity of innate behaviour – insights from the field of learning

In order to study instinct-memory interactions in the visual looming response, it is necessary to construct a paradigm involving plasticity of behaviour towards the visual looming stimulus. To achieve this, one may take inspiration from the broader field of learning and behavioural plasticity. Among the simplest ways to engage plasticity of behaviour to a particular stimulus is via repeated unreinforced presentation of the stimulus, as is done in habituation (Castellucci et al., 1970, Rankin et al., 2009, Thompson and Spencer, 1966) and extinction of a Pavlovian fear conditioned memory (Bouton, 2004, Bouton and King, 1983, Milad and Quirk, 2012). These phenomena, while methodologically similar, have key differences in terms of the history of the stimuli involved (novel stimulus vs. previously conditioned stimulus), the dynamics of the change in behaviour (e.g. context specificity), and potentially the neural mechanisms involved. I will summarise below what is known about these two paradigms of behavioural plasticity and how they may inform an experiment investigating behavioural plasticity to the visual looming stimulus specifically.

Habituation to a novel stimulus

Habituation is a non-associative learning process whereby an organism decreases its response to a repeated stimulus over time, without any sensory adaptation, sensory fatigue or motor fatigue (Rankin et al., 2009). Most commonly, the stimulus used is of neutral valence or only mildly rewarding or punishing (Das et al., 2011, Pilz et al., 2014, Rankin et al., 2009, Harris, 1943, Thompson and Spencer, 1966). It has even been argued by some authors that habituation cannot occur to biologically significant stimuli (Thorpe, 1956), although there are at least some examples of habituation to stressful paradigms, including restraint (Bhatnagar et al., 2002). Examples of habituation in rodents include reduced exploration of a novel context after repeated exposure, or a reduced startle response (measured as a whole body flinch) to an acoustic stimulus (Davis and Eaton, 1984).

There are 10 key characteristics of habituation learning which are highly conserved across species, as outlined by Rankin et al., (2009; see also table 1.1). However, despite the remarkably well conserved behavioural characteristics of habituation, there does not appear to be a single molecular, cellular or circuit level mechanism which defines behavioural habituation. Different habituation paradigms have been revealed to be mechanistically driven by decreases in intrinsic postsynaptic neural excitability (Kindt et al., 2007, Burrell et al., 2001), changes in the efficacies of specific synapses (Castellucci et al., 1970) and circuit level adaptations (Das et al., 2011, Ramaswami, 2014). Further, there is evidence of the involvement of multiple brain regions in rodents, including the hippocampus (Daenen et al., 2001, Patacchioli et al., 1989, Vianna et al., 2000). In some cases, habituation may rely on the activity of brain regions which were not even required for the initial behavioural response, suggesting it is can be an active learning process recruiting the activity of new brain regions rather than a form of passive inhibition or reduced intrinsic excitability (Bhatnagar et al., 2002). Furthermore, even distinct habituation timelines to the same stimulus may recruit different mechanisms, as evidenced by the inability of BK-knockout mice to perform short-term habituation to the acoustic startle stimulus, while long-term habituation to the same stimulus remains intact (Typlt et al., 2013).

Table 1.1. Common characteristics of habituation, adapted from Rankin et al. (2009).	
1	<i>"Repeated application of a stimulus results in a progressive decrease in some parameter of a response to an asymptotic level"</i>
2	<i>"If the stimulus is withheld after response decrement, the response recovers at least partially over the observation time ("spontaneous recovery")."</i>
3	<i>"After multiple series of stimulus repetitions and spontaneous recoveries, the response decrement becomes successively more rapid and/or more pronounced (known as potentiation of habituation)."</i>
4	<i>"Other things being equal, more frequent stimulation results in more rapid and/or more pronounced response decrement, and more rapid spontaneous recovery (if the decrement has reached asymptotic levels)."</i>
5	<i>"Within a stimulus modality, the less intense the stimulus, the more rapid and/or more pronounced the behavioural response decrement. Very intense stimuli may yield no significant observable response decrement."</i>
6	<i>"The effects of repeated stimulation may continue to accumulate even after the response has reached an asymptotic level (which may or may not be zero, or no response). This effect of stimulation beyond asymptotic levels can alter subsequent behaviour, for example, by delaying the onset of spontaneous recovery."</i>
7	<i>"Within the same stimulus modality, the response decrement shows some stimulus specificity."</i>
8	<i>"Presentation of a different stimulus results in an increase of the decremented response to the original stimulus (dishabituation)."</i>
9	<i>"Upon repeated application of the dishabituating stimulus, the amount of dishabituation produced decreases (this phenomenon can be called habituation of dishabituation)."</i>
10	<i>"Some stimulus repetition protocols may result in properties of the response decrement that last hours, days or weeks (long-term habituation)."</i>

The proposed experiment, repeated unreinforced presentation of a stimulus, bears many similarities with the process of habituation. However, the visual looming stimulus is more evocative than the stimuli typically used to study habituation. Further, the defensive behavioural responses associated with visual looming in rodents are not well studied in the context of habituation. Given this, we may look to the literature on extinction training of a fear conditioning memory for further insight on how information relating to a threatening stimulus may be updated. The visual looming response, being an innate behaviour, is of course inherently distinct from fear conditioned memories. However, one may hypothesise that the visual looming response arises from an innate association between the visual looming

Table 1.2. Contextual fear conditioning and the innate visual looming response.

Contextual fear conditioning	Innate visual looming response
Learned fear of context (CS) due to association with a foot-shock (US)	Innate fear of visual looming stimulus (US)
Defensive behavioural responses (freezing) to a negative valence stimulus	Defensive behavioural responses (freezing and fleeing) to a negative valence stimulus
Reflexive ¹ behaviour	Reflexive ¹ behaviour

stimulus and threat. As such, studying how a learned fear behaviour can be extinguished may still provide some valuable perspective in our study of the visual looming stimulus response.

Extinction to a fear conditioned stimulus

Like looming, fear conditioning evokes fear and defensive behaviour (usually freezing) upon the presentation of the appropriate sensory cues (Pavlov, 1927, Bouton and Bolles, 1980). Fear conditioning consists of a conditioned stimulus (CS), such as a context, tone, or other sensory cue, for which a particular association is to be learned. The association is with some reinforcing stimulus, known as the unconditioned stimulus (US), which usually has some innate valence, whether positive or negative. This could be something unpleasant, such as an electric shock (Schafe and LeDoux, 2000, Kim and Fanselow, 1992), or something rewarding such as food (Mishima et al., 1986), and will elicit a “reflexive”¹ behavioural response such as freezing or salivation. This is known as the unconditioned response (UCR). When the CS and US are repeatedly presented to the subject closely together (usually with the CS preceding the US), the CS becomes associated with the US and hence the subject begins performing the “reflexive” behavioural response to the CS instead. This is known as the conditioned response (CR).

So, how can a fear conditioning memory be updated? In the case of Pavlovian fear conditioning, this can be achieved via repeated presentation of the conditioned stimulus (CS) without any reinforcing stimulus, leading to the attenuation of the learned fear behaviour

¹ “Reflexive” here refers to predictable behavioural response, occurring automatically or innately in response to a stimulus, which may vary slightly depending on context but will ultimately fall into a predictable class of behaviours. This is distinct from sensorimotor reflexes, which are involuntary and nearly instantaneous movements in response to a stimulus, occurring via a reflex arc.

Table 1.3. Comparing common traits of extinction and habituation	
Extinction	Habituation
Associative learning process	Non-associative learning process
A previously learned Pavlovian response attenuates due to repeated unreinforced presentation of the CS	Response to a novel stimulus (e.g. a novel tone or context) attenuates with repeated presentation
US usually has an innate positive valence (e.g. food reward) or negative valence (electric shock)	The novel stimulus is usually of neutral valence (e.g. novel context) or slight negative valence (e.g. loud noise)
Spontaneously recovers	Spontaneously recovers
Context specific	May or may not be context-specific depending on the paradigm
Updating information on specific stimulus	Reduced attention towards specific stimulus
Recovers via re-presentation of the US	Recovers via re-presentation of a novel stimulus (dishabituation)
CS may be readily associated with the same or another US (CS remains a salient cue)	The stimulus is not readily associated with a US (stimulus becomes less salient)

(Pavlov, 1927, Bouton, 2004). This phenomenon is referred to as extinction. (Pavlov, 1927, Bouton, 2004). The extinguished response can be recovered either spontaneously with the passage of time (recovery) (Pavlov, 1927, Milad and Quirk, 2012), by a change in context (renewal) (Bouton and King, 1983), by another presentation of the US in a neutral or novel context (reinstatement) (Rescorla, 1975), or by re-pairing of the CS with the US (re-acquisition) (Rescorla, 2001). Memory engram studies have helped reveal what happens to a specific memory over the course of extinction. In the case of contextual fear conditioning (CFC), the memory engram of the context resides in the dentate gyrus (DG) of the hippocampus (Liu et al., 2012a, Wang et al., 2013, Ryan et al., 2015, Tonegawa et al., 2015), with a fear or shock component in the ventral CA1 area of the hippocampus (Zaki et al., 2022, Jimenez et al., 2020, Herbst et al., 2022, Kjelstrup et al., 2002). The activity of the

original engram is necessary during the process of extinction, implying that extinction is not a passive process (Khalaf et al., 2018, Autore et al., 2023). Furthermore, artificial stimulation of this engram in the absence of any reinforcing cues is sufficient to induce an

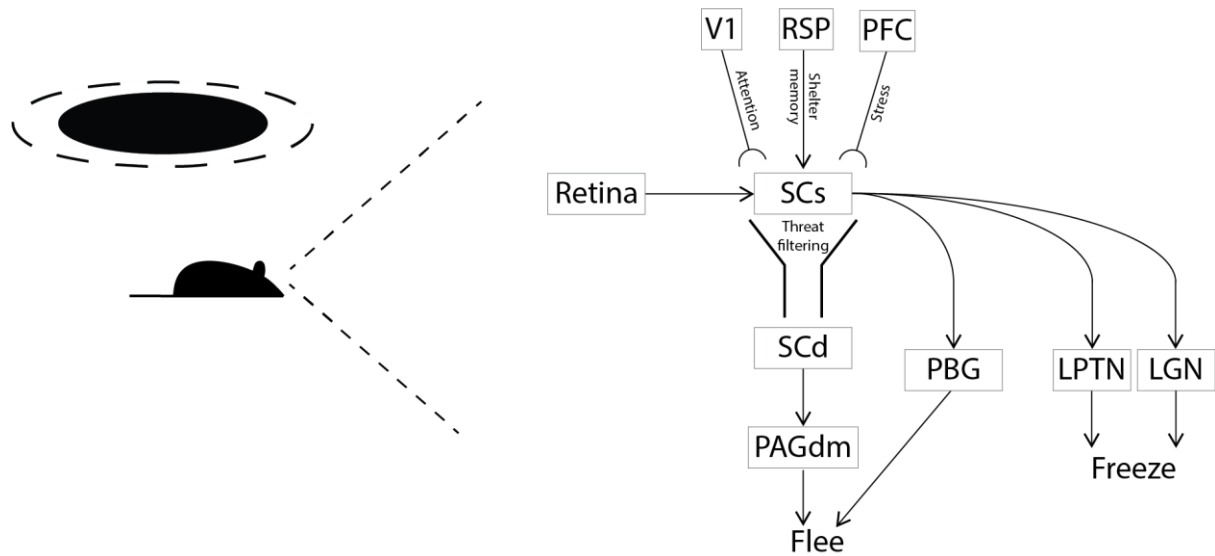


Figure 1.6 Circuitry underlying the innate behavioural responses to visual looming stimuli. Visual looming stimuli are detected by retinal ganglion cells and first recognised at the superficial layer of the superior colliculus. At the same time, activity of the superior colliculus is modulated by inputs from other brain areas, such as the visual cortex, retrosplenial cortex and prefrontal cortex. Information from the superficial SC is filter down to deeper layers of the SC, which are selective for salient and threatening stimuli. Sufficient activation of deep layers of the SC results in activation of the dorsomedial region of the PAG, which orchestrates a fleeing response. In addition to this, the SC transmits information to other brain areas, such as the PBG, LPTN and LGN, each of which is biased towards the stimulation of a certain behavioural response.

extinction-like phenotype (Park et al., 2022). Moreover, there is evidence for the existence of a second “extinction engram” which is active during context presentation following extinction, and competes with the original engram (Lacagnina et al., 2019). Spontaneous recovery, renewal and reinstatement emerge when the original fear memory engram outcompetes the extinction engram (Zaki et al., 2022).

Unlike fear conditioning, the visual looming response is an innate behaviour. As such, the visual looming stimulus is, by definition, a US. Similarly, the freezing/fleeing behaviour in response to the stimulus is an unconditioned response (UCR). However, one may hypothesise that the visual looming stimulus response consist of an innate association between the visual looming stimulus and threat responses. Under such a model, we may investigate what happens to the circuitry underlying the innate visual looming response which new information about the stimulus is received.

This thesis will examine the effect of repeated unreinforced presentation of the visual looming stimulus on the behavioural responses to the stimulus and to the circuitry underlying the behaviour. We will take inspiration from the literature of habituation and extinction to understand the dynamics of any behavioural adaptations which occur. Specifically, does the fear behaviour spontaneously recover with time or in a novel context? Can the behaviour be recovered by the presentation of a distracting stimulus such as an electric shock? And finally, should we reinstate the behavioural response, does this also reinstate the original midbrain circuitry or will the reinstated behavioural response then rely on distinct circuit mechanisms? The studies undertaken here will focus on the canonical circuitry implicated in the innate visual looming response and circuitry known to be involved in fear memories, habituation, extinction and reinstatement. Namely, the SC and PAG for looming (Evans et al., 2018, Lee et al., 2020, Wu et al., 2023), and the hippocampus for its well established role in learning, extinction and reinstatement (Zaki et al., 2022, Lacagnina et al., 2019, Marek et al., 2018, Xu et al., 2016).

1.4 Olfactory behavioural responses to trimethylamine

1.3.4 What is trimethylamine?

Trimethylamine (TMA) is a small, volatile tertiary amine molecule often described as having a “fishy” odour. It is most commonly found during putrefaction of decaying organic matter, but also as a result of the distressing condition Trimethylaminuria (also known as fish odour syndrome) whereby a person produces excess trimethylamine in their sweat and breath (Erkmen, 2016, Mitchell and Smith, 2001, Horowitz et al., 2014). It is aversive to humans, non-human primates and rats, presumably due to its association with poor gut health and putrefaction. However, it is attractive to *Mus musculus*, for which the males excrete the molecule in their urine and use it as a social signal (Li et al., 2013b, Mitchell and Smith, 2001, Lee et al., 2022, Cichy et al., 2021, Horowitz et al., 2014) (figure 1.7). Indeed, it is possible that TMA acts as an allomone for *Mus musculus* by deterring predators and competitors from their nest sites, with the social role of TMA evolving in parallel (Li et al., 2013b). In terms of responses of *Mus musculus* to TMA, it has been shown to be behaviourally attractive (Li et al., 2013b, Saraiva et al., 2016), affect aggressive behaviour in social interaction (Cichy et al.,

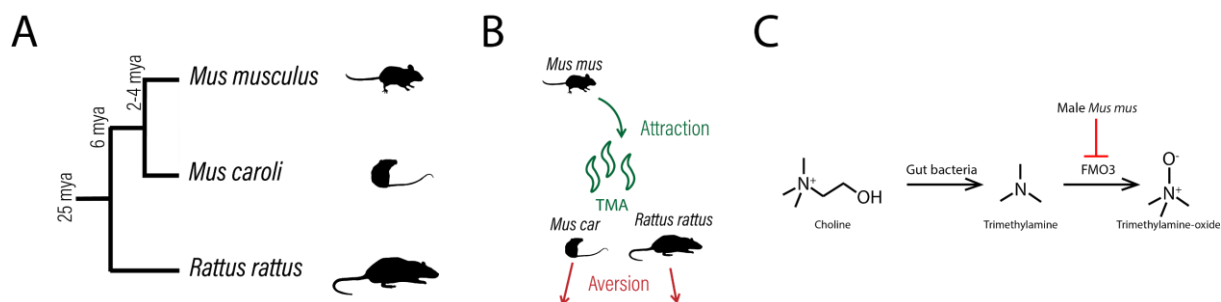


Figure 1.7 The evolution of TMA attraction in *Mus musculus*. (A) Phylogenetic tree for *Mus musculus*, *Mus caroli* and *Rattus rattus*. (B) Behavioural responses of *Mus musculus*, *Mus caroli* and *Rattus rattus*. (C) The enzyme FMO3, which oxidises TMA produced by gut bacteria into the odourless TMAO, is suppressed in male *Mus musculus*. *Mus caroli* is the closest relative of *Mus musculus* who do not produced TMA.

2021), and has anti-stress effects, preventing the release of corticotropin-releasing hormone in various stress assays (Lee et al., 2022).

TMA is produced by the action of commensal gut bacteria on dietary choline and L-carnitine (Hayward and Stadtman, 1959, Koeth et al., 2013, Zhang et al., 1999, Li et al., 2013b). In humans and rats, TMA is oxidised into the odourless Trimethylamine-N-Oxide (TMAO), predominantly in the liver, by the enzyme flavin-dependent monooxygenase 3 (FMO3) (Baker and Chaykin, 1962, Krueger and Williams, 2005, Li et al., 2013b). In male *Mus musculus*, the expression of the *fmo3* gene is silenced in the liver, with females expressing 1000 times as much FMO3 as males. Both sexes of *Rattus norvegicus* and other non-TMA-producing *Mus* species produce FMO3 in similar quantities as female *Mus musculus* (Li et al., 2013b).

The closest relative to *Mus musculus* which does not produce TMA is *Mus caroli* (Li et al., 2013b), whose ancestors diverged from *Mus musculus* about 2-4 million years ago (Silver, 1995). There is no published literature on the behavioural response of *Mus caroli* to TMA, but it is hypothesised that they will retain the ancestral response of TMA aversion which is present in other mammals (Li et al., 2013b, Mitchell and Smith, 2001, Horowitz et al., 2014).

The relatively recently evolved olfactory behavioural response of the *Mus* clade to TMA presents a unique opportunity to study the evolution of behaviour and the role of learning in this process. Firstly, it is a behaviour which is known to be innate, but due to the production of TMA by their parents juvenile mice will necessarily experience some learning towards this odorant in the early stages of their life. Secondly, we have access to a closely

related species which do not produce TMA and may act as a proxy for the “ancestral state” of TMA olfaction, allowing us to compare how the circuitry underlying TMA olfaction has been altered over the course of evolution. However, before doing this, we must review what we know about how the *Mus* brain detects and responds to TMA.

1.3.5 Circuitry underlying TMA olfaction

Mus species have a wide range of olfactory instincts to aid in behaviours such as social interactions, predator aversion and finding food (Ishii et al., 2017, Kondoh et al., 2016, Liberles, 2015, Pérez-Gómez et al., 2015, Kobayakawa et al., 2007, Li et al., 2013a, Root et al., 2014). The flow of information in the mouse olfactory system, from detection at olfactory receptor neurons, to glomeruli and onto higher brain regions is broadly well studied (Hashiguchi and Nishida, 2007, Jeanne et al., 2018, Johnson et al., 2012, Kobayakawa et al., 2007, Russo et al., 2020). However, there remains much to learn about where and how innate olfactory behavioural responses are encoded, and in particular how the brain responds to TMA olfaction.

Detection of TMA is mediated by the Trace-Amine Associated Receptor 5 (TAAR5) (Liberles and Buck, 2006, Li et al., 2013a). TAAR5 is a member of a the TAAR family of G-protein coupled receptors, a distinct family of chemosensory receptors which are phylogenetically distinct from the canonical olfactory receptors (OR) (Liberles and Buck, 2006, Borowsky et al., 2001, Liberles, 2015, Hashiguchi and Nishida, 2007, Grus and Zhang, 2008). TAARs are thought to have first appeared in vertebrates at the emergence of jawed fish around 400 million years ago (Hussain et al., 2009, Tessarolo et al., 2014, Libants et al., 2009). Based on studies of TAAR1, it was initially thought that the primary role of this family of receptors was to detect low-abundance neurotransmitters known as “trace-amines” (Lindemann and Hoener, 2005). It has since become clear that most TAARs function as chemosensory receptors in the main olfactory epithelium (MOE), with each *taar* gene being expressed in unique sensory neurons, as well as participating in axon guidance of these sensory neurons to unique and spatially typified glomeruli in the olfactory bulb (Liberles and Buck, 2006, Liberles, 2015, Johnson et al., 2012). The glomeruli of TAAR-expressing sensory neurons are typically located in the dorsomedial region of the olfactory bulb (Johnson et al., 2012, Pacifico et al., 2012).

The specific mapping of TAAR-circuitry beyond the olfactory bulb is currently uncharacterised. However, there has been some research into the mechanisms by which the anti-stress effect of TMA is mediated (Lee et al., 2021b). Lee and colleagues injected a retrograde pseudorabies virus into corticotropin release hormone neurons (CRHNs) of the paraventricular nucleus (PVN), and analysed TMA olfaction driven n-Fos expression 3 days post injection (coinciding with the time for the pseudorabies virus to travel retrogradely across a single synapse). They found a significant increase in the activity of GABAergic neurons of the ventromedial hypothalamus (VMH), and later went on to show that the activity of VMH GABAergic neurons are both necessary and sufficient to recapitulate the anti-stress effects of TMA. While these experiments propose a mechanism by which the downstream anti-stress effects of TMA may be mediated, they still leave a gap in the knowledge regarding how information transitions between glomerular activation and downstream targets. Further, while this provides insights into the anti-stress component of TMA behaviour, it does not explain other phenotypes such as attraction or social signalling. To infer what circuitry may be important here, we may consider the broader literature on the main olfactory system (summarised in figure 1.8).

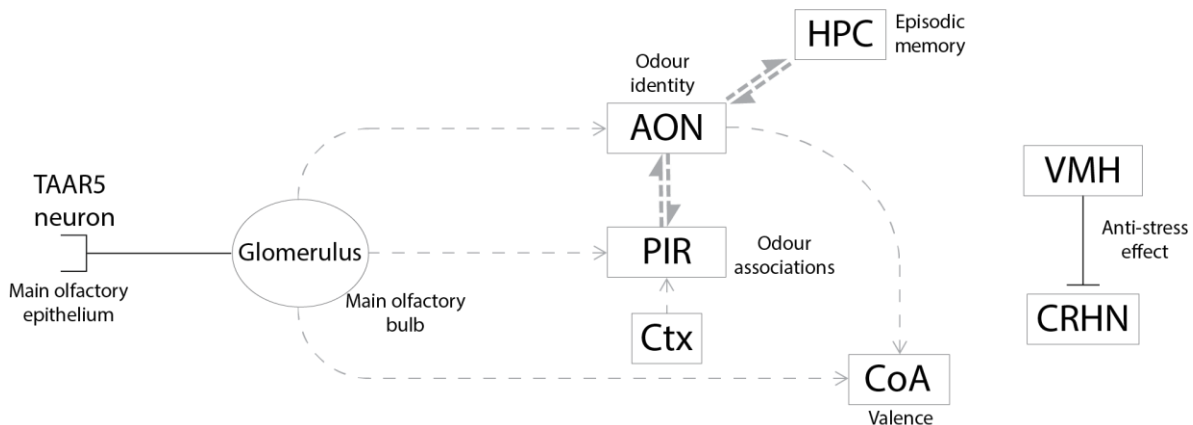


Figure 1.8 Circuitry underlying TMA olfaction. Circuitry known to be implicated in TMA olfaction is indicated by solid black lines. Connections which are known to exist but not known whether they are implicated in TMA olfaction specifically are indicated by dashed grey lines. TMA is initially detected by the TAAR5 receptor on TAAR5-expressing sensory neurons at the main olfactory epithelium. These sensory neurons converge onto a pair of specific glomeruli at the dorsomedial region of the main olfactory bulbs. The main olfactory bulb is known to have direct projections to areas throughout the olfactory cortex, including the AON, PIR and CoA.

It is known that TAAR5 glomeruli are found on the dorsomedial region of the main olfactory bulb (MOB), so what is known about the projections of the MOB to other brain regions? The anterior olfactory nucleus (AON; sometimes also referred to as anterior olfactory cortex) is the first region to receive projections from the MOB (Haberly, 2001, Brunjes et al., 2005, Cajal, 1909, Cajal, 1894). It has dense projections to and from the MOB and the piriform cortex (Brunjes et al., 2005, Russo et al., 2020) and is central to olfactory processing. Additionally, projections to and from the hippocampus appear to be involved in episodic olfactory memory formation (Aqrabawi and Kim, 2018). There is growing evidence that the AON is an important site for the olfactory “*gestalt*” – the representation of odour identity – which is important in coordinating downstream responses (Haberly, 2001). The AON is thought to play a role analogous to that of the hippocampus in memory recall by orchestrating responses of the hippocampus and cortex during recall of an odour related memory (Haberly, 2001, Aqrabawi and Kim, 2020, Aqrabawi and Kim, 2018, Tanaka et al., 2014, Nyberg et al., 2000).

The piriform cortex (PIR) is the largest cortical region that receives direct projections from the MOB. While initially viewed as the olfaction equivalent of a “primary cortex”, it is

now considered a secondary association area (with the AON displacing it from this position) (Haberly, 2001). The PIR receives projections from many other cortical areas, such as the locus coeruleus (Bouret and Sara, 2002), orbitofrontal cortex (Schoenbaum and Eichenbaum, 1995), entorhinal cortex (Luskin and Price, 1983) as well as the AON (Brunjes et al., 2005, Luskin and Price, 1983). It responds strongly to non-olfactory components of odour-discrimination tasks (Schoenbaum and Eichenbaum, 1995) and is thought to be important in forming new and abstract olfactory associations and relaying this information back to the AON (Haberly, 2001).

The cortical amygdala receives direct projections from the olfactory bulb which are spatially restricted and stereotyped, indicative of potentially genetically encoded circuits (Chen et al., 2022, Sosulski et al., 2011, Miyamichi et al., 2011, Wang et al., 2022). The cortical amygdala is thought to be involved in the innate valence of odours, both positive and negative (Root et al., 2014, Miyamichi et al., 2011, Howe et al., 2024). There is some evidence that valence encoding in the cortical amygdala may be topographically represented, with stimulation of the anterior portion of the posterolateral cortical amygdala inducing attraction and posterior regions inducing aversion (Howe et al., 2024). Appropriate valence-associated behaviours may then occur via distinct projections to specific brain areas. For example, cortical amygdala projections have been identified to the medial amygdala for attraction and nucleus accumbens for aversion (Howe et al., 2024). This is similar to the role of the basolateral amygdala in memory, which has divergent projections to the same regions, as well as the ventral hippocampus (Beyeler et al., 2018, Beyeler et al., 2016).

In the context of TMA olfaction in *Mus musculus* and *Mus caroli*, I hypothesise that *Mus caroli* will retain the ancestral state of TMA aversion, and this may be driven by negative valence encoding ensembles in the cortical amygdala. Meanwhile, *Mus musculus*, who are attracted to TMA, may do so via either cortical amygdala driven innate attraction, or by learning, potentially via the activity of the AON.

1.4 Aims and Objectives

The study of visual looming will focus on examining how an instinctual representation may change with learning experience. This study will use *in vivo* recordings to monitor how responses the deep SC and dorsal PAG to visual looming stimuli change over the course of learning. Further, it will use engram labelling technology to monitor how the responses of

specific ensembles change over the course of learning and aim to identify to what extent the original instinct *ingram* has changed.

We will undertake two sets of experiments as follows:

- i. Characterise plasticity of the looming response in *Mus musculus* in a repeated unreinforced exposure training paradigm (habituation).
- ii. Identify behavioural interventions that can recover an extinguished looming response.
- iii. Characterise how circuitry underlying innate looming responses is modified by learning.
- iv. Determine how the brain re-learns looming fear responses post learned attenuation of looming responses.

Meanwhile, the study of TMA olfaction will focus on how innate behaviour can evolve under the influence of learning, both behaviourally and in the circuitry underlying the response. This will involve characterizing the behavioural response to TMA to assess the relative contributions of innate and learned behaviour. Subsequently, comparisons will be drawn between the brain responses of *Mus musculus* and *Mus caroli*, with the goal of identifying brain regions where the response to TMA has been modified to drive the recently evolved behavioural response.

The aims of this project are:

- i. Characterise the role of learning in shaping the TMA olfactory response.
- ii. Characterise the behavioural response of *Mus caroli* to TMA.
- iii. Compare how the brains of *Mus musculus* and *Mus caroli* respond to TMA olfaction.
- iv. Identify neural ensembles responsible for driving TMA olfactory responses in *Mus musculus*.

These behavioural studies will also be complemented by a computational model, which will aim to understand how varying conditions of selection pressure and learning may influence the evolution of behaviour.

2. Materials and methods

2.1 Subjects

2.1.1 Husbandry

Mice were housed in groups of 3-6 in cages with enrichment, food and water ad libitum under a 12 h light/dark cycle. The animal room was kept at a constant temperature of 22°C. All experiments were performed during the light phase. All the procedures related to mouse care and behavioural experiments were carried out accordingly with Health Products Regulatory Authority (HPRA) Ireland guidelines and planned accordance with the principle of the 3R's (<https://nc3rs.org.uk/>). All animals were bred in-house.

Drosophila were reared on a standard cornmeal-yeast agar medium at 25°C and 70% relative humidity under 12h: 12h light: dark cycles. Male and female flies were sorted on CO₂ plates at least 5d prior to behaviour and 5-8d old flies were used for behavioural assessment.

2.1.2 Mouse lines

Strain	Jax ID
C57BL/6J	# 000664
Fostm2.1(icre/ERT2)Luo/J (TRAP2)	# 030323
B6.Cg-Gt(ROSA)26Sortm32(CAG-COP4*H134R/EYFP)Hze/J (Ai32)	# 024109
CAROLI/EiJ	# 000926

2.1.3 *Drosophila* lines

Strain	ID
<i>Drosophila melanogaster</i>	Canton-S
<i>Drosophila sechellia</i>	DSSC 14021-0248.28

2.1.3 Stereotactic surgery

Stereotactic surgery was performed at least two weeks before starting any behavioural procedures. Mice were anaesthetized with 500 mg kg⁻¹ avertin and head fixed on the stereotaxic instrument. Ophthalmic ointment was applied to eyes in order to prevent drying and intracutaneous lidocaine was injected in the skull. The surgical area was then washed with chlorohexidine and 70% ethanol three times before using a blade to perform a 1-1.5 cm incision through the midline of the scalp. After swabbing with increasing concentrations of ethanol, hydrogen peroxide was used to enhance visualisation of lambda and bregma. Animals underwent bilateral craniotomy and holes were drilled at the appropriate coordinates for the target region, detailed below. The viral mix was injected using a micro syringe pump (UMP3;WPI) and a 10 µL Hamilton syringe (701LT; Hamilton) at the appropriate depth for the target region. Injection of virus was at a rate of 60 nL/min. After completion of the injection, the needle was left in the injection site for an additional 5 minutes. For optogenetic experiments, a fiber implant (Doric) was fixed to the stereotaxic adapter and was lowered in the brain, 0.1-0.2mm above virus injection site. To secure the implant, an even layer of Metabond was applied and let dry for about 10-15 minutes. Dental cement was then used to secure the cap to the implant. The protective cap was provided by cutting the top portion of a black polypropylene microcentrifuge tube. Animals were then left in a warm box (29°C) until recovered from anaesthesia and then were returned to their homecage. On the day of the surgery and on the two following days mice were injected subcutaneously with meloxicam (0.075 mL/5g). Mice were allowed to recover for 14 days before any behavioural procedure was performed. Recovery was monitored with the use of a HPRA-approved recovery assessment table.

Target coordinates:

Region	AP from Bregma:	ML from Bregma:	DV from Bregma:
SCd	-4.5mm	+/-0.3mm	-1.65mm
PAGdm	-4.72mm	+/-0.15mm	-2mm
vCA1	-3.5mm	+/-3.5mm	-3.75-3.8mm
AON	+2.55mm	+/-1.35mm	-3.5mm
CoA	-1.7mm	+/-2.7mm	-5.8mm
MOB	+4mm	+/-0.7mm	-1.3-1.5mm

Viruses:

pAAV.Syn.GCaMP6f.WPRE.SV40 was a gift from Douglas Kim & GENIE Project (Addgene plasmid # 100837-AAV9 ; <http://n2t.net/addgene:100837> ; RRID:Addgene_100837)

pAAV-hSyn-DIO-HA-hM4D(Gi)-IRES-mCitrine was a gift from Bryan Roth (Addgene plasmid # 50455-AAV8 ; <http://n2t.net/addgene:50455> ; RRID:Addgene_50455)

pAAV-EF1a-double floxed-hChr2(H134R)-EYFP-WPRE-HGHpA was a gift from Karl Deisseroth (Addgene plasmid # 20298-AAV9 ; <http://n2t.net/addgene:20298> ; RRID:Addgene_20298)

AAV-TRE:mCherry-p2a-eNpHR was a gift from Mark Brimble.

AAV-GFP-CaM-uTEVp was a gift from Mark Brimble.

AAV-Nrxn3b-Nav1.6-MKII-f-hLOV1-TEVcs(ENLYFQ/M)-tTA-VP16 was a gift from Mark Brimble.

Virus	Addgene ref	Serotype	Titer	Dilution	Paper reference
AAV9-Syn-GCamp6f	#100837	AAV9	$\geq 1 \times 10^{13}$ vg/mL	1 in 2	(Chen et al., 2013)
AAV-TRE:mCherry-p2a-eNpHR (FLiCRE 1)	n/a	AAV-DJ	$\geq 1 \times 10^{13}$ vg/mL	undiluted	(Kim et al., 2020)
AAV-GFP-CaM-uTEVp (FLiCRE 2)	n/a	AAV-DJ	$\geq 1 \times 10^{13}$ vg/mL	undiluted	(Kim et al., 2020)
AAV-Nrxn3b-Nav1.6-MKII-f-hLOV1-TEVcs(ENLYFQ/M)-tTA-VP16 (FLiCRE 3)	n/a	AAV-DJ	$\geq 1 \times 10^{13}$ vg/mL	undiluted	(Kim et al., 2020)
pAAV-hSyn-DIO-HA-hM4D(Gi)-IRES-mCitrine	#50455	AAV8	$\geq 1 \times 10^{13}$ vg/mL	undiluted	n/a
AAV-EF1a-double floxed-hChr2(H134R)-EYFP-WPRE-HGHpA	#20298	AAV9	$\geq 1 \times 10^{13}$ vg/mL	1 in 4	n/a

Surgery attrition rates

Surgery performed	Target region	Surgeries performed	Successful surgery (verified histologically)
GCaMP expression + Fiber photometry	SC	27	16
GCaMP expression + Fiber photometry	PAG	11	7
FLiCRE (eNpHR)	vCA1	44	18
GCaMP expression + Miniscope imaging	AON	6	3
Optogenetics (virus + implant)	AON	26	17
Optogenetics (virus + implant)	CoA	20	16
DREADD inhibition (virus only)	MOB	11	7
DREADD inhibition (virus only)	AON	11	6
DREADD inhibition (virus only)	CoA	12	6

2.2 Behavioural procedures

2.2.1 Handling

Before starting each behavioural schedule, mice were handled for three minutes over three consecutive days to habituate them to the experimenter. On the experimental day, each animal was taken from the homecage and brought to the behavioural room in a transparent plastic cage. At the end of the behavioural procedure mice were returned to their homecage. The plastic cage was cleaned with trigene between each mouse.

2.2.2 Visual looming presentation

The standard looming behavioural arena consisted of a rectangular floor (46 cm width x 30 cm length) and a computer monitor on top (28cm above). For any experiments which required an electric foot-shock for reinstatement (and their controls), the entire paradigm was performed in the Med Associates chamber with a removable grid floor (24cm width x 21cm length; bars, 3.2-mm diameter; space, 7.9 mm apart). A red polycarbonate tube (7.5 cm diameter x 15 cm length) was

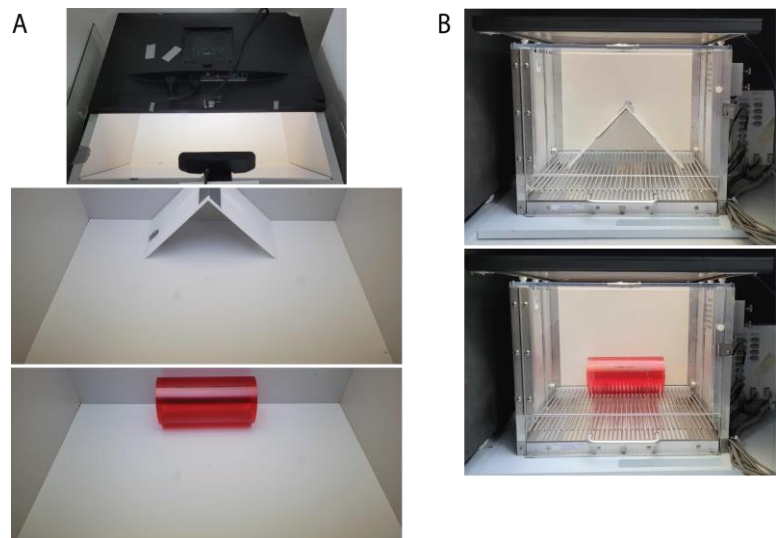


Figure 2.1 Visual looming apparatus. (A) Arena for the presentation of visual looming stimuli for experiments which do not require reinstatement. Mouse were supplied with either a triangle shelter when patch cords were required, or a tunnel shelter otherwise. (B) Arena for the presentation of visual looming stimuli for experiments which do require reinstatement (and their controls). Mouse were supplied with either a triangle shelter when patch cords were required, or a tunnel shelter otherwise.

positioned at the centre of the rear wall to provide a shelter. One day prior to experiments, mice were habituated to the arena for 5 min while the monitor displayed a white image. On experimental day, after 5 min of acclimation with a white monitor, the looming stimulus was

triggered when the animals were located in the half of the arena opposite the shelter. The stimulus consisted in black disc appearing in the centre of the monitor at a diameter of 2 degrees of visual angle, expanding to 20 degrees in 250 ms, remaining at that size for 250 ms before disappearing. This was repeated 15 times with 500 ms between repetitions. After 15 presentations, the mice were left in the arena with a white monitor for 1 min before being taken back to their home cage. The arena and the shelter were cleaned with Trigen between animals. Custom Python code using the PsychoPy library (Peirce, 2008) was used to present stimuli and record video of the animals. Time spent freezing and sheltering were scored using DeepLabCut tracking (Mathis et al., 2018). Sheltering was defined as having all body parts (excluding the tail) within the y-axis limits of the shelter, and at least half of all body parts (excluding the tail) contained within the x-axis limits of the shelter. Freezing was defined as periods in which no more than 1 body part was detected as moving a distance of 2mm or greater, for a time period of at least 0.5s. DeepLabCut behavioural scoring was verified by manual scoring in all wild type experiments.

2.2.3 Visual looming habituation

After habituation day and looming test 1 described above, mice were repeatedly exposed to three waves of 15 looming stimulus presentations, separated by 1 min each, or a white screen control for the same length of time on 6 consecutive days in the absence of the shelter. On the following days, mice underwent looming test 2, with the same parameters as test 1, the shelter being present.

2.2.4 Visual looming reinstatement

After completing the habituation paradigm, mice received an additional training day in the visual looming assay without a shelter. After a 5min habituation period, mice were either presented with a train of 15 looming stimuli paired with a co-terminating 0.75mA 2s foot-shock, or just the foot-shock without visual stimulus presentation. Mice were then test 24h later with a shelter.

2.2.5 Mouse vacuum-powered olfactory behaviour assay

The four-field behaviour chamber was designed to replicate the four-field behaviour chamber used by other groups (Root et al., 2014), and inspired by similar experiments performed in *Drosophila* (VET et al., 1983, Faucher et al., 2006, Semmelhack and Wang, 2009).

The chamber was an enclosed four quadrant arena with airflow out from the centre powered by a vacuum (KNF Laboport® mini-pump). Airflow at 120 ml/min was pumped int

o each quadrant via gas-mass flow controllers (Omega). Airflow exited the

chamber via a 20mm outlet in the centre of the floor. The chamber had a 76 cm diameter, a corner arc of 1.9 cm radius and a chamber height of 7.6 cm. The floor and the walls were made from polypropylene and the ceiling was clear, 1cm thick acrylic. Odour was applied by solenoid valves redirecting airflow through 100 ml glass bottles containing 10 ml of diluted odorant. Behaviour was recorded via a webcam mounted above the chamber. Custom software written in BONSAIrx, a reactive programming language (Lopes et al., 2015), controlled valve position, tracked the position of the mouse in real time at a rate of 30 Hz, and synchronised this information for writing to csv. The behaviour was further analysed offline using custom scripts written in python. Mice were placed in the chamber for 10 min experiments and tested only once per day. The first 5 min served as a baseline test for bias within the arena. The following 5 min were used to assess behavioural response to the odour. To control for any spatial bias, animals were always tested with a 5 min period of no odour. Further, the area to which odour was delivered was altered across experiments.

2.2.6 Mouse simple olfactory assay

A box with a sealed lid had two squares of 1cm x 1cm filter paper placed at each end. 20ul of 92mM TMA or 1mM BNZ was placed at each end of the arena. Animals were then placed into the arena, and their behaviour was recorded via a camera directly above the arena. The arena was divided into 3 sections – one at each end representing TMA and BNZ, and a central “neutral” zone. The time spent in each zone was counted and a preference for either TMA or BNZ was determined.

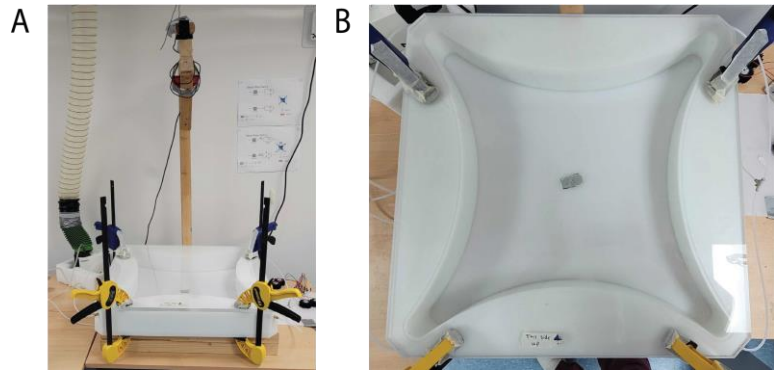


Figure 2.2 *Mouse vacuum-powered olfactory arena. (A) Front view of the olfactory arena. (B) Top view of the olfactory arena.*

2.3.7 *Drosophila* olfactory behavioural assay

The olfactory arena for behavioural assessment was constructed by Dr. Marcia Aranha and is based on the design of Aso et al. (Aso and Rubin, 2016). The arena consists of a circular arena of 11cm in diameter and 3mm height. The structure of the arena allows the creation of four sections (quadrants) of odour in the chamber. The flow rate on each section was maintained at 90ml/min by mass-flow controllers, and air was extracted from the central hole at 400ml/min. Odours were delivered to the arena by passing airflow through tubes containing diluted odours. Odour delivery was controlled by the action of solenoid valves. Behaviour was recorded using a camera (Point Grey FL3-U3 32S2M-CS with an 8mm fixed focal length lens (M0814-MP2, Computar) mounted above the chamber. Movies were acquired in the dark using infrared 850 nm LEDs and a Hoya 49mm R72 infrared filter. Valve position, video recoding, and real-time tracking were operated and synchronised by a custom workflow in Bonsai-rx (Lopes et al., 2015). Flies were starved for 18-20hr prior to experiment by placing in a vial with no food containing tissue paper soaked with 3ml water. The olfactory arena was maintained at a temperature of 22°C and humidity of 30-40%. Groups of 40 starved flies were placed in the arena and allowed to acclimatise for 4-5min before initiating the assay. Air was bubbled through paraffin oil (cat#18512, Sigma-Aldrich) or methyl hexanoate diluted in paraffin oil (MeHex; 5:1000; cat#259942, Sigma-Aldrich).

2.3 Engram labelling, *in vivo* recording and *in vivo* manipulation

2.3.1 TRAP2xAi32 engram labelling

TRAP2xAi32 were placed in the visual looming assay for a 5 minute acclimation period before presentation of any stimuli. Three rounds of 15 looming stimuli were presented at a frequency of 1 loom per second, with a 1 minute rest period between rounds. Immediately following the end of the exposure paradigm, mice were injected with 10mg/ml 4-hydroxytamoxifen (4-OHT) via intraperitoneal injection to transiently induce labelling in cells in a c-Fos-dependent manner.

2.3.2 FLiCRE engram labelling and stimulation

Mice were injected with a viral cocktail including AAV-TRE:mCherry-p2a-eNpHR, AAV-GFP-CaM-uTEVp, and AAV-Nrxn3b-Nav1.6-MKII-f-hLOV1-TEVcs(ENLYFQ/M)-tTA-VP16 in the ventral CA1. This viral cocktail allows expression of the “FLiCRE” labelling paradigm, which will result in expression of the inhibitory halodrhodopsin eNpHR upon coincidence of blue light (473nm) and calcium. For tagging of cells during the reinstatement training session, mice initiated the habituation-reinstatement training paradigm 3 weeks post-surgery. On the reinstatement training session, the blue laser was turned on for a 75s period either beginning 1 minute into the acclimation session, or at the onset of visual stimulus presentation. Light stimulation was delivered via continuous wave at a power of 5mW. The following day, mice underwent 2 rounds of visual stimulus presentation separated by at least 90s. On the first round, a yellow laser (595nm) was turned on to inhibit cells from 30s pre-stimulus until 30s post-stimulus onset. Laser stimulation was performed at 10Hz with 20ms pulses, at a power of 5mW. On the second round, no light was turned on.

For mice tagged during innate loom presentation, mice were presented with looming stimuli before any training commenced and tagged via the same protocol. Mice then commenced the habituation-reinstatement training paradigm, with two rounds of visual looming stimuli, receiving yellow light inhibition for the first round and no inhibition for the second.

2.3.3 TRAP2 engram labelling and stimulation

TRAP2 mice underwent stereotactic surgery to express a Cre-dependent channelrhodopsin-2 in the AON or the CoA. 2 weeks after surgery, mice were exposed to either clean air, TMA-odorised air, or TMT-odorised air for 5min, followed by immediate intraperitoneal injection of 10mg/ml 4-OHT. On the day of stimulation, mice were loaded into the chamber and the optical fibre implant was connected to a 450 nm laser diode fibre light source (Doric LDFLS 450/080). During the habituation session, the mouse was allowed to explore the chamber for 5 min, followed by a 5min stimulation stage. During the light-on epochs, the mouse received blue light stimulation at 20 Hz with a pulse width of 10ms whenever the mouse crossed a boundary at a halfway point in the arena.

2.3.4 TRAP2 engram inhibition

TRAP2 mice underwent stereotactic surgery to express a Cre-dependent inhibitory DREADD in the AON or CoA. 2 weeks after surgery, mice were exposed to either clean air, TMA-odorised air, or TMT-odorised air for 5min, followed by immediate intraperitoneal injection of 10mg/ml 4-OHT. 2 weeks later, mice were injected with either 2 mg/kg of clozapine-N-oxide in 0.9% w/v saline solution, or a saline control, 30 min prior to testing. Mice were then placed in the olfactory arena to assess their behavioural response to TMA. Mice were test again 1 week later with the opposite treatment (saline or CNO).

2.3.5 *In vivo* fiber photometry

A single-site, two-colour fiber photometry system (Doric Lenses) was used for *in vivo* fiber photometry, adapting the protocol of Silva et al. (2021). Recordings were taken of the 405nm isosbestic calcium-independent signal and the 465nm calcium-dependent GCaMP6f fluorescence using a 200µm optic fiber implant. The LED power was constant across animals and experimental sessions (200mW). The presence of visual looming stimuli was synchronised with fluorescence recordings via a transistor-transistor logic pulse from an Arduino module. Animals were habituated to patch chords in a familiar environment (not recorded) and in the looming environment (recorded) for 5min each over the two days prior to the commencement of the habituation-reinstatement training paradigm.

Acquired data files were processed using code adapted from (Martianova et al., 2019). A moving mean was applied to smooth the signal. And baseline correction was performed using the adaptive iteratively reweighted Penalised Least Squares (airPLS) algorithm to remove the slope and low frequency fluctuations in the signal. Each signal was normalised according to the mean and standard deviation. Using non-negative robust linear regression, the normalised 405 signal was fit to the 465 signal, in the format $y=a*x+b$. The parameters of this regression (a,b) were used to calculate new values for the 405 signal fitted to the 465 signal. Finally, the normalised fluorescence dF/F ($z\ dF/F$) is calculated as the normalised 465 signal subtracting the fitted normalised 405 signal.

2.3.6 *In vivo* miniscope calcium imaging

Calcium imaging was performed using a head-mounted calcium imaging system (deep-brain system, Doric Lenses). The onset of odour presentation, and the side of the arena on which the animal was position, was synchronised with fluorescence recordings via a transistor-

transistor logic pulse from an Arduino module, under the control of a custom workflow on Bonsai-rx (Lopes et al., 2015). The acquired sensor data was exported in TIFF format and analysed using Suite2p (Pachitariu et al., 2017). The activity of each cell was normalised to its own mean and standard deviation over the duration of the recording.

2.4 Histological Procedures and Quantification

2.4.1 Immunohistochemistry

Mice were injected intraperitoneally with a terminal dose of Sodium Pentobarbital (50 μ L) and perfused transcardially with PBS followed by 4% paraformaldehyde. Brains were then collected and left in 4% PFA at room temperature for 24 h, after which they were moved to PBS and stored at 4°C. Brains were cut using a vibratome into 50 μ m coronal slices for all experiments except for whole brain imaging experiments, which were cut into 100 μ m coronal slices.

For the immunostaining, slices were first washed in PBT 0.2% three times for 10 minutes. Then, they were incubated in a blocking solution (PBT 0.2% + NGS 10%) for 1 hour at room temperature. They were incubated with primary antibody (in PBT 0.2% + NGS 3%) overnight at 4°C while shaking. On the following day, slices underwent three 10 min washes in PBT-0.2% before incubation with secondary antibody (PBT 0.2% + NGS 3%) for two hours in the dark at room temperature. Then, after three additional 10 min washes in PBT 0.2%, slices were stained with DAPI (1:1000 in PBS) for 15 minutes at room temperature. Slices were finally mounted on superfrost slides using Vectashield-DAPI. The antibodies used for the c-Fos staining were rabbit anti-c-Fos (Synaptic Systems #226008 at 1:1000) and anti-rabbit Alexa-568 (Invitrogen) (1:500). Chr2-EYFP was stained using a primary chicken anti-GFP IgY fraction (Invitrogen) (1:500) and rabbit anti-chicken Alexa-488 IgG (Invitrogen) (1:500).

2.4.2 Image Acquisition and Analysis

Images were taken on a laser confocal microscope (Leica SP8 gated STED) with a 20x magnification unless otherwise stated. At least 4 images were taken for each region of interest per mouse. For each region of interest, density of cells was estimated by sampling cell density in the region of interest to calculate the number of DAPI cells per μ m². The total number of

cells in a region was calculated by multiplying this value by the total area of the region counted. The number of c-Fos positive cells was manually counted using ImageJ.

2.5 Statistical Analysis

2.5.1 Software and packages

Statistical analysis was performed using the pingouin and scipy.stats packages on Python. Data was tested for normality using the Shapiro-Wilk test. An unpaired Student's T-test was used to compare means of two independent groups that assume equal variance between groups, while the paired test was used for dependent groups. For data with more than two groups, an ANOVA was performed to determine if there was a significant difference. If so, and the data was normally distributed, post-hoc analyses were performed for pairwise comparisons. For data with multiple comparisons from the same subjects, False discovery rate correction via the Benjamini-Hochberg method was performed to calculate adjusted q values.

Statistical summaries for all experimental paradigms used in this thesis are provided below. The relevant statistical tests and N numbers for each paradigm are also reported in the legend of each figure.

2.5.2 Statistical Summaries

Figure 3.1.1														
Paradigm	N	Panel	Statistical summary											
Shelter vs. no shelter	Shelter n=9 No shelter n=8	3.1.1D	Paired t test	Group	N	Behaviour	p value (GPP)							
				No shelter	8 Freezing	0.00020304								
				Shelter	9 Freezing	0.00020303								
				No shelter	8 Sheltering	NaN - no sheltering occurs								
				Shelter	9 Sheltering	1.63E-07								
3.1.1F				No shelter	8 Top speed	0.2225086402914929								
				Shelter	9 Top speed	0.017430551237128538								
				No shelter vs. shelter	8 vs. 9	Latency to top speed	0.0810567361							
Figure 3.1.2														
Paradigm	Groups	Panel	Statistical summary											
No extinction	n = 8	3.1.2B	Repeated measures ANOVA	No extinction	Group	Behaviour	Source	p-unc	p-GG-corr					
					Freezing	Day	0.967112854	0.967112854						
					stage	5.47E-05	5.47E-05							
					Day * stage	0.89935451	0.89935451							
					Sheltering	Day	0.760833365	0.760833365						
					stage	6.88E-06	6.88E-06							
			Post-hoc Paired t-test	No extinction	Group	Behaviour	Contrast	Day	A	B	Paired	Parametric	p-unc	
					Freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.967112854		
					Day * stage	Test 1	Hab	Loom	TRUE	TRUE	5.47E-05			
					Day * stage	Test 2	Hab	Loom	TRUE	TRUE	5.44E-05			
					Sheltering	Day	-	Test 1	Test 2	TRUE	TRUE	0.760833365		
					Day * stage	Test 1	Hab	Loom	TRUE	TRUE	6.88E-06			
Extinction	n = 14	3.1.2C	Repeated measures ANOVA	Extinction	Group	Behaviour	Source	p-unc	p-GG-corr					
					Freezing	Day	0.000594793	0.000594793						
					stage	0.000437728	0.000437728							
					Day * stage	0.000835883	0.000835883							
					Sheltering	Day	0.001209707	0.001209707						
					stage	0.000367704	0.000367704							
			Post-hoc Paired t-test	Extinction	Group	Behaviour	Contrast	Day	A	B	Paired	Parametric	p-unc	
					Freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.000594793		
					Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000437728			
					Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.000135353			
					Sheltering	Day	-	Test 1	Test 2	TRUE	TRUE	0.001209707		
					Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000367704			
Post-extinction renewal Same context vs novel context	Ctrl (same context) n = 7 Novel context n = 7	3.1.2G	Repeated measures ANOVA	Control	Group	Behaviour	Source	p-unc	p-GG-corr					
					Freezing	stage	0.000133771	0.000133771						
					test	0.000367574	0.000367574							
					test * stage	0.000025707	0.000126023							
					Sheltering	stage	0.000133771	0.000133771						
					test	0.000367574	0.000367574							
			Renewal	Freezing	test	0.000133771	0.000804411							
				stage	0.000278014326513	0.000278014326513								
				test * stage	0.00454699957124186	0.009336266								
				Sheltering	test	0.000133771	0.000804411							
				stage	0.000278014326513	0.000278014326513								
				test * stage	0.00454699957124186	0.009336266								
Tukey's post-hoc comparison	Control	Freezing	Group	Behaviour	Contrast	test	A	B	Paired	Parametric	p-unc			
			test	-	Test 1	Test 2	TRUE	TRUE	0.000679451					
			test	-	Test 1	Test 3	TRUE	TRUE	0.001288036					
			test	-	Test 2	Test 3	TRUE	TRUE	0.3862531146616503					
			stage	-	Post-loom	Pre-loom	TRUE	TRUE	0.000167574					
			test * stage	Test 1	Post-loom	Pre-loom	TRUE	TRUE	0.004592255					
		Renewal	test * stage	Test 2	Post-loom	Pre-loom	TRUE	TRUE	0.094132787					
			test * stage	Test 3	Post-loom	Pre-loom	TRUE	TRUE	0.60364505101363					
			test	-	Test 1	Test 2	TRUE	TRUE	0.0196555717999645					
			test	-	Test 1	Test 3	TRUE	TRUE	0.000404069					
			test	-	Test 2	Test 3	TRUE	TRUE	0.18239226069584716					
			stage	-	Post-loom	Pre-loom	TRUE	TRUE	0.03027677801426496					
Tukey's post-hoc comparison	Sheltering	Freezing	Group	Behaviour	Contrast	test	A	B	Paired	Parametric	p-unc			
			test	-	Test 1	Test 2	TRUE	TRUE	0.000403998					
			test	-	Test 1	Test 3	TRUE	TRUE	0.10304617317954051					
			test	-	Test 2	Test 3	TRUE	TRUE	0.094132787					
			stage	-	Post-loom	Pre-loom	TRUE	TRUE	0.01523948066245484					
			test * stage	Test 1	Post-loom	Pre-loom	TRUE	TRUE	0.00020709					
		Renewal	test * stage	Test 2	Post-loom	Pre-loom	TRUE	TRUE	0.00616723					
			test * stage	Test 3	Post-loom	Pre-loom	TRUE	TRUE	0.6871048222230481					
			stage	-	Post-loom	Pre-loom	TRUE	TRUE	0.004493984					
			test * stage	Test 1	Post-loom	Pre-loom	TRUE	TRUE	0.00020709					
			test * stage	Test 2	Post-loom	Pre-loom	TRUE	TRUE	0.2973597560666319					
			test * stage	Test 3	Post-loom	Pre-loom	TRUE	TRUE	0.4647431763464115					
Tukey's post-hoc comparison	Sheltering	Freezing	Group	Behaviour	Contrast	test	A	B	Paired	Parametric	p-unc			
			test	-	Test 1	Test 2	TRUE	TRUE	0.16460558046629113					
			test	-	Test 1	Test 3	TRUE	TRUE	0.008190299					
			test	-	Test 2	Test 3	TRUE	TRUE	0.1267816873903435					
			stage	-	Post-loom	Pre-loom	TRUE	TRUE	0.00004758					
			test * stage	Test 1	Post-loom	Pre-loom	TRUE	TRUE	0.00782161					
		Renewal	test * stage	Test 2	Post-loom	Pre-loom	TRUE	TRUE	0.1573830297454827					
			test * stage	Test 3	Post-loom	Pre-loom	TRUE	TRUE	0.557688605					
			Freezing	test	2.521001196790564e-05									
			Sheltering	test	2.55029126363608e-05									
			Group	Behaviour	Source	p-unc								
			Freezing	test	2.521001196790564e-05									
Post-extinction 3 week test	n = 7	3.1.2I	Repeated measures ANOVA	No extinction	Group	Behaviour	Source	p-unc	p-GG-corr					
					Freezing	Day	0.000594793	0.000594793						
					stage	0.000437728	0.000437728							
					Day * stage	0.000835883	0.000835883							
					Sheltering	Day	0.001209707	0.001209707						
					stage	0.000367704	0.000367704							
			Post-hoc Paired t-test	No extinction	Group	Behaviour	Contrast	Day	A	B	Paired	Parametric	p-unc	
					Freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.000594793		
					Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000437728			
					Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.000135353			
					Sheltering	Day	-	Test 1	Test 2	TRUE	TRUE	0.001209707		
					Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000367704			

Figure 3.1.3

Paradigm	Groups	Panel	Statistical summary												
Reinstatement "Loom only"	n = 9	3.1.3C	eated measures ANO	Group	Behaviour	Source	p-unc								
				Loom only	freezing	Day	0.008373486								
						stage	0.00583578								
					sheltering	Day * stage	0.003265095								
						Day	1.69E-07								
						stage	0.000368281								
						Day * stage	4.39E-05								
			Paired t-test	Group	Behaviour	Contrast	Day	A	B	Paired	Parametric	p-unc			
				Loom only	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.007642784			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.044002242			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.582592397			
						stage	-	Hab	Loom	TRUE	TRUE	0.00583578			
						Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.002946847			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.247110662			
					sheltering	Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.841812804			
						Day	-	Test 1	Test 2	TRUE	TRUE	1.09E-05			
						Day	-	Test 1	Test 3	TRUE	TRUE	9.21E-05			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.762777283			
						stage	-	Hab	Loom	TRUE	TRUE	0.000368281			
						Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000800653			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.233465538			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.013515795			
						eated measures ANO	Shock only	freezing	Day	0.000191972					
									stage	0.000133815					
								sheltering	Day * stage	0.000101113					
									Day	0.005128279					
				stage	0.073885801										
Day * stage	1.67E-05														
Paired t-test	Shock only	freezing		Day	-		Test 1	Test 2	TRUE	TRUE	0.002003293				
				Day	-		Test 1	Test 3	TRUE	TRUE	0.56496045				
				Day	-		Test 2	Test 3	TRUE	TRUE	0.000399513				
				stage	-	Hab	Loom	TRUE	TRUE	0.000133815					
				Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000237513					
				Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.883202238					
		sheltering	Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.872506833						
			Day	-	Test 1	Test 2	TRUE	TRUE	0.008573097						
			Day	-	Test 1	Test 3	TRUE	TRUE	0.03121385						
			Day	-	Test 2	Test 3	TRUE	TRUE	0.629229075						
			stage	-	Hab	Loom	TRUE	TRUE	0.073885801						
			Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000293601						
			Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.207899103						
			Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.092454249						
			Repeated measures ANOVA	Loom + shock	freezing	Day	2.88E-05								
						stage	0.002563859								
					sheltering	Day * stage	0.015944905								
						Day	0.000194912								
stage	0.00481064														
Day * stage	0.008395561														
Post-hoc Paired t-test	Loom + shock	freezing		Day	-	Test 1	Test 2	TRUE	TRUE	0.002772623					
				Day	-	Test 1	Test 3	TRUE	TRUE	0.005945004					
				Day	-	Test 2	Test 3	TRUE	TRUE	0.000800727					
				stage	-	Hab	Loom	TRUE	TRUE	0.002563859					
		sheltering		Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.006236161					
				Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.777704276					
				Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.011267876					
				Day	-	Test 1	Test 2	TRUE	TRUE	0.000621686					
Reinstatement "Loom + shock"	n = 10	3.1.3G	Post-hoc Paired t-test	Loom + shock	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002772623			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.005945004			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000800727			
						stage	-	Hab	Loom	TRUE	TRUE	0.002563859			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.006236161			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.777704276			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.011267876			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.000621686			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.01285209			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.096593839			
Reinstatement "Loom only"	n = 12	3.1.3E	Paired t-test	Shock only	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002003293			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.56496045			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000399513			
						stage	-	Hab	Loom	TRUE	TRUE	0.000133815			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000237513			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.883202238			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.872506833			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.008573097			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.03121385			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.629229075			
Repeated measures ANOVA	n = 10	3.1.3G	Post-hoc Paired t-test	Loom + shock	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002772623			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.005945004			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000800727			
						stage	-	Hab	Loom	TRUE	TRUE	0.002563859			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.006236161			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.777704276			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.011267876			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.000621686			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.01285209			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.096593839			
Reinstatement "Loom + shock"	n = 10	3.1.3G	Post-hoc Paired t-test	Loom + shock	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002772623			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.005945004			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000800727			
						stage	-	Hab	Loom	TRUE	TRUE	0.002563859			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.006236161			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.777704276			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.011267876			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.000621686			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.01285209			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.096593839			
Reinstatement "Loom only"	n = 12	3.1.3E	Paired t-test	Shock only	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002003293			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.56496045			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000399513			
						stage	-	Hab	Loom	TRUE	TRUE	0.000133815			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000237513			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.883202238			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.872506833			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.008573097			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.03121385			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.629229075			
Repeated measures ANOVA	n = 10	3.1.3G	Post-hoc Paired t-test	Loom + shock	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002772623			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.005945004			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000800727			
						stage	-	Hab	Loom	TRUE	TRUE	0.002563859			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.006236161			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.777704276			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.011267876			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.000621686			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.01285209			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.096593839			
Reinstatement "Loom + shock"	n = 10	3.1.3G	Post-hoc Paired t-test	Loom + shock	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002772623			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.005945004			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000800727			
						stage	-	Hab	Loom	TRUE	TRUE	0.002563859			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.006236161			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.777704276			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.011267876			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.000621686			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.01285209			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.096593839			
Reinstatement "Loom only"	n = 12	3.1.3E	Paired t-test	Shock only	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002003293			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.56496045			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000399513			
						stage	-	Hab	Loom	TRUE	TRUE	0.000133815			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000237513			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.883202238			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.872506833			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.008573097			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.03121385			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.629229075			
Repeated measures ANOVA	n = 10	3.1.3G	Post-hoc Paired t-test	Loom + shock	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002772623			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.005945004			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000800727			
						stage	-	Hab	Loom	TRUE	TRUE	0.002563859			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.006236161			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.777704276			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.011267876			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.000621686			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.01285209			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.096593839			
Reinstatement "Loom + shock"	n = 10	3.1.3G	Post-hoc Paired t-test	Loom + shock	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002772623			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.005945004			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000800727			
						stage	-	Hab	Loom	TRUE	TRUE	0.002563859			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.006236161			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.777704276			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.011267876			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.000621686			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.01285209			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.096593839			
Reinstatement "Loom only"	n = 12	3.1.3E	Paired t-test	Shock only	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002003293			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.56496045			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000399513			
						stage	-	Hab	Loom	TRUE	TRUE	0.000133815			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.000237513			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.883202238			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.872506833			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.008573097			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.03121385			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.629229075			
Repeated measures ANOVA	n = 10	3.1.3G	Post-hoc Paired t-test	Loom + shock	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002772623			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.005945004			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000800727			
						stage	-	Hab	Loom	TRUE	TRUE	0.002563859			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.006236161			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.777704276			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.011267876			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.000621686			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.01285209			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.096593839			
Reinstatement "Loom + shock"	n = 10	3.1.3G	Post-hoc Paired t-test	Loom + shock	freezing	Day	-	Test 1	Test 2	TRUE	TRUE	0.002772623			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.005945004			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.000800727			
						stage	-	Hab	Loom	TRUE	TRUE	0.002563859			
					sheltering	Day * stage	Test 1	Hab	Loom	TRUE	TRUE	0.006236161			
						Day * stage	Test 2	Hab	Loom	TRUE	TRUE	0.777704276			
						Day * stage	Test 3	Hab	Loom	TRUE	TRUE	0.011267876			
						Day	-	Test 1	Test 2	TRUE	TRUE	0.000621686			
						Day	-	Test 1	Test 3	TRUE	TRUE	0.01285209			
						Day	-	Test 2	Test 3	TRUE	TRUE	0.096593839			
Reinstatement "Loom only"	n = 12	3.1.3E	Paired t-test	Shock only	freezing	Day	-	Test 1	Test 2						

Figure 3.1.4

Paradigm	Groups	Panel	Statistical summary																			
SC Fiber photometry Extinction-reinstatement ("loom + shock") x freezing	Test 1 n = 6 Test 2 n = 7 Test 3 n = 8	3.1.4E	Logistic regression - free	Day	Stage	Model	Method	converged	Covariance Type	No. Observations	Df Residuals	Df Model	LLR p-value	P value								
				Test 1	Pre-loom	Logit	MLE	True	nonrobust	5398	5396	1	0.268	0.269422918217307								
				Test 1	During-loom	Logit	MLE	True	nonrobust	5402	5400	1	1.919e-27	5.882458113637909e-25								
				Test 2	Pre-loom	Logit	MLE	True	nonrobust	6297	6295	1	0.091e-06	2.7500433801055693e-06								
				Test 2	During-loom	Logit	MLE	True	nonrobust	6302	6300	1	1.675e-11	7.678335236597451e-11								
				Test 3	Pre-loom	Logit	MLE	True	nonrobust	7199	7197	1	1.404e-94	4.1029465297969904e-80								
		Test 3	During-loom	Logit	MLE	True	nonrobust	7204	7202	1	1.584e-118	1.8033566914020652e-104										
SC Fiber photometry Extinction-reinstatement ("loom + shock") x speed		3.1.4F	Linear regression - speed	Day	Stage	Slope	Intercept	R value	P value	Standard error												
				Test 1	Pre-loom	-0.002102363	0.06800793	-0.010860935	0.42498458377540715	0.00263499												
				Test 1	During-loom	0.006198679	0.030634675477651075	0.13908438743044507	0.615346160786267e-2	0.000600596												
				Test 2	Pre-loom	0.017702138	0.066045581	0.094557021	5.5279260651416805e-	0.002349004												
				Test 2	During-loom	0.021360081	0.073876412	0.13390791831571183	1.3140062994041382e-	0.001991578												
				Test 3	Pre-loom	0.042901297881931026	0.10410702680547672	0.26855518084197316	5.6279382353854037e-	0.001813872												
Test 3		During-loom	0.0251128	0.039288158	0.19653170519573915	1.1907167257676379e-	0.001476327															
SC Fiber photometry Extinction-reinstatement ("loom + shock") Area under curve		3.1.4G	Repeated measures ANOVA	Group	Brain region	Source	p-unc															
								Ext-Reinst	SC	Day	0.1176237114396693											
										Stage	0.036200921901578434											
										Day * Stage	0.015904218											
			Post-hoc Paired t-test	Group	Brain region	Contrast	Day	A	B	Paired	TRUE	Parametric	p-unc									
														Day	-	Test 1	Test 2	TRUE	TRUE	0.1297552028826691		
														Day	-	Test 1	Test 3	TRUE	TRUE	0.1814766742369408		
														Day	-	Test 2	Test 3	TRUE	TRUE	0.9900995042727259		
														Stage	-	Post-loom	Pre-loom	TRUE	TRUE	0.036200922		
														Day * Stage	Test 1	Post-loom	Pre-loom	TRUE	TRUE	0.024920059084087055		
														Day * Stage	Test 2	Post-loom	Pre-loom	TRUE	TRUE	0.13466375582340545		
														Day * Stage	Test 3	Post-loom	Pre-loom	TRUE	TRUE	0.44580762790066597		
SC Fiber photometry Extinction-reinstatement ("loom + shock") Area under curve		3.1.4H	Repeated measures ANOVA	Group	Brain region	Source	p-unc															
								Ext-Reinst	SC	Day	0.034846508107436204											
										Stage	0.034774704											
										Day * Stage	0.8171988053670012											
			Post-hoc Paired t-test	Group	Brain region	Contrast	Day	A	B	Paired	TRUE	Parametric	p-unc									
														Day	-	Test 1	Test 2	TRUE	TRUE	0.2060624550643965		
														Day	-	Test 1	Test 3	TRUE	TRUE	0.026872572372312137		
														Day	-	Test 2	Test 3	TRUE	TRUE	0.16213304643933493		
Stage		-	Post-loom	Pre-loom	TRUE	TRUE	0.034774704															
Day * Stage		Test 1	Post-loom	Pre-loom	TRUE	TRUE	0.030751863															
Day * Stage		Test 2	Post-loom	Pre-loom	TRUE	TRUE	0.1949672029085395															
Day * Stage	Test 3	Post-loom	Pre-loom	TRUE	TRUE	0.2830050476481987																

Figure 3.1.5

Paradigm	Groups	Panel	Statistical summary												
SC Fiber photometry Extinction-reinstatement ("shock only") x freezing	Test 1 n = 8 Test 2 n = 8 Test 3 n = 8	3.1.5E	Logistic regression freezing	Day	Stage	Model	Method	converged	Covariance Type	No. Observations	Df Residuals	Df Model	LLR p-value	P value	
				Test 1	Pre-loom	Logit	MLE	True	nonrobust	7176	7174	1	0.1091	0.10741279476660381	
				Test 1	During-loom	Logit	MLE	True	nonrobust	7201	7199	1	1.082e-17	1.3893649945947876e-15	
				Test 2	Pre-loom	Logit	MLE	True	nonrobust	7125	7123	1	5.409e-05	3.916040774787719e-05	
				Test 2	During-loom	Logit	MLE	True	nonrobust	7202	7200	1	0.4683	0.4666882803019444	
				Test 3	Pre-loom	Logit	MLE	True	nonrobust	6274	6272	1	2.019e-39	5.493788534403359e-36	
			Test 3	During-loom	Logit	MLE	True	nonrobust	6302	6300	1	0.9981	0.9981581521430841		
SC Fiber photometry Extinction-reinstatement ("shock only") x speed		3.1.5F	Linear regression speed	Day	Stage	Slope	Intercept	R value	P value	Standard error					
				Test 1	Pre-loom	0.009512401	0.07925918	0.053258160361842416	6.362093360768877e-0	0.002105748					
				Test 1	During-loom	0.006598948	0.051578425	0.070068612	2.645360815045876e-0	0.001107252					
				Test 2	Pre-loom	-0.040516968	0.088783601	-0.107606907	8.396845576027225e-2	0.004435435					
				Test 2	During-loom	0.016076553	0.062926457	0.0659931339	2.139080037745942e-0	0.002867404					
				Test 3	Pre-loom	0.030791065799842084	0.082225021	0.14238299332425522	8.956018127848934e-3	0.002702814					
			Test 3	During-loom	0.00172102	0.091768199	0.010457275326549914	0.40653298686161147	0.002073354						
SC Fiber photometry Extinction-reinstatement ("shock only") Area under curve		Test 1 n = 8 Test 2 n = 8 Test 3 n = 8	3.1.5G	Repeated measures ANOVA	Group	Brain region	Source	p-unc							
							Day	0.12798363730451914							
							Stage	0.0682028							
							Day * Stage	0.076219559							
					Group	Brain region	Contrast	Day	A	B	Paired	Parametric	p-unc		
							Day	-	Test 1	Test 2	TRUE	TRUE	0.12477745397400264		
				Post-hoc Paired t-test			Day	-	Test 1	Test 3	TRUE	TRUE	0.081060501		
							Day	-	Test 2	Test 3	TRUE	TRUE	0.7925421229274338		
							Stage	-	Post-loom	Pre-loom	TRUE	TRUE	0.0682028		
							Day * Stage	Test 1	Post-loom	Pre-loom	TRUE	TRUE	0.082220005		
							Day * Stage	Test 2	Post-loom	Pre-loom	TRUE	TRUE	0.5223504102110655		
							Day * Stage	Test 3	Post-loom	Pre-loom	TRUE	TRUE	0.25135548898536886		
SC Fiber photometry Extinction-reinstatement ("shock only") Area under curve		Test 1 n = 8 Test 2 n = 8 Test 3 n = 8	3.1.5H	Repeated measures	Group	Brain region	Source	p-unc							
							Day	0.6763816352289567							
							Stage	0.094959021							
							Day * Stage	0.23051061462447045							
					Group	Brain region	Contrast	Day	A	B	Paired	Parametric	p-unc		
							Day	-	Test 1	Test 2	TRUE	TRUE			
				Post-hocPaired t-test			Day	-	Test 1	Test 3	TRUE	TRUE			
							Day	-	Test 2	Test 3	TRUE	TRUE			
							Stage	-	Post-loom	Pre-loom	TRUE	TRUE			
							Day * Stage	Test 1	Post-loom	Pre-loom	TRUE	TRUE			
						Day * Stage	Test 2	Post-loom	Pre-loom	TRUE	TRUE				
						Day * Stage	Test3	Post-loom	Pre-loom	TRUE	TRUE				

Figure 3.1.6																									
Paradigm	Groups	Panel	Statistical summary																						
PAG Fiber photometry Extinction- reinstatement ("loom + shock") x freezing	Test 1 n = 7 Test 2 n = 7 Test 3 n = 7	3.1.6E	Logistic regression - free	Day	Stage	Model	Method	converged	Covariance Type	No. Observations	Df Residuals	Df Model	LLR p-value	P value											
				Test 1	Pre-loom	Logit	MLE	True	nonrobust	6277	6275	1	1	0.1701	0.17005641043403297										
				Test 1	During-loom	Logit	MLE	True	nonrobust	6297	6295	1	1	1.169e-28	1.3335589843270606e-27										
				Test 2	Pre-loom	Logit	MLE	True	nonrobust	6297	6295	1	1	0.1009	0.10418721544230111										
				Test 2	During-loom	Logit	MLE	True	nonrobust	6283	6281	1	1	0.4459	0.4460128059221591										
				Test 3	Pre-loom	Logit	MLE	True	nonrobust	6299	6297	1	2	1.41e-06	2.3571539398350317e-06										
Test 3		During-loom	Logit	MLE	True	nonrobust	6303	6301	1	3	2.99e-51	2.2524569277928276e-48													
PAG Fiber photometry Extinction- reinstatement ("loom + shock") x speed		3.1.6F	Linear regression - spe	Day	Stage	Slope	Intercept	R value	P value	Standard error															
				Test 1	Pre-loom	-0.022668434	0.079018735	-0.088333566	2.370219741652797e-1	0.003226918															
				Test 1	During-loom	-0.000223751	0.047850996603907466	-0.001138158	0.9280495722905718	0.002477792															
				Test 2	Pre-loom	0.003971997	0.053486110752752634	0.019787219	0.11640818453135998	0.002529538															
				Test 2	During-loom	0.02767085	0.062326792	0.092209324	2.422023778613419e-1	0.003770327															
				Test 3	Pre-loom	0.022651241916001173	0.082426045	0.065662901	1.8281554470019868e-1	0.004337773															
Test 3		During-loom	0.030041270863716066	0.054242155911813174	0.11409651097114482	1.0258586004771172e-1	0.003295306																		
PAG Fiber photometry Extinction- reinstatement ("loom + shock") Area under curve		Test 1 n = 7 Test 2 n = 7 Test 3 n = 7	3.1.6G	Repeated measures ANOVA	Group	Brain region	Source	p-unc	p-unc	p-unc	p-unc	p-unc	p-unc	p-unc	p-unc										
																Ext-Reinst	PAG	Day	0.056260949						
																		Stage	0.015941500697818144						
																		Day * Stage	0.015242548454071596						
			Post-hoc Paired t-test	Ext-Reinst	PAG	Day	Test 1	A	B	Paired	Parametric	p-unc	0.089578513												
						Day	-	Test 1	Test 3	TRUE	TRUE	0.028788051													
						Day	-	Test 2	Test 3	TRUE	TRUE	0.979386311													
						Stage	-	Post-loom	Pre-loom	TRUE	TRUE	0.015941500697818224													
						Day * Stage	Test 1	Post-loom	Pre-loom	TRUE	TRUE	0.028159968368124778													
						Day * Stage	Test 2	Post-loom	Pre-loom	TRUE	TRUE	0.7177924427210977													
						Day * Stage	Test 3	Post-loom	Pre-loom	TRUE	TRUE	0.2937535943963293													
						Day	-	Post-loom	Pre-loom	TRUE	TRUE														
PAG Fiber photometry Extinction- reinstatement ("loom + shock") Area under curve		3.1.6H 2 SECONDS VRESION	Repeated measures	Group	Brain region	Source	p-unc	p-unc	p-unc	p-unc	p-unc	p-unc	p-unc	p-unc	p-unc										
																Ext-Reinst	PAG	Day	0.5971501114658254						
																		Stage	0.1599193273211252						
																		Day * Stage	0.003024998						
			Post-hoc Paired t-test	Ext-Reinst	PAG	Day	Test 1	A	B	Paired	Parametric	p-unc	0.7489892206976565												
						Day	-	Test 1	Test 3	TRUE	TRUE	0.26727833372584453													
						Day	-	Test 2	Test 3	TRUE	TRUE	0.43396145103143047													
						Stage	-	Post-loom	Pre-loom	TRUE	TRUE	0.15991932732112496													
						Day * Stage	Test 1	Post-loom	Pre-loom	TRUE	TRUE	0.017006713													
						Day * Stage	Test 2	Post-loom	Pre-loom	TRUE	TRUE	0.9552904691889352													
						Day * Stage	Test 3	Post-loom	Pre-loom	TRUE	TRUE	0.049173404													
						Day	-	Post-loom	Pre-loom	TRUE	TRUE														

Figure 3.1.7

Paradigm	Groups	Panel	Statistical summary
Re-exposure vs no re-exposure EYFP+/DAPI		3.1.7D	Independent t test False Discovery rate correction Benjamini-Hochberg method
			Regions
			SCs
			SCi
			SCd
			PAGdm
			PAGdl
			PAGl
			PAGvl
			dDG
			dCA1
			dCA3
			vDG
Re-exposure vs no re-exposure c-Fos+/DAPI	Re-exposure n = 6 No re-exposure n = 6	3.1.7E	Independent t test False Discovery rate correction Benjamini-Hochberg method
			Regions
			SCs
			SCi
			SCd
			PAGdm
			PAGdl
			PAGl
			PAGvl
			dDG
			dCA1
			dCA3
			vDG
Re-exposure vs no re-exposure c-Fos+EYFP+/EFYP+		3.1.7F	Independent t test False Discovery rate correction Benjamini-Hochberg method
			Regions
			SCs
			SCi
			SCd
			PAGdm
			PAGdl
			PAGl
			PAGvl
			dDG
			dCA1
			dCA3
			vDG
Extinction vs innate EYFP+/DAPI		3.1.7H	Independent t test False Discovery rate correction Benjamini-Hochberg method
			Regions
			SCs
			SCi
			SCd
			PAGdm
			PAGdl
			PAGl
			PAGvl
			dDG
			dCA1
			dCA3
			vDG
Extinction vs innate c-Fos+/DAPI	Extinction n = 7 No extinction n = 8	3.1.7I	Independent t test False Discovery rate correction Benjamini-Hochberg method
			Regions
			SCs
			SCi
			SCd
			PAGdm
			PAGdl
			PAGl
			PAGvl
			dDG
			dCA1
			dCA3
			vDG
Extinction vs innate c-Fos+EYFP+/EFYP+		3.1.7J	Independent t test False Discovery rate correction Benjamini-Hochberg method
			Regions
			SCs
			SCi
			SCd
			PAGdm
			PAGdl
			PAGl
			PAGvl
			dDG
			dCA1
			dCA3
			vDG

Reinstatement vs innate EYFP+/DAPI		3.1.7L	Independent t test False Discovery rate correction Benjamini- Hochberg method	Regions	p value	q value
				SCs	0.7278475321665046	0.9220627062379426
				SCi	0.6573578535121726	0.9220627062379426
				SCd	0.8659787621503706	0.9381436589962348
				PAGdm	0.4910466420940053	0.7979507934027585
				PAGdl	0.4622931742949245	0.7979507934027585
				PAGl	0.2209150729808163	0.5743791897501224
				PAGvl	0.18569153539361277	0.5743791897501224
				dDG	0.9668546130937996	0.9668546130937996
				dCA1	0.7802069052782591	0.9220627062379426
				dCA3	0.17985872164309344	0.5743791897501224
				vDG	0.081016116	0.5743791897501224
				vCA1	0.11120210585903322	0.5743791897501224
				vCA3	0.3288001419791533	0.7124003076214988
Reinstatement vs innate c-Fos+/DAPI	Reinstatement n = 11 No training n = 8	3.1.7M	Independent t test False Discovery rate correction Benjamini- Hochberg method	Regions	p value	q value
				SCs	0.5952691473224321	0.5955307325420469
				SCi	0.35827055182228773	0.5486019586079843
				SCd	0.5955307325420469	0.5955307325420469
				PAGdm	0.21028305920943316	0.5486019586079843
				PAGdl	0.34948739764466996	0.5486019586079843
				PAGl	0.14380848728412512	0.5486019586079843
				PAGvl	0.5824392621820726	0.5955307325420469
				dDG	0.42200150662152636	0.5486019586079843
				dCA1	0.15311808111287856	0.5486019586079843
				dCA3	0.3851928955336734	0.5486019586079843
				vDG	0.3317996896872233	0.5486019586079843
				vCA1	0.19071410260570698	0.5486019586079843
				vCA3	0.32916314744434483	0.5486019586079843
Reinstatement vs innate c-Fos+EYFP+/EFYP+		3.1.7N	Independent t test False Discovery rate correction Benjamini- Hochberg method	Regions	p value	q value
				SCs	0.029655560117093548	0.077104456
				SCi	0.5410407681546296	0.7033529986010185
				SCd	0.4823469416593801	0.6967233601746601
				PAGdm	0.003958401	0.031131214845479552
				PAGdl	0.017822328986661373	0.057922569
				PAGl	0.8910629917873062	0.8910629917873062
				PAGvl	0.8260898186115868	0.8910629917873062
				dDG	0.010819607135972138	0.046884964
				dCA1	0.094602749	0.19707800848571091
				dCA3	0.21595489328972545	0.35092670159580386
				vDG	0.6646674787957823	0.7855161113041064
				vCA1	0.004789418	0.031131214845479552
				vCA3	0.10611892764615202	0.19707800848571091

Figure 3.1.10													
Paradigm	Groups	Panel	Statistical summary										
FLICRE Reinst Pre-loom label	n = 6	3.1.10B	Repeated measures ANOVA	Group	Behaviour	Source	p-unc						
				Pre-loom label	Day	7.192268483815474e-05							
					stage	0.001287594							
					freezing	0.005878219							
			Post-hoc Paired t-test	Group	Behaviour	Contrast	Day	A	B	Paired	Parametric	p-unc	
				Pre-loom label	Day	-	-	Test 1	Test 2	TRUE	TRUE	0.017614846	
					Day	-	-	Test 1	Test 3A	TRUE	TRUE	0.2734725642992363	
					Day	-	-	Test 1	Test 3B	TRUE	TRUE	0.045341393	
					Day	-	-	Test 2	Test 3A	TRUE	TRUE	0.00533366	
					Day	-	-	Test 2	Test 3B	TRUE	TRUE	0.000981815	
					Day	-	-	Test 3A	Test 3B	TRUE	TRUE	0.15148521600430853	
					stage	-	-	Hab	Loom	TRUE	TRUE	0.001287594	
					Day * stage	Test 1	-	Hab	Loom	TRUE	TRUE	0.008719466	
					Day * stage	Test 2	-	Hab	Loom	TRUE	TRUE	0.13744093126543616	
					Day * stage	Test 3A	-	Hab	Loom	TRUE	TRUE	0.00590659	
					Day * stage	Test 3B	-	Hab	Loom	TRUE	TRUE	0.00355757	
				freezing									
				Group	Behaviour	Source	p-unc						
				Reinst Loom label	Day	0.000361276							
				freezing	Day * stage	0.000234455							
				Day * stage	0.001036142								
FLICRE Reinst Loom label	n = 6	3.1.10C	Repeated measures ANOVA	Group	Behaviour	Source	p-unc						
				Reinst Loom label	Day	0.000361276							
					stage	0.000234455							
					freezing	0.001036142							
			Post-hoc Paired t-test	Group	Behaviour	Contrast	Day	A	B	Paired	Parametric	p-unc	
				Reinst Loom label	Day	-	-	Test 1	Test 2	TRUE	TRUE	0.022605063	
					Day	-	-	Test 1	Test 3A	TRUE	TRUE	0.4147860976540058	
					Day	-	-	Test 1	Test 3B	TRUE	TRUE	0.073669665	
					Day	-	-	Test 2	Test 3A	TRUE	TRUE	0.0136713893920647	
					Day	-	-	Test 2	Test 3B	TRUE	TRUE	0.001295082	
					Day	-	-	Test 3A	Test 3B	TRUE	TRUE	0.008736435	
					stage	-	-	Hab	Loom	TRUE	TRUE	0.000234455	
					Day * stage	Test 1	-	Hab	Loom	TRUE	TRUE	0.000216745	
					Day * stage	Test 2	-	Hab	Loom	TRUE	TRUE	0.14331992967699592	
					Day * stage	Test 3A	-	Hab	Loom	TRUE	TRUE	0.9262998427528641	
					Day * stage	Test 3B	-	Hab	Loom	TRUE	TRUE	0.009442845	
				freezing									
				Group	Behaviour	Source	p-unc						
				Innate Loom label	Day	4.7848317816832336e-08							
				stage	0.001038074								
				Day * stage	7.739303105027773e-07								
FLICRE Innate Loom label	n = 6	3.1.10E	Repeated measures ANOVA	Group	Behaviour	Source	p-unc						
				Innate Loom label	Day	4.7848317816832336e-08							
					stage	0.001038074							
					freezing	7.739303105027773e-07							
			Post-hoc Paired t-test	Group	Behaviour	Contrast	Day	A	B	Paired	Parametric	p-unc	
				Innate Loom label	freezing	Day	-	Test 1A	Test 1B	TRUE	TRUE	0.23322005495637857	
						Day	-	Test 1A	Test 2A	TRUE	TRUE	0.006714798	
						Day	-	Test 1A	Test 2B	TRUE	TRUE	0.008999953	
						Day	-	Test 1A	Test 3A	TRUE	TRUE	0.37179134426702887	
						Day	-	Test 1A	Test 3B	TRUE	TRUE	0.017756991	
						Day	-	Test 1B	Test 2A	TRUE	TRUE	0.005622553	
						Day	-	Test 1B	Test 2B	TRUE	TRUE	0.007728357	
						Day	-	Test 1B	Test 3A	TRUE	TRUE	0.2471256363291327	
						Day	-	Test 1B	Test 3B	TRUE	TRUE	0.022718095736254842	
						Day	-	Test 2A	Test 2B	TRUE	TRUE	0.22561197442320247	
						Day	-	Test 2A	Test 3A	TRUE	TRUE	0.093157638	
						Day	-	Test 2A	Test 3B	TRUE	TRUE	0.000285891	
						Day	-	Test 2B	Test 3A	TRUE	TRUE	0.050262758	
						Day	-	Test 2B	Test 3B	TRUE	TRUE	0.000261743	
						Day	-	Test 3A	Test 3B	TRUE	TRUE	0.000554969	
						stage	-	Hab	Loom	TRUE	TRUE	0.001038074	
						Day * stage	Test 1A	Hab	Loom	TRUE	TRUE	0.009670664	
						Day * stage	Test 1B	Hab	Loom	TRUE	TRUE	0.009345021	
						Day * stage	Test 2A	Hab	Loom	TRUE	TRUE	0.3226455406268388	
						Day * stage	Test 2B	Hab	Loom	TRUE	TRUE	0.3064955270597267	
						Day * stage	Test 3A	Hab	Loom	TRUE	TRUE	0.058612419	
						Day * stage	Test 3B	Hab	Loom	TRUE	TRUE	0.000394122	
FLICRE labelling GFP/DAPI		3.1.10F	ANOVA	Source	p-unc								
				Condition	0.28650822523804065								
				A	B	p-tukey							
				Innate Loom label	Reinst Hab Label	0.2615766464477448							
FLICRE labelling mCherry/GFP	Reinst pre-loom label n = 6	3.1.10G	ANOVA	Source	p-unc								
				Condition	0.03373282								
				A	B	p-tukey							
				Innate Loom label	Reinst Hab Label	0.070936424							
FLICRE labelling mCherry/DAPI	Reinst loom label n = 6	3.1.10H	ANOVA	Source	p-unc								
				Condition	0.056224548323591285								
				A	B	p-tukey							
				Innate Loom label	Reinst Hab Label	0.068783755							
	Innate loom label n = 6		ANOVA	Source	p-unc								
				Condition	0.056224548323591285								
				A	B	p-tukey							
				Innate Loom label	Reinst Loom Label	0.11648291337598093							
			ANOVA	Source	p-unc								
				Condition	0.056224548323591285								
				A	B	p-tukey							
				Innate Loom label	Reinst Loom Label	0.9538277341289385							

Figure 4.1.1

Paradigm	Groups	Panel	Statistical summary									
TMA Concentration assay	46mM n = 9 92mM n = 15 184mM n = 9 368mM n = 7	4.1.1C	ANOVA	Source	p-unc							
				Concentration	0.000136184							
				stage	0.48235095928838956							
				Concentration * stage	2.8142272365752042e-05							
			Post-hoc Paired t-test	Contrast	Concentration	A	B	Paired	Parametric	alternative	p-unc	
				Concentration	-		46	92	FALSE	TRUE	two-sided	0.14311339528920608
				Concentration	-		46	184	FALSE	TRUE	two-sided	0.011950487386598792
				Concentration	-		46	368	FALSE	TRUE	two-sided	0.16507377887874397
				Concentration	-		92	184	FALSE	TRUE	two-sided	0.072555148
				Concentration	-		92	368	FALSE	TRUE	two-sided	0.00513786
				Concentration	-		184	368	FALSE	TRUE	two-sided	0.000484901
				stage	-	Hab		Odour	TRUE	TRUE	two-sided	0.5862556988468711
				Concentration * stage	46	Hab		Odour	TRUE	TRUE	two-sided	0.46846941498360517
				Concentration * stage	92	Hab		Odour	TRUE	TRUE	two-sided	0.9133013692122448
				Concentration * stage	184	Hab		Odour	TRUE	TRUE	two-sided	0.030149683
				Concentration * stage	368	Hab		Odour	TRUE	TRUE	two-sided	0.008804989
TMT Concentration assay	7mM n = 5 70mM n = 7 210mM n = 9 350mM n = 8	4.1.1E	ANOVA	Source	p-unc							
				Concentration	0.056450029172053766							
				stage	0.10019077701541364							
				Concentration * stage	0.073722102							
			Post-hoc Paired t-test	Contrast	Concentration	A	B	Paired	Parametric	alternative	p-unc	
				Concentration	-		7	70	FALSE	TRUE	two-sided	0.078874061
				Concentration	-		7	210	FALSE	TRUE	two-sided	0.8399178718151155
				Concentration	-		7	350	FALSE	TRUE	two-sided	0.3809566173430423
				Concentration	-		70	210	FALSE	TRUE	two-sided	0.10652570317190739
				Concentration	-		70	350	FALSE	TRUE	two-sided	0.026876528
				Concentration	-		210	350	FALSE	TRUE	two-sided	0.607017345
				stage	-	Hab		Odour	TRUE	TRUE	two-sided	0.079803023
				Concentration * stage	7	Hab		Odour	TRUE	TRUE	two-sided	0.649455022
				Concentration * stage	70	Hab		Odour	TRUE	TRUE	two-sided	0.23526460117123263
				Concentration * stage	210	Hab		Odour	TRUE	TRUE	two-sided	0.1344678976333205
				Concentration * stage	350	Hab		Odour	TRUE	TRUE	two-sided	0.02195152
Mus musculus and Mus caroli Urinary TMA NMR analysis	male Mus mus n = 8 female Mus mus n = 5 male Mus car n = 6 female Mus car n = 5	4.1.1H	ANOVA	Source	p-unc							
				Species	7.748112864273483e-08							
				Sex	2.8823778140465696e-07							
				Species * Sex	2.2001037249060276e-05							
			Post-hoc Paired t-test	Contrast	Sex	A	B	Paired	Parametric	alternative	p-unc	
				Species	-	Mus car	Mus mus	FALSE	TRUE	two-sided	0.000865071	
Simple odour test Mus mus vs. Mus caroli	Mus mus n = 7 Mus car n = 6	4.1.1K	Student's unpaired t test	Sex	-	F	M	FALSE	TRUE	two-sided	0.000977902	
				Species * Sex	Mus car	F	M	FALSE	TRUE	two-sided	0.013376804486778516	
				Species * Sex	Mus mus	F	M	FALSE	TRUE	two-sided	2.2073158138630236e-05	
				Group	T statistic (GFP)	df (GFP)	p value (GFP)					
				Mus mus vs. Mus car	-5.949447379	11	9.599953030435478e-05					

Figure 4.1.2

Paradigm	Groups	Panel	Statistical summary						
Mus musculus Doxycycline diet Urinary TMA NMR analysis	Mus mus male standard diet n = 8 Mus mus female standard diet n = 5 Mus mus male doxycycline diet n = 5 mus female doxycycline diet n = 3	4.1.2C	ANOVA	Source	p-unc				
				Sex	2.6242828181320835e-05				
				Diet	0.003107052				
				Sex * Diet	1.0072197553702913e-05				
			Post-hoc Paired t-test	Contrast	Sex	A	B	Paired	Parametric
				Sex	-	F	M	FALSE	TRUE
				Diet	-	Dox	Standard	FALSE	TRUE
				Sex * Diet	F	Dox	Standard	FALSE	TRUE
				Sex * Diet	M	Dox	Standard	FALSE	TRUE
Mus musculus Experience-dependent behaviour Males	WT n = 9 Dox whole life n = 15 Dox until weaning n = 7 Single housed n = 10	4.1.2F	ANOVA	Source	p-unc				
				Experiment	1.6570943848594322e-05				
				stage	0.17387256576567817				
				Experiment * stage	0.000499402				
			Post-hoc Student's t-test	Contrast	Experiment	A	B	Paired	Parametric
				Experiment	-	D-R curve	Dox fed	FALSE	TRUE
				Experiment	-	D-R curve	Dox until weaning	FALSE	TRUE
				Experiment	-	D-R curve	Single house	FALSE	TRUE
				Experiment	-	Dox fed	Dox until weaning	FALSE	TRUE
				Experiment	-	Dox fed	Single house	FALSE	TRUE
				Experiment	-	Dox until weaning	Single house	FALSE	TRUE
				stage	-	Hab	Odour	TRUE	TRUE
				Experiment * stage	D-R curve	Hab	Odour	TRUE	TRUE
				Experiment * stage	Dox fed	Hab	Odour	TRUE	TRUE
				Experiment * stage	Dox until weaning	Hab	Odour	TRUE	TRUE
				Experiment * stage	Single house	Hab	Odour	TRUE	TRUE
Mus musculus Experience-dependent behaviour Females	WT n = 30 Dox whole life n = 18 Dox until weaning n = 15	4.1.2G	ANOVA	Source	p-unc				
				Experiment	0.4461864255402156				
				stage	0.12632598859518993				
				Experiment * stage	0.2104479632716461				
			Post-hoc Student's t-test	Contrast	Experiment	A	B	Paired	Parametric
				Experiment	-	D-R curve	Dox fed	FALSE	TRUE
				Experiment	-	D-R curve	Dox until weaning	FALSE	TRUE
				Experiment	-	Dox fed	Dox until weaning	FALSE	TRUE
				stage	-	Hab	Odour	TRUE	TRUE
				Experiment * stage	D-R curve	Hab	Odour	TRUE	TRUE
Mus musculus Females - estrus cycle	Not estrus n = 10 Estrus n = 5	4.1.2H	ANOVA	Source	p-unc				
				Estrus cycle	0.001214777				
				stage	0.2489572299382162				
				Estrus cycle * stage	0.019330233				
			Post-hoc Student's t-test	Contrast	Estrus cycle	A	B	Paired	Parametric
				Estrus cycle	-	Estrus	Not estrus	FALSE	TRUE
				stage	-	Hab	Odour	TRUE	TRUE
				Estrus cycle * stage	Estrus	Hab	Odour	TRUE	TRUE

Figure 4.1.3						
Paradigm	Groups	Panel	Statistical summary			
Looming response during TMA olfaction	H2O n = 3	4.1.3B	Student's paired t test	Behaviour	Group	p value (GFP)
				Freezing	H2O	0.43164331680962076
	TMA	0.070121383				
	TMA n = 3	4.1.3C		Behaviour	Group	p value (GFP)
Sheltering				H2O	0.064119926	
	TMA	0.6729686723138515				
Looming response during TMT olfaction	H2O n = 3	4.1.3E		Behaviour	Group	p value (GFP)
				Freezing	H2O	0.3411040138265118
	TMT	0.5541145047154159				
	TMT n = 3	4.1.3F		Behaviour	Group	p value (GFP)
Sheltering				H2O	0.5003514209218167	
	TMT	0.32312581645916116				

Figure 4.1.4						
Paradigm	Groups	Panel	Statistical summary			
Mus musculus c-Fos expression following olfaction H2O vs. TMA	H2O n = 5 TMA n = 6	4.1.4C	Student's paired t test	Regions	p value (GFP)	q value (GFP)
				AON (avg)	0.001266409	0.002849419
				PIR	1.7753253902566608e-06	1.5977928512309948e-06
				PAA	0.015198588	0.022797882
				CoA	2.2364964360254038e-06	0.000100642
				NLOT	0.054058383594753304	0.069503636
				CA1	0.00123532	0.002849419
				CA2	0.6538868622498912	0.6538868622498912
				CA3	0.002015815	0.003628467
				DG	0.3810393080602861	0.42866922156782183
Mus caroli c-Fos expression following olfaction H2O vs. TMA	H2O n = 3 TMA n = 3	4.1.4D	Student's paired t test	Regions	p value (GFP)	q value (GFP)
				AON	0.036570207	0.12435313544072613
				PIR	0.08290209	0.12435313544072613
				MOB	0.5591719352389627	0.629068427
				CoA	0.024670344	0.12435313544072613
				PAA	0.7401402257907863	0.7401402257907863
				CA3	0.055178619348085704	0.12435313544072613
				CA2	0.077707442	0.12435313544072613
				CA1	0.075330891	0.12435313544072613
				DG	0.3787676526466157	0.48698698197422025

Figure 4.1.7												
Paradigm	Groups	Panel	Statistical summary									
AON engram optogenetics	Clean air labelling n = 6 TMA labelling n = 6 TMT labelling n = 5	4.1.7C	ANOVA	Source	p-unc							
				Treatment	0.8157726986591112							
				stage	0.042357807							
				Treatment * stage	0.18107256274851674							
			Post-hoc Student's t-test	Contrast	stage	A	B	Paired	Parametric	alternative	p-unc	
				stage	-	Hab	Opto	TRUE	TRUE	two-sided	0.047339484675512924	
				Treatment	-	H2O	TMA	FALSE	TRUE	two-sided	0.899659787	
				Treatment	-	H2O	TMT	FALSE	TRUE	two-sided	0.6599438466652952	
				Treatment	-	TMA	TMT	FALSE	TRUE	two-sided	0.6356113930009725	
				stage * Treatment	Hab	H2O	TMA	FALSE	TRUE	two-sided	0.15403165412976974	
				stage * Treatment	Hab	H2O	TMT	FALSE	TRUE	two-sided	0.6168661904764925	
				stage * Treatment	Hab	TMA	TMT	FALSE	TRUE	two-sided	0.656682101	
				stage * Treatment	Opto	H2O	TMA	FALSE	TRUE	two-sided	0.26085423486933895	
				stage * Treatment	Opto	H2O	TMT	FALSE	TRUE	two-sided	0.892373178	
				stage * Treatment	Opto	TMA	TMT	FALSE	TRUE	two-sided	0.20037719710425314	
Figure 4.1.8												
Paradigm	Groups	Panel	Statistical summary									
CoA engram optogenetics	Clean air labelling n = 6 TMA labelling n = 5 TMT labelling n = 5	4.1.8C	ANOVA	Source	p-unc							
				Treatment	0.1761850773157133							
				stage	0.584266848							
				Treatment * stage	0.2278968997317546							
			Post-hoc Student's t-test	Contrast	stage	A	B	Paired	Parametric	alternative	p-unc	
				stage	-	Hab	Opto	TRUE	TRUE	two-sided	0.6507014382486364	
				Treatment	-	H2O	TMA	FALSE	TRUE	two-sided	0.15098001735474456	
				Treatment	-	H2O	TMT	FALSE	TRUE	two-sided	0.38150919784026927	
				Treatment	-	TMA	TMT	FALSE	TRUE	two-sided	0.5194658936076681	
				stage * Treatment	Hab	H2O	TMA	FALSE	TRUE	two-sided	0.15795752328493876	
				stage * Treatment	Hab	H2O	TMT	FALSE	TRUE	two-sided	0.19512353529564508	
				stage * Treatment	Hab	TMA	TMT	FALSE	TRUE	two-sided	0.9651979122204822	
				stage * Treatment	Opto	H2O	TMA	FALSE	TRUE	two-sided	0.36205797988129357	
				stage * Treatment	Opto	H2O	TMT	FALSE	TRUE	two-sided	0.188805104	
				stage * Treatment	Opto	TMA	TMT	FALSE	TRUE	two-sided	0.11372498830285797	

Figure 4.1.9											
Paradigm	Groups	Panel	Statistical summary								
MOB engram DREADD inhibition	Saline n = 7	4.1.9C	ANOVA	Source	p-unc	p-GG-corr					
				Treatment	0.026580742849515605	0.026580742849515605					
				stage	0.7918875843761616	0.7918875843761616					
				Treatment * stage	0.012078841336786113	0.012078841336786113					
	CNO n = 7	Post-hoc Student's t-test	Contrast	Treatment	A	B	Paired	Parametric	alternative	p-unc	
			Treatment	-	CNO	Saline	TRUE	TRUE	two-sided	0.026580742849515695	
			stage	-	Hab	Odour	TRUE	TRUE	two-sided	0.7918875843761617	
			Treatment * stage	CNO	Hab	Odour	TRUE	TRUE	two-sided	0.020338282048797902	
			Treatment * stage	Saline	Hab	Odour	TRUE	TRUE	two-sided	0.090638186	
Figure 4.1.10											
Paradigm	Groups	Panel	Statistical summary								
AON engram DREADD inhibition	Saline n = 6	4.1.10C	ANOVA	Source	p-unc	p-GG-corr					
				Treatment	0.768487833	0.768487833					
				stage	0.41860135581575403	0.41860135581575403					
				Treatment * stage	0.001114662	0.001114662					
	CNO n = 6	Post-hoc Student's t-test	Contrast	Treatment	A	B	Paired	Parametric	alternative	p-unc	
			Treatment	-	CNO	Saline	TRUE	TRUE	two-sided	0.7684878334574072	
			stage	-	Hab	Odour	TRUE	TRUE	two-sided	0.4186013558157541	
			Treatment * stage	CNO	Hab	Odour	TRUE	TRUE	two-sided	0.007992069	
			Treatment * stage	Saline	Hab	Odour	TRUE	TRUE	two-sided	0.025851503353522133	
Figure 4.1.11											
Paradigm	Groups	Panel	Statistical summary								
CoA engram DREADD inhibition	Saline n = 6	4.1.11C	ANOVA	Source	p-unc	p-GG-corr					
				Treatment	0.2749374699742894	0.2749374699742894					
				stage	0.17397470740315377	0.17397470740315377					
				Treatment * stage	0.018369375725115777	0.018369375725115777					
	CNO n = 6	Post-hoc Student's t-test	Contrast	Treatment	A	B	Paired	Parametric	p-unc		
			Treatment	-	CNO	Saline	TRUE	TRUE	0.2749374699742896		
			stage	-	Hab	Odour	TRUE	TRUE	0.1739747074031534		
			Treatment * stage	CNO	Hab	Odour	TRUE	TRUE	0.4935225008806593		
			Treatment * stage	Saline	Hab	Odour	TRUE	TRUE	0.018653491		

2.6 Computational modelling

2.6.1 Simple model

The baseline innate preference of 200 “flies” was established by random selection from a normal distribution with mean of -0.5 and standard deviation 0.2. The “choice” made by flies is dependent on their innate preference. Randomness in the choice is modelled by adding a randomly selected number from a normal distribution with mean 0 and standard deviation 0.2. At this point, flies with a value greater than zero have approached the side with a high chance of survival and flies with a value less than zero have approached the side with a lower chance of survival. The survival rates of each arm are used to randomly select survivors from each side, and these survivors are randomly selected for cloning until population size reaches the initial value of 200. For flies which receive “training”, they receive a boost to their innate score before making a choice. Each iteration of the simulation ran for 150 generations and was repeated 50 times.

2.6.2 Polygenic model with costly learning

The polygenic model was similar to the simple model, but instead of the flies baseline preference being constructed via a randomly assigned single number, flies were randomly assigned 5 discrete values representing genes. Each gene has 5 variants. The flies innate preference was calculated as the mean of those 5 genes. An additional single number, sampled from a uniform distribution between 0 and 1, was assigned to each fly. This value was the “plasticity gene” and served as a multiplier for training strength. In this model, costly learning was simulated by randomly selecting trained flies to be “killed”. A general learning risk, LR, was multiplied by the value of the plasticity gene for each fly to determine the chance of each individual fly being killed.

Sexual reproduction was modelled via random pairwise selection of survivors, and the offspring randomly received a copy of each innate gene from both parents. The plasticity gene of the offspring was the average of the plasticity genes of both parents.

Each iteration of the simulation ran for 150 generations and was repeated 50 times.

3. Plasticity of the innate visual looming response

This chapter will investigate the interaction of instinct and memory in the mature brain using the innate defensive responses to visual looming stimuli as a model behaviour. It will investigate plasticity of visual looming fear responses by performing repeated unreinforced exposure of the visual looming stimulus (habituation) and investigating known properties of habituation such as context-specificity and spontaneous recovery. It will also take inspiration from the fear conditioning extinction literature and investigate the possibility of reinstatement of the behaviour. It will seek to understand what happens to the innate circuitry during the process of habituation learning, and whether reinstatement of fear responses to the exact same visual stimulus will recover the activity of innate circuitry or if new circuitry will be developed.

3.1 Results

3.1.1 Visual looming fear responses are flexible

To establish a baseline understanding of the behavioural dynamics of the visual looming response, mice were presented with a train of 15 visual looming stimuli at a frequency of 1 stimulus per second, either with or without the presence of a shelter (a red Perspex tunnel; figure 3.1.1A). When there is no shelter present, mice tend to freeze (figure 3.1.1B). When a shelter is present, mice rapidly flee to the shelter immediately or after a brief period of freezing, before continuing to freeze while inside the shelter (figure 3.1.1C). Mice exhibit a significant increase in freezing over a 30s period from the beginning of looming stimulus onset compared with the 30s pre-stimulus period, regardless of the presence of a shelter (figure 3.1.1D), but will remain in the shelter if it is present (figure 3.1.1C,E). When a shelter is present, mice show a significant increase in top speed relative to 30s pre-stimulus, and reach their top speed sooner than mice which do not have a shelter, indicating flight behaviour (figure 3.1.1F-G). In summary, mice will flee to shelter when it is present, in combination with freezing behaviour. When there is no shelter, mice rely on freezing as the primary response to looming stimuli.

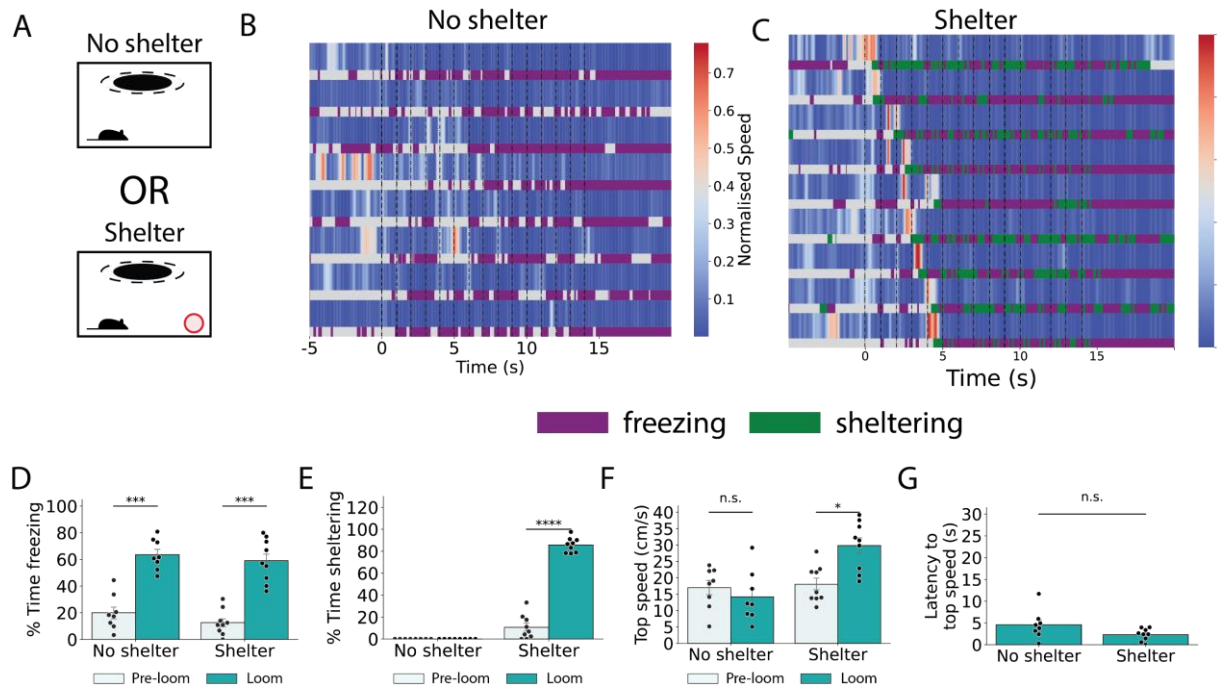


Figure 3.1.1 Innate behavioural responses to visual looming stimuli. (A) Mice were exposed to visual looming stimuli either with or without a shelter. (B) Heatmap indicating the speed of mice, with each blue-red row indicating the speed of a single mouse ($n = 8$ mice). Beneath the speed, a bar indicates whether the individual mouse was detected as freezing (purple) or sheltering (green). Looming stimulus onset is indicated by the dashed black lines, with first stimulus at time=0s. (C) As in B, but with a shelter provided ($n = 9$ mice). (D) Freezing behaviour of mice 30s pre- versus 30s post-stimulus onset both with and without the presence of a shelter (Student's t test; No shelter, $n = 8$ mice; Shelter, $n = 9$ mice). (E-G) As in D, but for time spent in shelter (E), top speed before and after stimulus presentation (F), and time taken to reach top speed following stimulus presentation (G). n.s. $p \geq 0.05$, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$

3.1.2 Habituation of visual looming fear responses is long-lasting and not context-specific

To investigate plasticity of behavioural responses to visual looming stimuli, repeated unreinforced presentation of the visual looming stimulus was performed (figure 3.1.2A). Mice were initially exposed to a single round of 15 looming stimuli to establish a baseline response to the stimulus (test 1). A shelter was provided in the form of a red perspex tunnel. Then, for 6 consecutive days, mice were exposed to 6 rounds of 15 looms in the absence of a shelter, each spread 1 minute apart (habituation training). A control group was placed into the arena for the same length of time each day but without presentation of the visual looming stimulus. Following this, mice were again tested in the presence of a shelter to determine their response following this experience (test 2). On test 1 mice demonstrated a significant increase in both freezing and sheltering following looming relative to pre-stimulus presentation (figure 3.1.2B-E). On test 2, there was no significant increase in freezing or sheltering following stimulus presentation. Meanwhile, control mice which were placed into the context in the absence of the looming stimulus but for the same amount of time maintained freezing and sheltering responses to the visual looming stimulus (figure 3.1.2B). These results demonstrate that innate fear responses to the visual looming response are plastic, and can be attenuated via repeated presentation of the stimulus without any concurrent reinforcing stimulus.

Next, the properties of contextual specificity and spontaneous recovery post-behavioural adaptation were investigated. Investigating contextual specificity, mice were placed in a novel context following training and tested as before (figure 3.1.2F). In this case, mice retained similarly low levels of freezing and sheltering in both the familiar context and the novel context (figure 3.1.2G). To determine whether fear responses spontaneously recover, mice were tested 3 weeks after the end of the repeated exposure training protocol (figure 3.1.2H). Mice maintained significantly lower levels of freezing and sheltering on test 3 relative to test 1 (figure 3.1.2I). The results show that the habituation-like phenotype of the visual looming response is resistant to recovery in a novel context and lasts at least 3 weeks without spontaneous recovery.

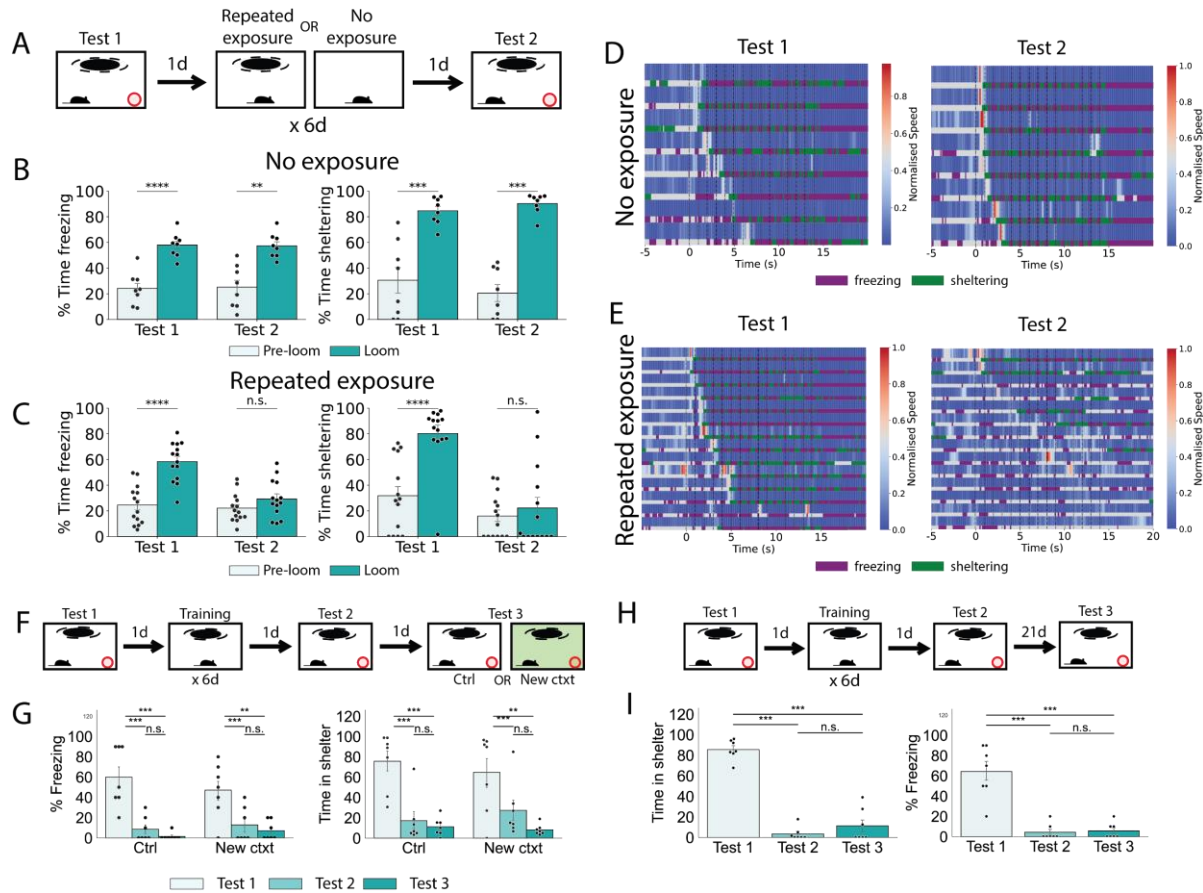


Figure 3.1.2 Habituation of visual looming defensive responses. (A) Repeated exposure training paradigm (habituation). (B) Freezing and sheltering behaviour of the no-exposure cohort, 30s pre- and post-stimulus onset (Student's paired t test; $n = 8$ mice). (C) as in B, but for the repeated exposure cohort ($n = 14$ mice). (D-E) Time-series data of speed, which each row indicating a single trial. The speed, indicated by the blue-red colour scheme, is underlined by bars indicating behavioural response at the same time. Sheltering is indicated in green, and freezing is indicated in purple. (F) Habituation-renewal assessment paradigm. (G) Freezing and sheltering behaviour across test days in the habituation-renewal paradigm (Tukey's pairwise comparisons; ctrl, $n = 7$ mice; new context, $n = 7$ mice). (H) Habituation-spontaneous recovery assessment paradigm. (I) Freezing and sheltering behaviour in the habituation-spontaneous recovery paradigm (Tukey's pairwise comparison; $n = 7$ mice). n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

3.1.3 Fear responses to visual looming stimuli may be reinstated by pairing the visual looming stimulus with an electric footshock

Next, the ability to reinstate fear responses to the visual looming stimulus was investigated. Following completion of the repeated exposure training paradigm, mice received a further training day where they were exposed to either the looming stimulus on its own (“loom only”), a 2s electric footshock on its own (“shock only”), or the looming stimulus paired with a co-terminating 2s electric footshock (“loom + shock”) (figure 3.1.3A). In the loom only cohort, the additional training day had no further effect on freezing or sheltering behaviour (figure 3.1.3B-C). The shock only cohort demonstrated a significant increase in freezing on test 3 in general, but this effect was not stimulus-specific. This suggests that the mice may have formed a generalised fear memory. There was no effect on sheltering behaviour in the shock only group (figure 3.1.3D,E). Finally, the loom + shock cohort showed an increase in freezing on test 3, with a significant increase in freezing following stimulus presentation compared to pre-loom (figure 3.1.3F,G). Again, there was no impact on sheltering behaviour in the loom + shock group. As such, specific fear to the visual looming stimulus can be reinstated by pairing the visual stimulus with an electric footshock. Either the loom or shock on their own are not sufficient to reinstate loom-specific fear responses. Notably, this behavioural paradigm results in a bias towards freezing behaviour over sheltering following reinstatement.

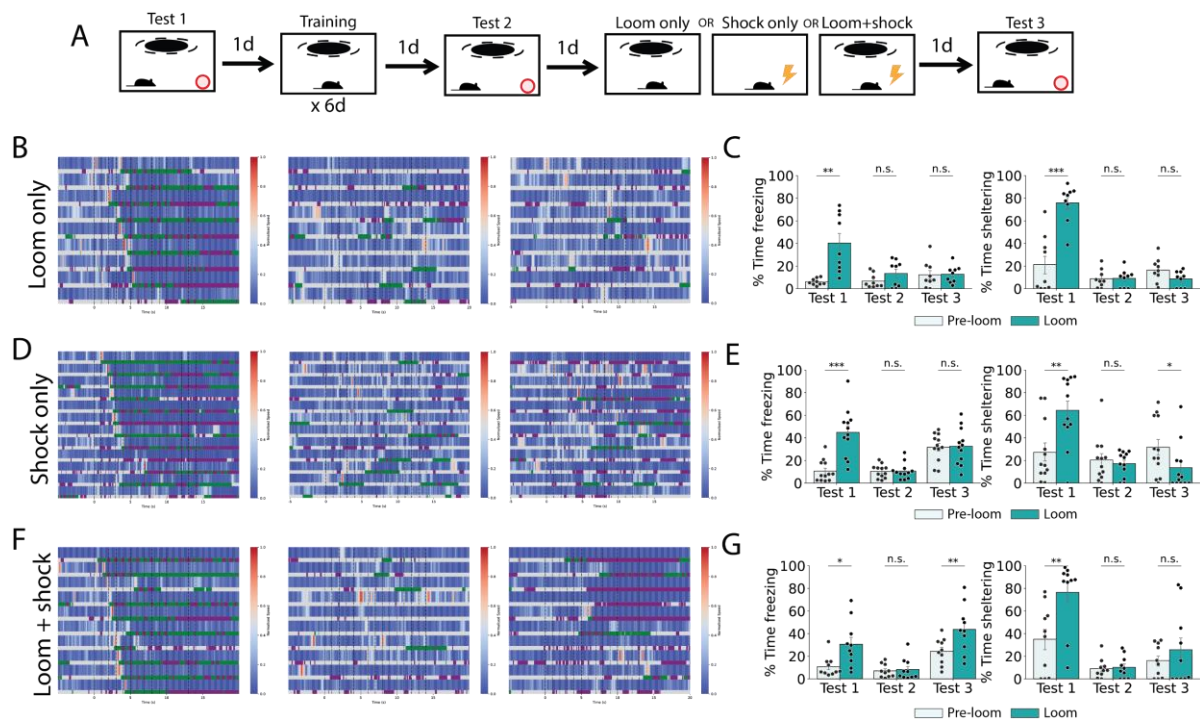


Figure 3.1.3 Re-learning of visual looming fear response. (A) Habituation-reinstatement training paradigm. (B) Mice which receive only the looming stimulus on “reinstatement” training day ($n = 9$ mice). Heatmap indicates the speed of the mouse in response to the looming stimulus, with sheltering and freezing indicated below in green and purple respectively. Each row indicates a single trial. (C) Freezing and sheltering responses of mice from the “loom only” cohort (Repeated measures ANOVA and post-hoc Student’s paired T test). (D-E) as B,C, but for mice which receive only the shock stimulus on “reinstatement” day ($n = 12$ mice). (F-G) as B,C, but for mice which receive the looming stimulus paired with the shock stimulus on “reinstatement” day ($n = 10$ mice). n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.1.4 Fiber photometry reveals attenuation of innate midbrain responses following habituation which do not recover following reinstatement.

Defensive behavioural responses to visual looming stimuli are known to be mediated by the accumulation of threat information in the deep SC which triggers the dorsal PAG to initiate a behavioural response (Evans et al., 2018). To understand how activity in these regions changes, fiber photometric recordings were performed to monitor the fluorescence of virally-induced GCaMP protein, as a correlate of neural activity, during visual looming stimulus presentation. Recordings were performed in either the deep SC or the dorsal PAG. On Test 1, visual looming stimuli reliably drive increases in activity of the deep SC (figure 3.1.4A). This activity has a linear, negative correlation with freezing but is not mutually exclusive with freezing (figure 3.1.4D,E). Further, this activity has a positive correlation with the speed of the mouse (figure 3.1.4F). On Test 2, there is a marked reduction in activity in response to visual looming stimuli (figure 3.1.4B-I). Deep SC activity maintains a negatively correlated relationship with freezing, although the positive correlation between activity and speed is reduced. On Test 3, there is a small recovery of the deep SC response to visual looming stimuli, but this effect only persists for a brief period. The area under the curve of the fluorescence during looming stimuli relative to pre-stimulus is significantly increased on test 1, but not on test 2 or 3 (figure 3.1.4G). When this measure is restricted to only the first 2s of stimulus presentation, there is a trend in recovery of test 3 responses but the effect is not significant. Furthermore, a fast fourier transform demonstrates the presence of a strong 1Hz signal in the fiber photometric recordings during test 1 stimulus presentation, coinciding with the frequency of looming stimulus presentation (figure 3.1.4I). Following repeated exposure this peak is reduced and does not recover following reinstatement.

In terms of the relationship between deep SC activity and behavioural responses during test 3, logistic regression of deep SC activity with freezing behaviour produces an inverse sigmoidal relationship between freezing and GCaMP fluorescence. This indicates that their occurrence is more mutually exclusive than in previous test days. The positive correlation between deep SC activity and speed is maintained. This, alongside the observation of freezing responses initiating following the termination of deep SC spike activity during the first 2s (3.1.4 D, right hand side), suggests that the now predominant freezing response is not driven by deep SC activity.

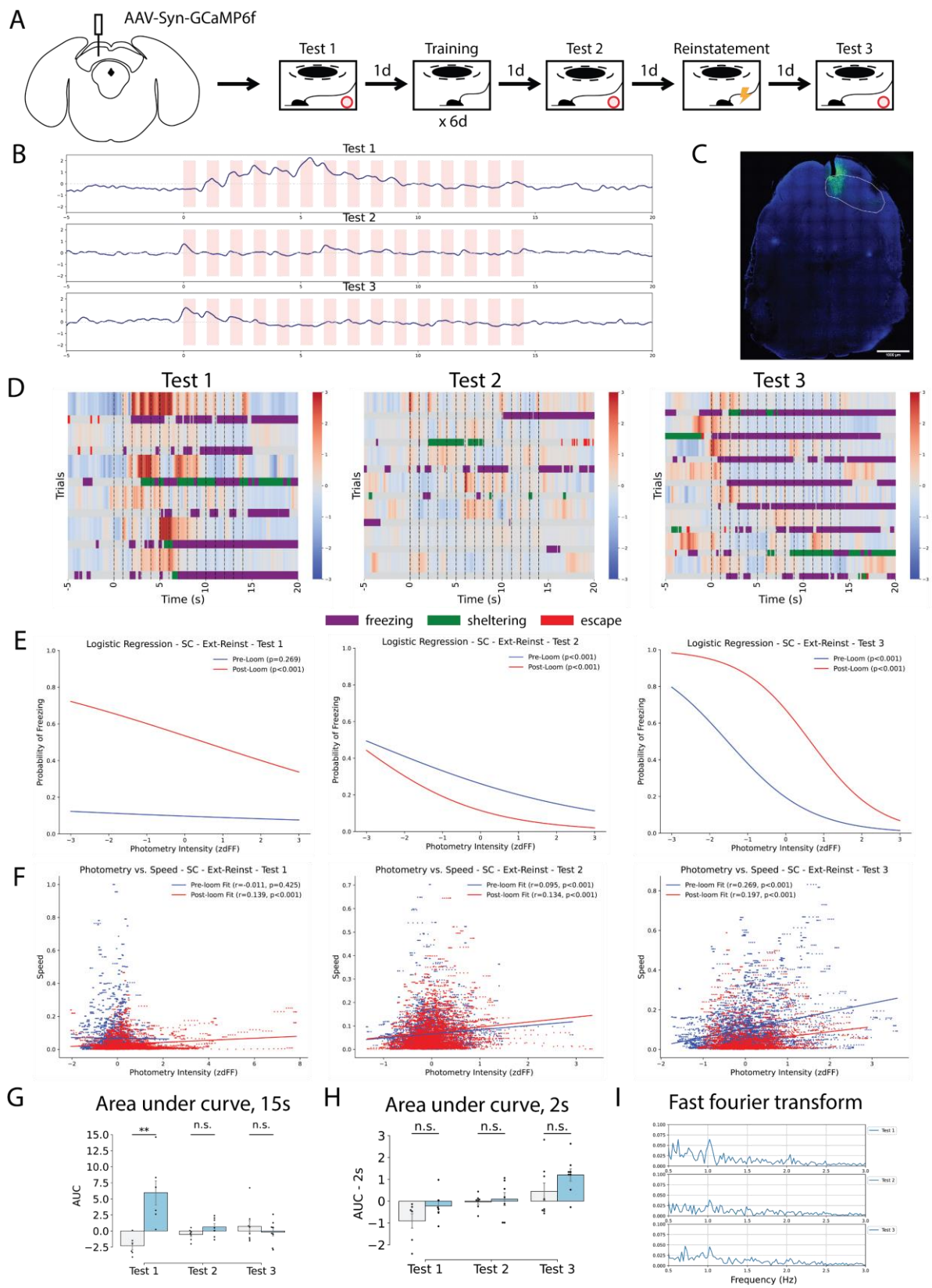


Figure 3.1.4 Fiber photometric recordings of GCaMP signal in the deep SC for mice undergoing habituation-reinstatement with pairing of the loom and shock stimuli. (A) Habituation-reinstatement and recording paradigm. (B) Mean traces of calcium activity in the deep SC on each test day (Test 1, $n = 6$ mice; Test 2, $n = 7$ mice; Test 3, $n = 8$ mice). (C) Example slice showing viral expression and implant track in the deep SC. (D) Heatmaps indicating GCaMP fluorescence relative to looming stimulus presentation (dashed black lines). Behavioural response is indicated below each trace, with freezing in purple, sheltering in green and escape in red. (E) Logistic regression of freezing with GCaMP signal, 15s pre- and post-stimulus onset, for each test day. (F) Linear regression of speed with GCaMP signal, 15s pre- and post-stimulus onset, for each day. (G) Area under curve of GCaMP signal 15s pre- and post-stimulus onset, for each day (Student's T test). (H) Area under curve of GCaMP signal 2s pre- and post-stimulus onset, for each day (Student's T test). (I) Fast Fourier transform of the GCaMP signal during stimulus presentation on each day. The most ethologically relevant peak occurs is at 1Hz, which is the frequency of looming stimulus presentation. $n.s. p \geq 0.05$, $**p < 0.01$, $***p < 0.001$

Mice that underwent the “shock only” training paradigm show slightly different activity in the deep SC. While on test 1 and 2 their activity and its relationship with behaviour is similar to that of the previous cohort (figure 3.1.5A-D), there is no indication of any partial recovery of activity as was observed with the “shock + loom” cohort (figure 3.1.5B,D,H). Additionally, there is no significant relationship between deep SC activity and freezing behaviour or top speed during stimulus presentation on test 3 (figure 3.1.5E,F). However, there is an inverse sigmoidal relationship between freezing and GCaMP fluorescence pre-stimulus only, concurrent with the previous observation that “shock only” treatment results in a generalised freezing response independent of the looming stimulus (figure 3.1.5E).

Together, these data complement the finding that only the shock + loom cohort recover a behavioural response towards visual looming stimuli, however this recovered behavioural response, which is biased towards freezing over fleeing, has a negative relationship with deep SC activity.

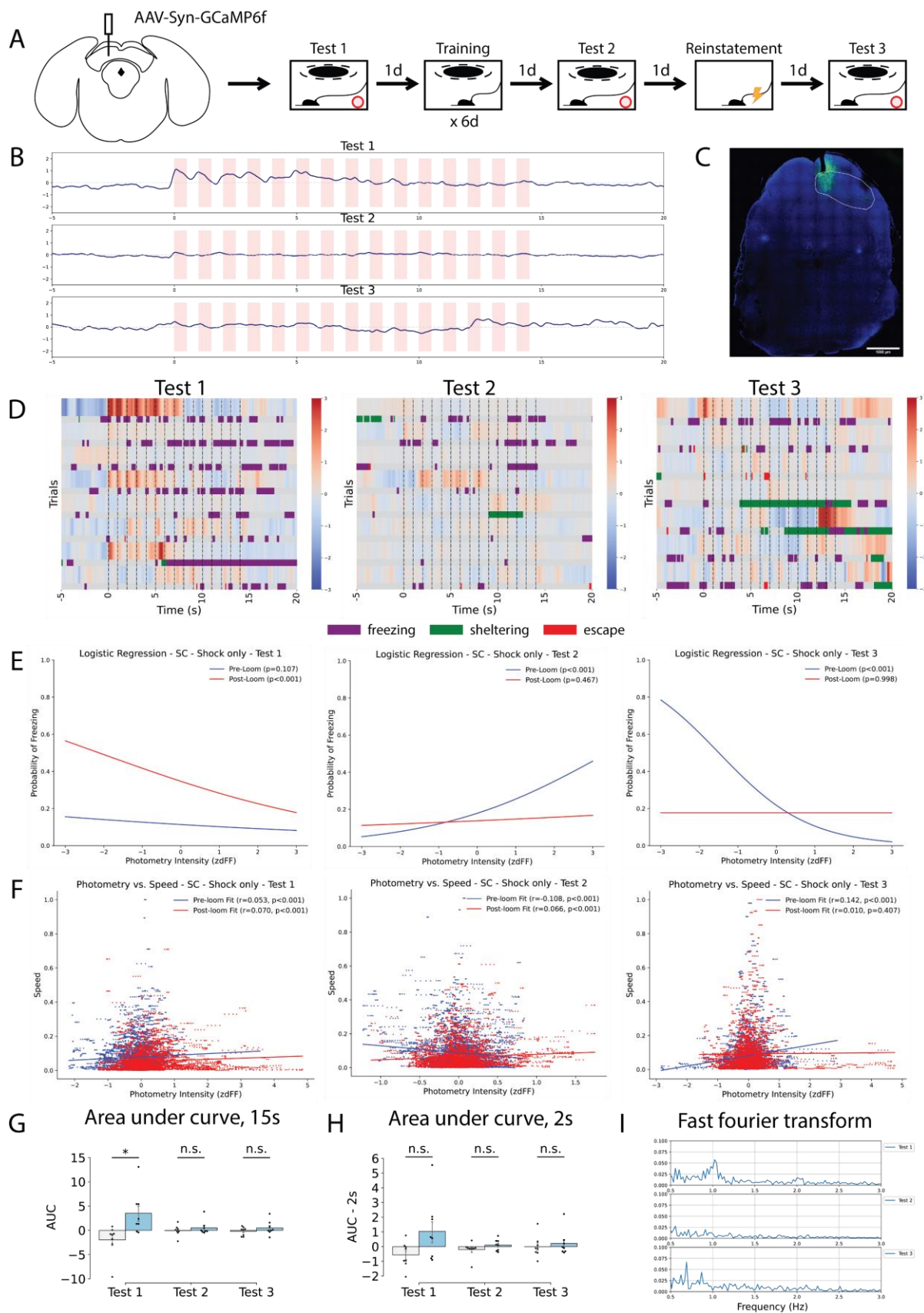


Figure 3.1.5 Fiber photometric recordings of GCaMP signal in the deep SC for mice undergoing habituation-reinstatement with only the shock stimulus on reinstatement day. (A) Habituation-reinstatement and recording paradigm. (B) Mean traces of calcium activity in the deep SC on each test day (Test 1, $n = 8$ mice; Test 2, $n = 8$ mice; Test 3, $n = 8$ mice). (C) Example slice showing viral expression and implant track in the deep SC. (D) Heatmaps indicating GCaMP fluorescence relative to looming stimulus presentation (dashed black lines). Behavioural response is indicated below each trace, with freezing in purple, sheltering in green and escape in red. (E) Logistic regression of freezing with GCaMP signal, 15s pre- and post-stimulus onset, for each test day. (F) Linear regression of speed with GCaMP signal, 15s pre- and post-stimulus onset, for each day. (G) Area under curve of GCaMP signal 15s pre- and post-stimulus onset, for each day (Student's t test). (H) Area under curve of GCaMP signal 2s pre- and post-stimulus onset, for each day (Student's T test). (I) Fast Fourier transform of the GCaMP signal during stimulus presentation on each day. The most ethologically relevant peak occurs is at 1Hz, which is the frequency of looming stimulus presentation. *n.s.* $p \geq 0.05$, * $p < 0.05$, *** $p < 0.001$

In the PAG, there is also a strong response to visual looming stimuli on test 1 (figure 3.1.6A-D). This response attenuates on test 2, and remains suppressed on test 3 (figure 3.1.6B,G), with no evidence of a brief response akin to what was observed in the SC (figure 3.1.6H). Again, the test 1 response occurs in synchrony with looming stimulus presentation (figure 3.1.6A) and has a strong peak at 1Hz when decomposed via fast Fourier transform (figure 3.1.6I). Dorsal PAG activity on test 1 has a negatively correlated relationship with freezing during stimulus presentation, which decays on test 2 and test 3. Meanwhile, it has a positive correlation with speed, albeit not as strong as what was observed in the SC (figure 3.1.6E).

In summary, the deep SC and dorsal PAG exhibit strong responsiveness to visual looming stimulus presentation in the naïve mouse. The activity in the deep SC correlates strongly with speed, while being negatively correlated with freezing. PAG activity is also positively correlated with speed but to a lesser extent, and has a positive correlation with freezing. Habituation training results in an attenuation of deep SC and dorsal PAG responses to visual looming stimuli. Following reinstatement training, a shock paired with a loom, but not a shock on its own, causes a brief return of deep SC responses. However, this response lasts approximately only 2s out of the 15s duration of stimulus presentation, and tends to reduce during freezing which is the dominant behavioural response during reinstated looming fear responses, as characterised by an inverse sigmoidal relationship. Meanwhile, the dorsal PAG response is suppressed following habituation, and shows no evidence of any recovery.

While the relationship between dorsal PAG activity with freezing is positive during test 1, it switches to an inverse sigmoidal relationship by test 3. Together, this data illustrates that visual looming stimuli drive activity in the deep SC and dorsal PAG initially, which correlate with flight behaviour in the case of the deep SC, and both flight and freezing in the PAG. Following habituation-reinstatement, this response is generally absent, and in both regions correlates negatively with freezing behaviour, which has become the dominant behavioural response.

As such, the habituation-reinstatement training paradigm de-couples visual looming fear responses from the canonical deep SC-PAG pathway.

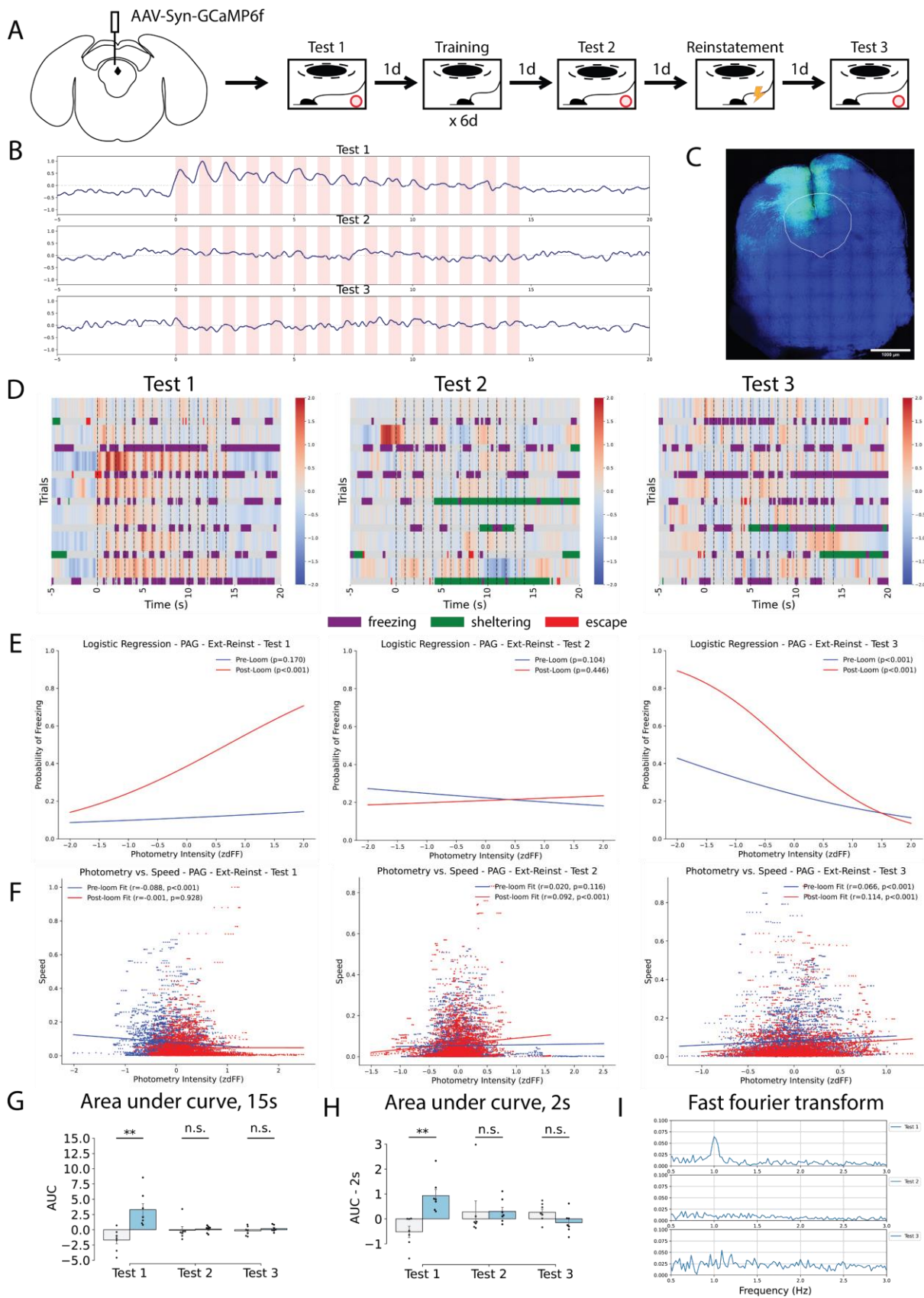


Figure 3.1.6 Fiber photometric recordings of GCaMP signal in the dorsal PAG for mice undergoing habituation-reinstatement with pairing of the loom and shock stimuli. (A) Habituation-reinstatement and recording paradigm. (B) Mean traces of calcium activity in the dorsal PAG on each test day (Test 1, $n = 7$ mice; Test 2, $n = 7$ mice; Test 3, $n = 7$ mice). (C) Example slice showing viral expression and implant track in the dorsal PAG. (D) Heatmaps indicating GCaMP fluorescence relative to looming stimulus presentation (dashed black lines). Behavioural response is indicated below each trace, with freezing in purple, sheltering in green and escape in red. (E) Logistic regression of freezing with GCaMP signal, 15s pre- and post-stimulus onset, for each test day. (F) Linear regression of speed with GCaMP signal, 15s pre- and post-stimulus onset, for each day. (G) Area under curve of GCaMP signal 15s pre- and post-stimulus onset, for each day (Student's T test). (H) Area under curve of GCaMP signal 2s pre- and post-stimulus onset, for each day (Student's T test). (I) Fast Fourier transform of the GCaMP signal during stimulus presentation on each day. The most ethologically relevant peak occurs is at 1Hz, which is the frequency of looming stimulus presentation. *n.s.* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$

3.1.5 Habituation and reinstatement of visual looming fear results in altered c-Fos expression in the midbrain and hippocampus

Fiber photometry indicated a decoupling of visual looming fear responses from the deep SC-dorsal PAG pathway following habituation-reinstatement. To determine the response of a broader range of brain areas to visual looming stimuli, c-Fos expression patterns were analysed. TRAP2xAi32 mice were used to tag cells in a c-Fos-dependent manner following exposure to visual looming stimuli (figure 3.1.7A,B). Mice then underwent one of several behavioural paradigms: looming stimulus re-exposure, looming habituation training, or looming habituation-reinstatement training. On the last day of the paradigm, mice were perfused 60min following looming stimulus exposure, with brains sliced and stained for endogenous c-Fos. Thus, GFP positive cells indicate those cells which are active following innate visual looming stimulus response, c-Fos positive cells are those which are active following post-training visual looming stimulus response, and GFP & c-Fos double positive cells are those which are active for both the innate looming response and the post-training looming response.

In the re-exposure paradigm (figure 3.1.7C-F), mice were tagged following innate visual looming responses, and then 1 week later were either returned to the arena with no visual looming stimuli presented (no re-exposure) or with visual looming stimuli presented (re-exposure). Following re-exposure, no significant difference in overall c-Fos expression throughout the SC, PAG or hippocampus was observed compared with no re-exposure

controls, although there is a trend of elevated c-Fos expression throughout the SC (figure 3.1.7E). However, there is a significant increase in the reactivation of cells tagged during innate loom responses in the SCs, ventral CA1 and ventrolateral PAG (figure 3.1.7F). Additionally, there is a trend of increased reactivation of cells in other PAG subregions as well as the deep SC. This data illustrates that, while overall c-Fos levels show little change following looming stimulus presentation, there are specific cells within the SC, PAG and ventral CA1 which are reliably active during visual looming stimulus presentation compared with no stimulus presentation.

In the habituation training paradigm, mice were also tagged following the innate visual looming response, and then underwent repeated unreinforced presentation of the visual looming stimulus (figure 3.1.7G-J). Control mice were placed into the arena for the same length of time each day, but without any looming stimulus presentation. After training, both groups of mice were re-exposed to the visual looming stimulus. Hence, the c-Fos and GFP reactivation levels in these mice should correspond to those of the innate visual looming response in control mice, and the habituated visual looming response in habituation mice. In habituated mice, there was a significant decrease in overall c-Fos expression levels in the deep SC, dorsolateral PAG and lateral PAG relative to control mice (figure 3.1.7I). Furthermore, there was a persistent trending decrease in c-Fos levels throughout the SC and PAG, but not the hippocampus. There was no change in the reactivation of GFP cells in the SC following habituation, but there was a significant decrease in the reactivation of the dorsal PAG (figure 3.1.7J). Together this suggests that, while overall responses of the SC decrease following habituation, specific loom-responsive cells in the SC may remain active following habituation training. However, the specific loom-responsive cells in the dorsal PAG cease to respond to looming stimuli.

In the reinstatement paradigm, mice were again tagged following innate looming behavioural responses, and then either underwent the “loom + shock” habituation-reinstatement training paradigm, or a control paradigm where mice were placed in the arena each day for the same length of time without any looming stimuli being presented (figure 3.1.7K-N). In this experiment, c-Fos levels and GFP reactivation compare the reinstated behavioural response with the innate behavioural response. The overall c-Fos expression level throughout the SC and PAG is not significantly different between reinstatement and controls (figure 3.1.7M). This suggests that overall c-Fos expression levels recover in the SC and PAG,

compared with the decrease which was observed following habituation. Following reinstatement however, there was a significant decrease in the reactivation of innate-tagged cells in the SCs, PAGdm and PAGdl compared with control mice (figure 3.1.7H). The reduction in PAGdm/PAGdl may result from the similarly observed reduction following habituation, but the reduced reactivation of the SCs is unique to the reinstated condition. Additionally, there is a significant increase in the reactivation of the innate-tagged ventral CA1 cells in reinstated mice compared with control.

Together, these data illustrate an innate visual looming response with increased activity of specific ensembles in the SC and ventral CA1. Following habituation there is reduced activity in the SC and PAG, while the ventral CA1 is unaffected. With reinstatement, there is a recovery of overall c-Fos expression in the SC and PAG, but the activity of initially tagged innate loom-responsive cells does not recover. Meanwhile, there is increased reactivation of the initially tagged ventral CA1 loom-responsive cells.

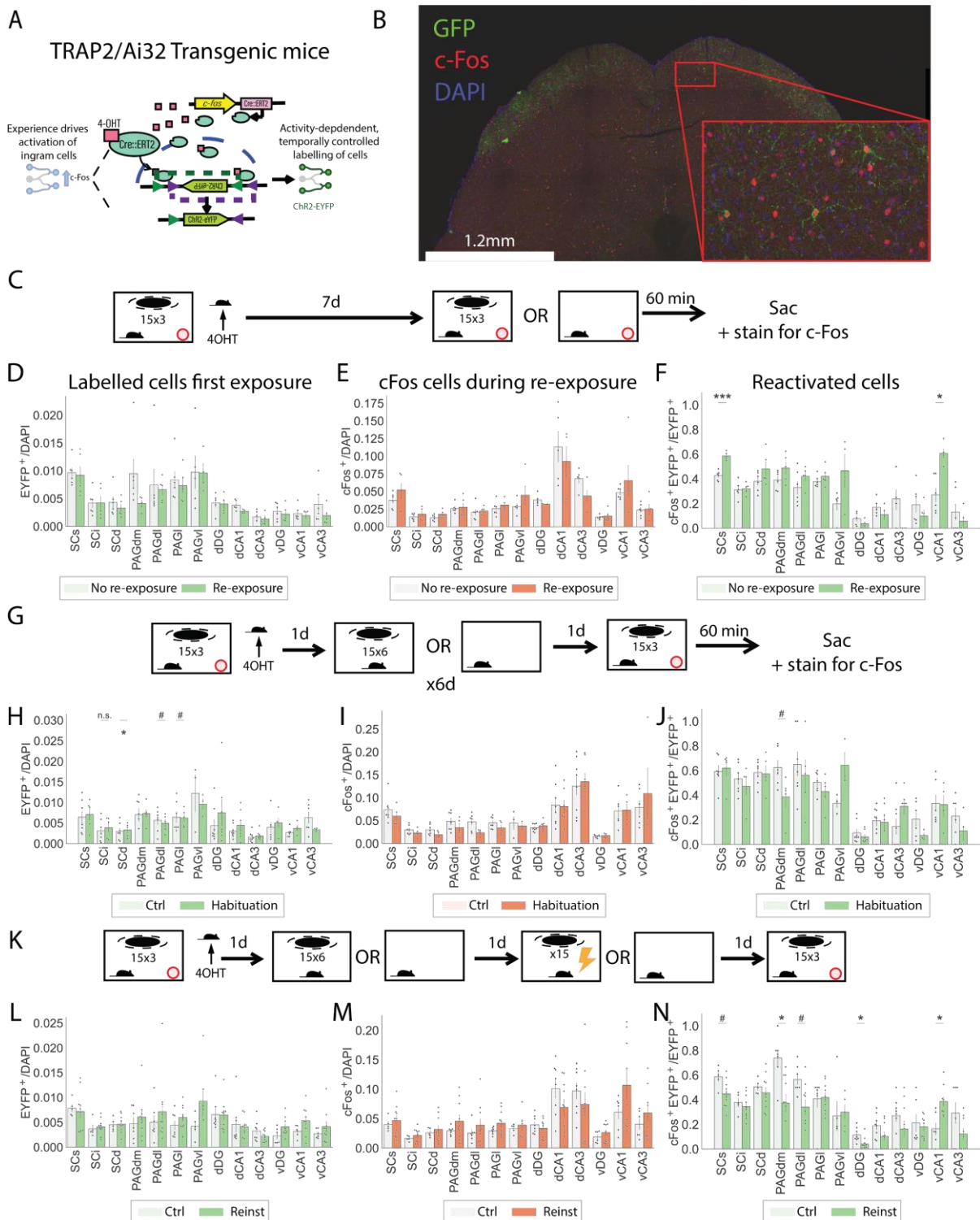


Figure 3.1.7 Investigating experience-dependent c-Fos expression and engram reactivation throughout the SC, PAG and hippocampus during looming stimulus presentation. (A) Engram labelling strategy. (B) Example slice showing labelled candidate engram cells (GFP) and cells expressing c-Fos protein (red). (C) Behavioural timeline for the investigation of looming stimulus-induced c-Fos expression in the absence of learning experience (Re-exposure, $n = 6$ mice; No re-exposure, $n = 6$ mice). (D) Number of engram cells tagged on day 1 in each experimental group (Student's unpaired t test with False discovery rate correction). (E) Number of c-Fos positive cells on day 2 in each experimental group, as a correlate of overall activity for each brain region (Student's paired t test with False discovery rate correction). (F) Proportion of day 1 engram cells which are reactivated on day 2 in each brain region (Student's unpaired t test with False discovery rate correction). (G) Behavioural timeline for the investigation of looming stimulus-induced c-Fos expression following habituation training, compared with no-training controls (Habituation, $n = 7$ mice; No habituation, $n = 8$ mice). (H-J) As D-F, but for mice undergoing habituation training. (K) Behavioural timeline for the investigation of looming stimulus-induced c-Fos expression following habituation-reinstatement training compared with no-training controls (Reinstatement, $n = 11$ mice; No training $n = 8$ mice). (L-N) As D-F, but for mice undergoing habituation-reinstatement training. *n.s.* $p \geq 0.05$, # $p < 0.05$, * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$; where p is the Student's t test P value, and q is the false discovery rate-adjusted P value.

While the low temporal resolution of c-Fos expression limits the ability to investigate its correlation with the rapid behavioural responses to visual looming stimuli, it is still possible to investigate its correlation with behavioural metrics such as top speed during looming stimulus, or percentage time freezing during looming stimulus presentation (figure 3.1.8). During the innate looming response, overall c-Fos expression and GFP reactivation correlates positively with the top speed during loom presentation in the SC, while in the ventral CA1 overall c-Fos and GFP reactivation correlate positively with freezing levels (figure 3.1.8A-C). Following habituation, the correlation of these regions with top speed and freezing levels is reduced (figure 3.1.8D-F). With reinstatement, the correlation of deep SC and dorsal PAG overall c-Fos with top speed is partially recovered, however not the correlation of GFP reactivation (figure 3.1.8G-I). Meanwhile, overall c-Fos in the ventral CA1 does not correlate with freezing, but reactivation of tagged cells in the ventral CA1 does correlate positively with freezing. In summary, innate behavioural responses show correlation with the SC, PAG and vCA1, while reinstated behavioural responses lose their correlation with the SC and maintain correlations with some PAG subregions and the vCA1.

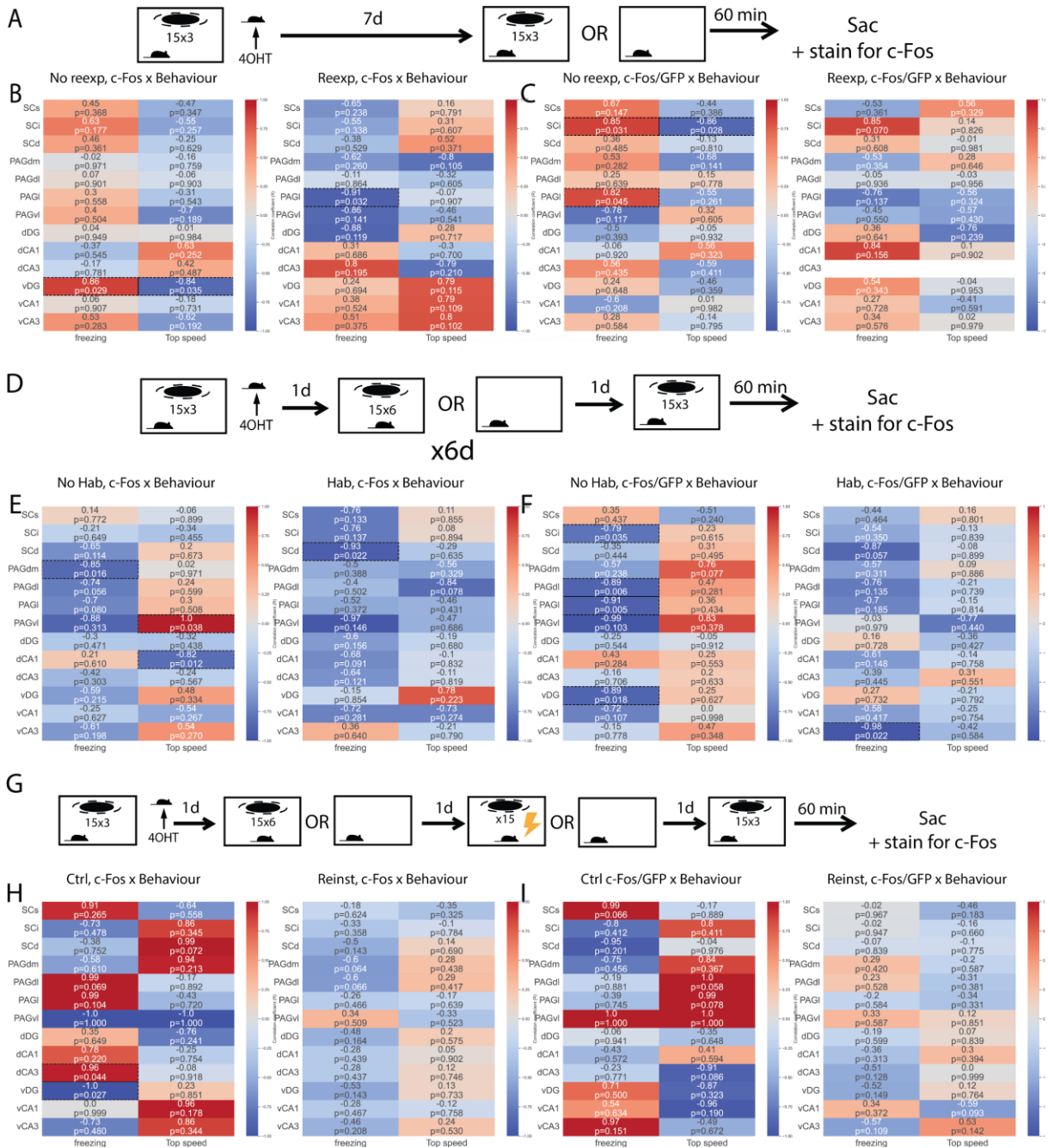


Figure 3.1.8 Correlation of overall c-Fos expression and engram reactivation with behavioural responses. (A) Behavioural timeline for the investigation of looming stimulus-induced c-Fos expression in the absence of learning experience (Re-exposure, $n = 6$ mice; No re-exposure, $n = 6$ mice). (B) Pearson's correlation of c-Fos expression in each brain region with freezing, latency to top speed, and top speed for mice which are not re-exposed to the looming stimulus, or mice re-exposed to the looming stimulus. (C) As in B, but for engram reactivation. (D) Behavioural timeline for the investigation of looming stimulus-induced c-Fos expression following habituation training, compared with no-training controls (Habituation, $n = 7$ mice; No habituation, $n = 8$ mice). (E-F) As B-C, but for mice undergoing habituation training. (G) Behavioural timeline for the investigation of looming stimulus-induced c-Fos expression following habituation-reinstatement training compared with no-training controls. (H-I) As B-C, but for mice undergoing habituation-reinstatement training (Reinstatement, $n = 11$ mice; No training $n = 8$ mice). n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

3.1.6 Loom responsive cells in vCA1 are necessary for learned but not innate loom responses

Based on the increased reactivation of innate-loom tagged ventral CA1 ensembles, alongside the established role of the ventral CA1 in the fear conditioning reinstatement (Jimenez et al., 2020), the role of the ventral CA1 in visual looming fear reinstatement was investigated. To determine whether specific loom-responsive cells in the ventral CA1 are necessary in the reinstated visual looming response, the FLiCRE labelling system (Kim et al., 2020) was used. This labelling paradigm relies on intracellular calcium alongside artificially-supplied blue light (473nm) to induce labelling of neurons (figure 3.1.9). As a result, this system produces activity-dependent labelling with a temporal precision on the order of seconds.

Taking advantage of this high temporal precision, ventral CA1 ensembles were tagged either pre-stimulus or during loom and shock presentation of the reinstatement training session (figure 3.1.10A), inducing the expression of an inhibitory Halorhodopsin which is activated by yellow light (595nm). Then, 24h later, mice were exposed to two rounds of looming stimulus presentation; first with laser stimulation at 595nm to inhibit tagged cells and second with no light-induced inhibition. For pre-loom tagged cells, there was a significant increase in freezing following loom-presentation regardless of whether the laser was turned on (test 3A) or off (test 3B) (figure 3.1.10B). However, for cells tagged during loom and shock presentation, there was no increase in freezing whilst these cells were inhibited, but a

significant increase in freezing when mice were presented the stimulus again without inhibition just 90s later (figure 3.1.10C). Furthermore, there was no significant difference in the number of cells tagged during these two sessions (figure 3.1.10F), despite their differing effect on behaviour. This indicates that there is an ensemble of cells in the ventral CA1 which is activated specifically during presentation of the looming and shock stimuli, which is necessary for recall of the reinstated fear 24h later.

Since looming and shock stimulus are presented together, this ventral CA1 ensemble may contain both loom-responsive and shock-responsive cells. Next, the role of solely loom-responsive ventral CA1 cells were investigated – both in innate and learned visual looming responses. Loom-responsive cells in the ventral CA1 were tagged using the FLiCRE system in the naïve mouse. Then, mice underwent the habituation-reinstatement training paradigm as usual, except in this case each test day contained two rounds of looming stimulus presentation. During the first round, loom-responsive cells were optogenetically inhibited, and during the second round there was no inhibition applied (figure 3.1.10D).

During Test 1, there was a significant increase in freezing following loom-presentation for both rounds, indicating that loom-responsive cells in the ventral CA1 are not necessary for innate fear responses to visual looming stimuli. On Test 2, there was no increase in freezing on either round, indicating that the loom-responsive cells are not necessary for recall of the habituation memory. On Test 3, there was no significant increase in freezing while loom-responsive cells were inhibited, but there was a significant increase in freezing to the looming stimulus 90s later when these cells were no longer being inhibited. As such, there are cells in the ventral CA1 which are active during innate visual looming responses but are not required for innate responses. However, these same cells are later necessary for recall of the reinstated visual looming fear responses. Furthermore, while there is no significant difference between the number of cells tagged during pre-loom or during-loom on the reinstatement session, there are significantly more cells tagged during the innate loom response.

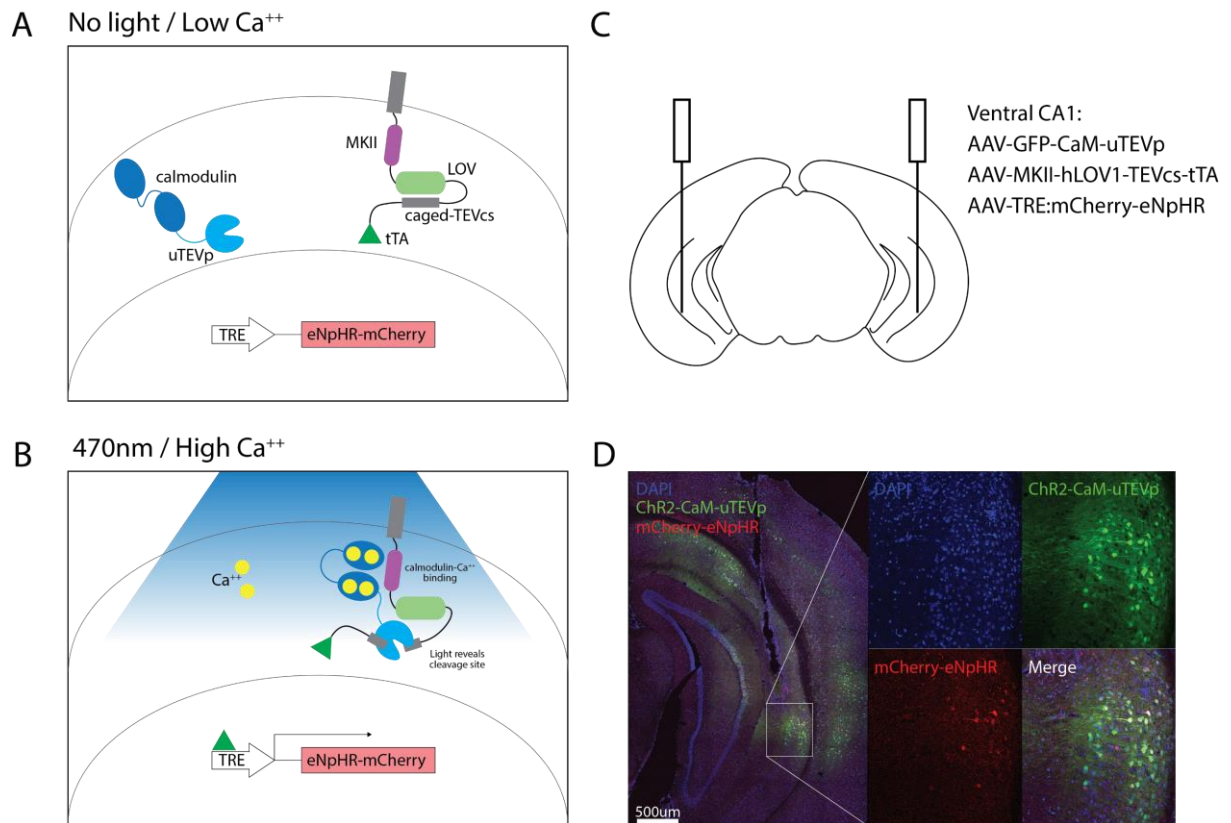


Figure 3.1.9 FLiCRE – a calcium and light-dependent labelling paradigm for activity dependent labelling of cells with high temporal precision. (A) In the absence of calcium, calmodulin-bound uTEVp is dispersed throughout the cytoplasm of the cell. Meanwhile, in the absence of light, TEVcs (TEV cutting site) remains inaccessible to uTEVp. (B) In the presence of calcium, calmodulin will bind to MKII, bringing the uTEVp protein with it. In the presence of 470nm light, conformation changes to the LOV protein reveal the TEVcs, which is cleaved by uTEVp. This frees the tTA protein, which travels to the nuclear membrane and bind with the TRE protein, driving expression of eNpHR-mCherry. The end result is expression of eNpHR-mCherry only when there is coincident Calcium activity and the presence of light. (C) Viral cocktail to implement the FLiCRE system in the ventral CA1. (D) example slice demonstrating implant track and viral expression. Cells infected with the FLiCRE system will express GFP, while cells which are tagged during labelling will express mCherry.

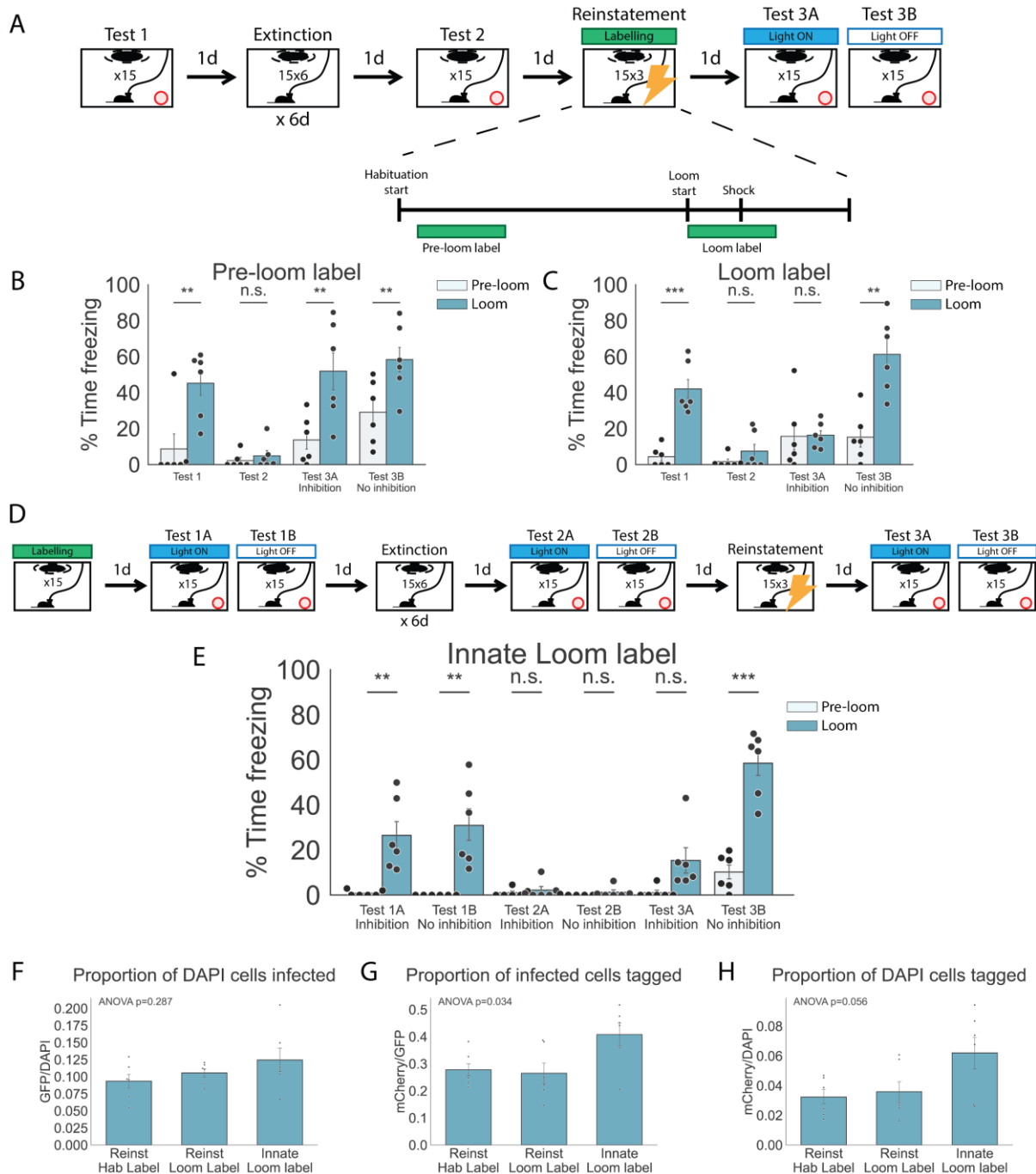


Figure 3.1.10 Loom-responsive cells active during reinstatement training and during innate loom presentation are necessary for recall of the reinstated looming response, but not for the innate looming response. (A) Behavioural timeline to investigate the role of ventral CA1 ensembles that are active during context presentation alone (pre-loom labelling) and during looming and shock stimuli presentation (loom labelling). (B) Freezing responses pre- and post-looming stimulus on each test day for the pre-loom labelled cohort (repeated measures ANOVA with post-hoc comparisons; $n = 6$ mice). (C) Freezing responses pre- and post-looming stimulus onset on each test day for the loom-labelled cohort (repeated measures ANOVA with post-hoc comparisons; $n = 6$ mice). (D) Behavioural timeline to investigate the role of ventral CA1 ensembles that are active during naïve looming stimulus presentation. (E) Freezing responses pre- and post-looming stimulus on each test day for the innate loom labelled cohort (repeated measures ANOVA with post-hoc comparisons; $n = 6$ mice). (F) The number of cells infected and tagged across the three labelling conditions. n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

3.2 Discussion

Summary

This project has established a novel behavioural paradigm to investigate how the brain can facilitate adaptive behaviour towards a highly innate threatening visual stimulus. The innate visual looming response is susceptible to behavioural plasticity – habituation via repeated unreinforced exposure. This habituated response can generalise to other contexts and persists for at least 3 weeks. Over the course of the habituation training paradigm, there is an attenuation of the deep SC and dorsal PAG activity which drives escape behaviour. This activity does not recover following reinstatement of looming stimulus fear via pairing it with an electric foot-shock. Meanwhile, there is an ensemble of cells in the vCA1 which responds to looming stimuli upon naïve presentation. These innately loom-responsive cells are not necessary for innate loom responses but are necessary for adaptive behavioural responses later following reinstatement. This study illustrates how the brain can handle the challenge of creating an innate behaviour which is important for survival, while still allowing adaptive behavioural responses to the stimulus via experience-dependent learning.

Innate looming responses are adaptable

The visual looming habituation-reinstatement paradigm builds on a literature whereby the innate visual looming response has been shown to be adaptable and hence influenced by

memory. Previous studies have shown the memory of shelter location modulates flight behaviour via projections from the retrosplenial cortex to the SC (Campagner et al., 2023). Mice can also remember areas where looming stimuli were previously presented to later avoid those areas (Evans et al., 2018), as well as learning to fear a tone previously paired with looming stimuli (albeit to a much lesser degree than with an electric shock) (Heinemans and Moita, 2022). Additionally, mice which are exposed to a looming stimulus of gradually increasing contrast from sub-detection levels are shown to not respond to the looming stimulus at full contrast (Lenzi et al., 2022, Mederos et al., 2024). In the habituation-reinstatement paradigm established in this project, we show a transition from looming fear to complete attenuation of behavioural responses for the first time. The next advancement achieved in this behavioural paradigm is the reinstatement of the behavioural response to the specific looming stimulus. At this point in time, no other published literature attempts to recover visual looming fear responses following learned attenuation. This advancement allows comparison of innate and learned fear responses to the exact same stimulus within a single subject.

The habituation-like phenotype characterised here has some distinctions from both classical habituation and fear conditioning extinction. For example, it persists at least 3 weeks after training and generalises to other contexts. As such, one may consider this a form of long-term habituation. Notably, the behavioural attenuation was resistant to dishabituation, as an electric foot-shock on its own was not sufficient to recover behavioural responses to the looming stimulus. Meanwhile, an electric foot-shock paired with the visual looming stimulus was sufficient to recover fear responses to visual looming stimuli. This learning occurred with only a single pairing of the looming stimulus with the shock, so is learned quite readily despite the otherwise persistent behavioural attenuation. This is also in contrast with the slow time course over which the habituation training occurs.

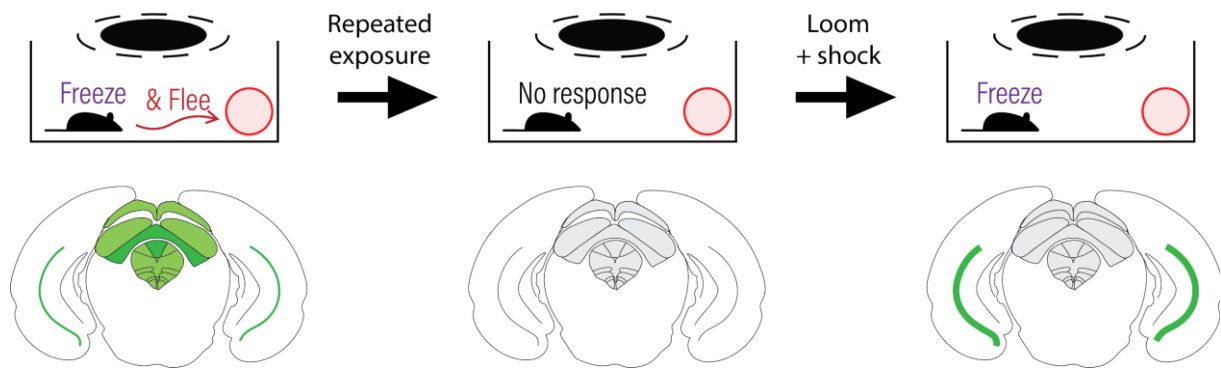


Figure 3.2.1 Visual looming habituation and reinstatement results in reduced SC and PAG activity and produces vCA1-dependent fear responses. Initial SC and PAG driven loom responses include freezing and fleeing. Naïve looming also activates vCA1 ensembles. Following habituation training, mice cease responding to the looming stimulus. When the stimulus is then paired with an electric foot-shock, mice recover fear responses to the stimulus which are now dependent on the initially tagged vCA1 ensemble.

A novel role for the hippocampus in enabling adaptive visual looming responses

In this study, it has been discovered that, alongside the canonical circuitry of the SC and PAG, there are cells in the ventral CA1 which respond to visual looming stimuli and are reliably reactivated upon subsequent re-exposure to visual looming stimuli. This finding establishes a novel role of the hippocampus in behavioural responses to visual looming. These vCA1 loom-responsive ensembles are not necessary for evoking innate defensive behavioural responses, but do become necessary for learned responses following the habituation-reinstatement paradigm (figure 3.3.10E). Meanwhile, the SC and PAG responses which are known to drive visual looming fear responses (Evans et al., 2018) become much less responsive to visual looming stimuli and in fact have an inverse relationship with freezing. As such, it seems that the behavioural response to visual looming stimuli has transitioned from SC-dependency to vCA1-dependency. However, what is the role of the vCA1 loom-responsive ensembles in the first place?

The vCA1 is implicated in a wide range of emotionally-salient rapidly forming memories, including fear conditioning (Jimenez et al., 2020, Shpokayte et al., 2022), social memory (Okuyama et al., 2016) and reward (Ruediger et al., 2012, Shpokayte et al., 2022). Therefore, these loom-responsive stimuli in the vCA1 may aid in rapid adaptation of behaviour in response to looming stimuli, such as avoiding areas where looming stimuli were previously

presented (Evans et al., 2018, Burnett et al., 2024). Additionally, while the vCA1 ensemble may form rapidly, it is evidently long lasting given its role in the reinstated behavioural response.

The ability of the vCA1 to facilitate rapid learning may complement a less plastic SC. This is reflected in the many looming stimulus exposures required to extinguish innate loom responses, and the lack of recovery of the SC following reinstatement. This organisation may enable protection of the SC circuitry, conserving circuitry which is important for survival, while still enabling plastic responses via the vCA1. With sufficient training, the relatively slow plasticity of the SC leads to attenuation of SC responses, and reinstatement then relies on vCA1.

In summary, these vCA1 ensembles loom-responsive ensembles may be the result of innately-constructed but spontaneously assigned stimulus representation of the salient visual looming stimulus which are important for enabling adaptive behaviour.

How might visual information reach the hippocampus?

The question of how this specific information may reach the hippocampus remains the subject of future research. However, it is well established that there is extensive exchange of information between the SC and hippocampus, likely through polysynaptic pathways (Natsume et al., 1999, Routtenberg and Taub, 1973, Benavidez et al., 2021). So, what are the potential pathways by which looming stimulus information could reach the hippocampus?

While there are no known direct connections between the superior colliculus and the hippocampus, there are several brain regions which receive projections from the superior colliculus that themselves project to the hippocampus, or para-hippocampal areas such as the perirhinal or entorhinal cortices. For example, the ventral midline thalamus (vMT) receives projections from the SC to the subnuclei the nucleus Reuniens (Re) and the xiphoid nucleus (Xi), each of which have been shown to promote different aspects of looming-response behaviours (Salay et al., 2018). Meanwhile, the vMT, and in particular the Re, has been shown to project directly to the hippocampal CA1 in addition to other limbic structures (Vertes et al., 2006). Furthermore, the hippocampal CA1 also has direct projections back to the Re (McKenna and Vertes, 2004), and this bilateral communication has been proposed to play a role in exchanging information between subcortical and limbic structures to enable adaptive behavioural responses via learning (Vertes et al., 2006). Indeed, the vCA1 ensembles do appear to be involved in allowing adaptive behavioural responses to the visual looming

stimulus, so perhaps the vMT is involved in relaying the looming threat information to the hippocampus. Moreover, the ability of specific cells in the vMT to drive behaviour may serve as a mechanism by which the vCA1 could trigger looming defensive behaviour.

In addition to this 2-step pathway, there are other mechanisms by which information can travel from the SC to the hippocampus. For example, the SC can transmit information to the parietal cortex (particularly the retrosplenial cortex) via the LPTN (Shang et al., 2018, Kondo et al., 2022, Reep et al., 1994, Campagner et al., 2023), which then relays information to the entorhinal cortex (van Groen and Michael Wyss, 1990, Sugar et al., 2011) which forms projections to the hippocampal CA1 (Amaral and Witter, 1989, Van Strien et al., 2009, Iijima et al., 1996). The visual cortex is another structure which may be involved in both innate and learned looming behaviour. In innate behaviour, the visual cortex is known to modulate the magnitude of defensive behavioural response to looming via projections to the SC (Zhao et al., 2014, Harvey and Worthington, 1990). Furthermore, it can indirectly interact with the hippocampus via the relaying of information to the postrhinal and entorhinal cortices (Furtak et al., 2007, Witter et al., 2000, Burwell and Amaral, 1998).

Given this information, it is clear that the hippocampus receives highly processed information, which is important in both memory formation and recall and spatial navigation. The looming stimulus is a highly salient, threatening stimulus which is likely important in both of these tasks, and hence is relevant information to be transmitted to the hippocampus for adaptive behaviour in the short and long term. Later, following reinstatement, these same loom-responsive ensembles may become updated with shock information and help drive visual looming-evoked fear responses.

What do we learn about the interaction of instinct and memory?

The visual looming fear response is mediated by innate circuitry in the SC and PAG which enables rapid defensive behavioural responses. Given the importance of this behaviour, it is no surprise that such an innate circuit evolved. However, there is also the need to maintain plasticity of behaviour to adapt to novel circumstances. As such, the brain solves this challenge by storing looming information in the hippocampus, which may be updated as new experiences provide new information. This organisation may be the result of the specific selection pressure looming is under, where execution of appropriate behavioural responses is needed immediately with little-to-no opportunities for learning. However, even this highly

innate behaviour which is so important to survival maintains mechanisms to allow plasticity of behaviour.

Remaining questions and future directions

The involvement of the vCA1 in visual looming fear responses leads to many questions. For example, what role do these ensembles play in innate loom responses, and in secondary defensive responses such as avoiding loom-associated contexts? Additionally, do these vCA1 ensembles recruit the same downstream regions in the reinstated response as the SC does in innate behaviour? And, out of the potential circuit mechanisms listed above, how does the vCA1 transmit and receive looming stimulus information?

One additional question which remains is what the precise nature of information carried by these loom-responsive ensembles is. Is the information here relating to the specific looming stimulus, or is it a general threat measure? There is already a precedent that different stimuli are represented by distinct ensembles in the vCA1. For example, unique ensembles can represent different contexts in the vCA1 (Ortega-de San Luis et al., 2023), and even similar nociceptive stimuli delivered by slightly different modalities show distinct representations in the vCA1 (Wang et al., 2024). As such, it stands to reason there must be some degree of information specificity to this vCA1 ensemble, but this remains to be demonstrated empirically.

Further questions remain as to what would happen if repeated reinstatement trainings were performed, or a stronger unconditioned stimulus was used to reinforce the behaviour. If the SC does indeed engage in slow plasticity, could sufficient training recover SC responses? Furthermore, would this recovery depend on the vCA1 activity? Future experiments should seek to further understand the relative roles of the SC and vCA1 in enabling plasticity of behaviour over varying time courses. Additionally, the role of the vCA1 ensembles in pre-habituation looming responses should be investigated. For example, are these vCA1 ensembles necessary for associating specific contexts with looming stimuli?

4. Trimethylamine olfactory responses in the *Mus* clade

This chapter aims to understand how a new behaviour can evolve by investigating the recently evolved role of TMA as an attractive social signal in *Mus musculus*. It characterises the degree to which this behavioural response depends on learning experience and internal state, as well as assessing the sex-dependent component of the behaviour. Furthermore, this chapter investigates the circuitry underlying TMA olfaction, with the aim of establishing a hypothesis as to the changes that have occurred between *Mus musculus* and *Mus caroli*.

4.1 Results

4.1.1 TMA olfactory response is concentration-dependent

In order to investigate olfactory behaviour in *Mus musculus*, a vacuum-powered olfactory arena was constructed based on the designs of Root et al., 2014. Mice freely explore the symmetrical arena, which receives air into four corners at a flow rate of 220ml/min. The air lines, under the control of a solenoid valve, toggle between a bottle containing water or a bottle containing odorant providing spatial and temporal control over odour presentation (figure 4.1.1A). Any spatial bias of the mice may be attributed to the presence of the odour in a given region (figure 4.1.1B).

To determine what concentration of TMA is most attractive, the spatial bias of mice within the arena was tested over a range of concentrations (figure 4.1.1C,D). A low concentration benzaldehyde (BNZ, 1mM) was included as a control, to ensure that odour investigation was as a result of attraction as opposed to novelty or lack of stimulus in the opposite half of the arena. Attraction of mice to TMA gradually increases as TMA concentration increases from 46mM, up to a maximum preference at 184mM. At 368 mM, mice were repelled from the TMA half of the arena. As a comparison, olfactory behaviour towards the predator odour TMT was also assessed over varying concentrations (figure 4.1.1E,F). Here peak attraction occurred at a concentration of 70mM, but this was not

significantly different from the pre-odour period. At 210mM and 250mM, TMT was aversive to mice.

4.1.2 *Mus caroli* do not produce TMA in their urine and are repelled by TMA

To investigate how TMA production varies across sex and species, urine from male and female *Mus musculus* and *Mus caroli* was collected for analysis by NMR-H1 spectroscopy. In accordance with previous reports (Li et al., 2013b), TMA concentration was significantly increased in male *Mus musculus* compared with female *Mus musculus*, as well as both sexes of *Mus caroli* (figure 4.1.1G,H). To determine whether *Mus caroli* retained the ancestral state of TMA-aversion, *Mus caroli* were assessed in a simple arena with 20μL of odorant placed at each end (figure 4.1.1I,J).

Mus caroli were found to be repelled by TMA at the same concentration which is attractive to *Mus musculus* (figure 4.1.1K). As such, it may be concluded that *Mus caroli* retain the ancestral response of TMA aversion.

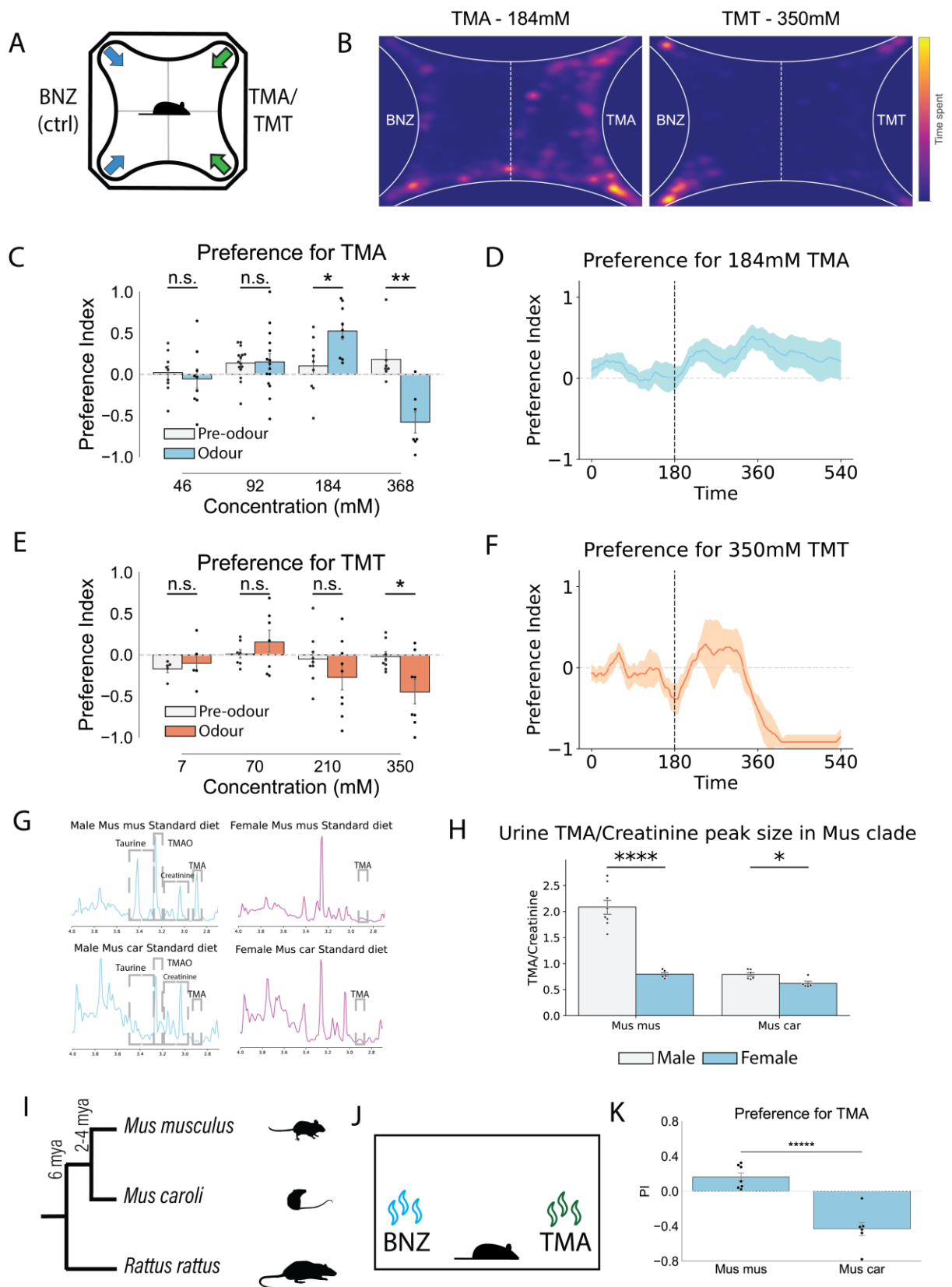


Figure 4.1.1 TMA response of the *Mus* clade is concentration-dependent and varies across species. (A) Schematic of arena used to assess olfactory valence. (B) Heatmap of attractive TMA response at 184mM ($n = 9$), and aversive TMT response at 350mM. (C) Response of *Mus musculus* to TMA over various concentrations (Student's paired T test; 46mM, $n = 9$; 92mM, $n = 15$; 184mM, $n = 9$; 368mM, $n = 7$). (D) Timetrace indicating how ever preference index evolves over time ($n = 9$, shaded area represents standard error of the mean). Odour presentation indicated by a vertical dashed line. (E-F) As in C-D, but for TMT (7mM, $n = 5$; 70mM, $n = 7$; 210mM, $n = 9$; 350mM, $n = 8$). (G) NMR H_1 spectra for male and female *Mus musculus* and *Mus caroli*. Dashed boxes indicate characteristic peaks locations for the labelled molecules (male *Mus mus*, $n = 8$; female *Mus mus*, $n = 5$; male *Mus car*, $n = 6$; female *Mus car*, $n = 5$). (H) TMA levels, normalised to creatinine, in both sexes of *Mus musculus* and *Mus caroli*. (I) Phylogenetic relationship of *Mus musculus*, *Mus caroli* and *Rattus rattus*. (J) Simple olfactory behavioural assay, with 20uL of odorant placed at each end. (K) *Mus caroli* and *Mus musculus* preference index in the simple olfactory behavioural assay (Students t test; *Mus mus*, $n = 7$; *Mus car*, $n = 6$). n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

4.1.3 *Mus musculus* TMA response depends on experience, social state, and sex

Male *Mus musculus* produce TMA in their urine, and therefore there may be a learned component to *Mus musculus* TMA responses. To inspect the learned component of TMA behavioural responses, mice were fed diet containing the broad-spectrum antibiotic doxycycline. This antibiotic ablates the gut bacteria which produce TMA (figure 4.1.2A,B). Mice raised by parents fed doxycycline were then either weaned onto a continued doxycycline diet, or a diet which did not contain doxycycline to prevent further inhibition of their gut microbiome. To confirm that doxycycline influences urinary TMA levels, TMA was collected from male and female *Mus musculus* fed a doxycycline diet (figure 4.1.2A-C). Doxycycline diet resulted in a significant decrease in urinary TMA levels in male mice compared to standard diet, although did not remove it completely (figure 4.1.2C). In female mice, doxycycline diet also results in a small but significant decrease in TMA from the already low levels.

Male and female mice across the various environmental conditions were tested for TMA attraction (figure 4.1.2F-G). Male mice raised on doxycycline that remain on the diet post-weaning show no attraction to TMA. Similarly, mice raised on doxycycline but switched to a standard diet after weaning show no attraction to TMA. Together, this suggests that mice which are not exposed to normal levels of TMA during the juvenile period do not develop TMA attraction, even if they later produce TMA themselves. To further understand the implications

of TMA as a social signal, male mice raised in a standard diet cage were then singly housed from the point of weaning. When the response of these single housed males was tested, they showed aversion to TMA, indicating that chronic social isolation induces TMA aversion (figure 4.1.2F).

As urinary TMA concentration is a sexually dimorphic phenotype, the sex-dependent response to TMA was investigated. The response of female mice to TMA was tested, followed by visual assessment of their stage of the estrus cycle. On average, female mice show no significant attraction or aversion to TMA. However, when the stage of the estrus cycle is considered, it was found that mice in estrus are attracted to TMA, while mice not in estrus were repelled by TMA (figure 4.1.2H). These results suggest that TMA is recognised as a male-specific signal by mice.

Previous reports have shown that TMA can reduce stress responses in mice during stress-inducing behavioural paradigms (Lee et al., 2022). To investigate if TMA can alter behavioural responses to innately threatening stimuli, mice were presented with visual looming stimuli either in the presence of TMA or H₂O (figure 4.1.3A-C). The presence of TMA prevented an increase in sheltering and freezing responses to the visual looming stimulus relative to pre-stimulus levels (figure 4.1.3A,B). To determine if other innately salient odours can also impact behavioural response to visual looming stimuli, the innately stress-inducing predator odour TMT was presented alongside a lower contrast looming stimulus which is insufficient to induce behavioural responses on its own (figure 4.1.3D-F). In this paradigm, there was no significant increase in either freezing or sheltering levels for either the TMT or H₂O exposed groups (figure 4.1.3E,F).

In summary, these results demonstrate that TMA is a male-specific social signal for *Mus musculus* which relies on infantile exposure to induce appropriate behavioural responses. The odour has sex-specific responses which depend on internal states, including stage of the reproductive cycle for females and social drive for males. Additionally, the odorant can alter behavioural responses to other stimuli as a result of its anti-stress effect. Meanwhile, *Mus caroli* retain the ancestral response of TMA aversion.

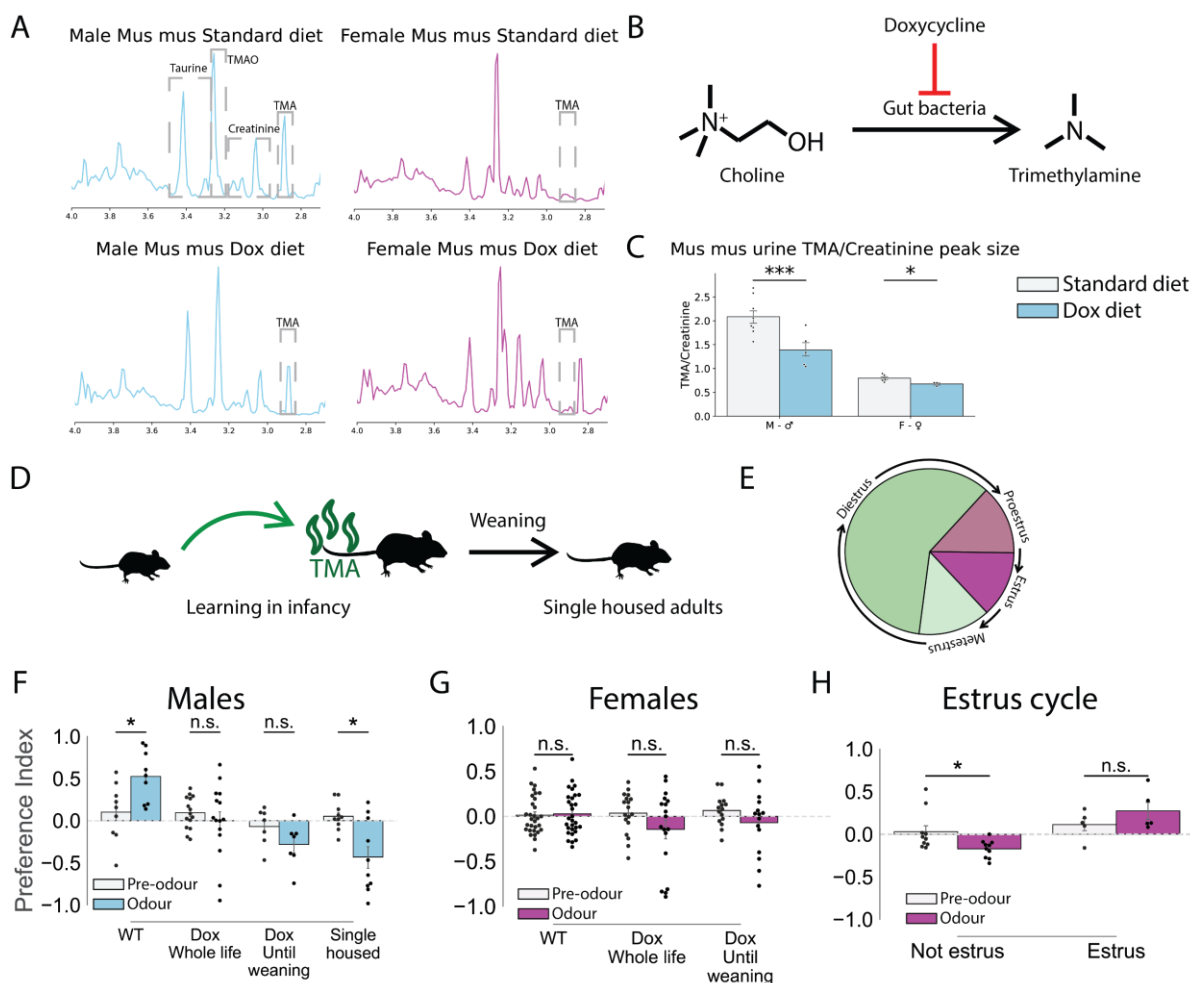


Figure 4.1.2 Mus musculus TMA response varies depending on exposure in infancy, social experience and sex. (A) NMR-H1 spectra of urine from male and female *Mus musculus* fed standard diet or diet containing the broad-spectrum antibiotic doxycycline (male *Mus mus* standard diet, $n = 8$; female *Mus mus* standard diet, $n = 5$; male *Mus mus* Doxycycline diet, $n = 5$; female *Mus mus* Doxycycline diet, $n = 3$). (B) Schematic illustrating how the antibiotic effects of doxycycline prevent TMA production by ablating gut bacteria. (C) Impact of doxycycline on urinary TMA in male and female mice, normalised to creatinine (Student's T test). (D) Schematic illustrating the single housing intervention, where mice are singly housed from the point of weaning. (E) Illustration of the estrus cycle. Female mice in estrus are more receptive to mating. (F) Experience dependent TMA behaviour in *Mus musculus* (Student's paired t test; WT $n = 9$; Dox whole life, $n = 15$; Dox until weaning, $n = 7$; Single housed, $n = 10$). (G) As in f, but for female mice (Student's paired t test; WT $n = 30$; Dox whole life, $n = 18$; Dox until weaning, $n = 15$). (H) Effect of estrus cycle state in female mice (Student's paired t test; Not estrus, $n = 10$; estrus, $n = 5$). n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

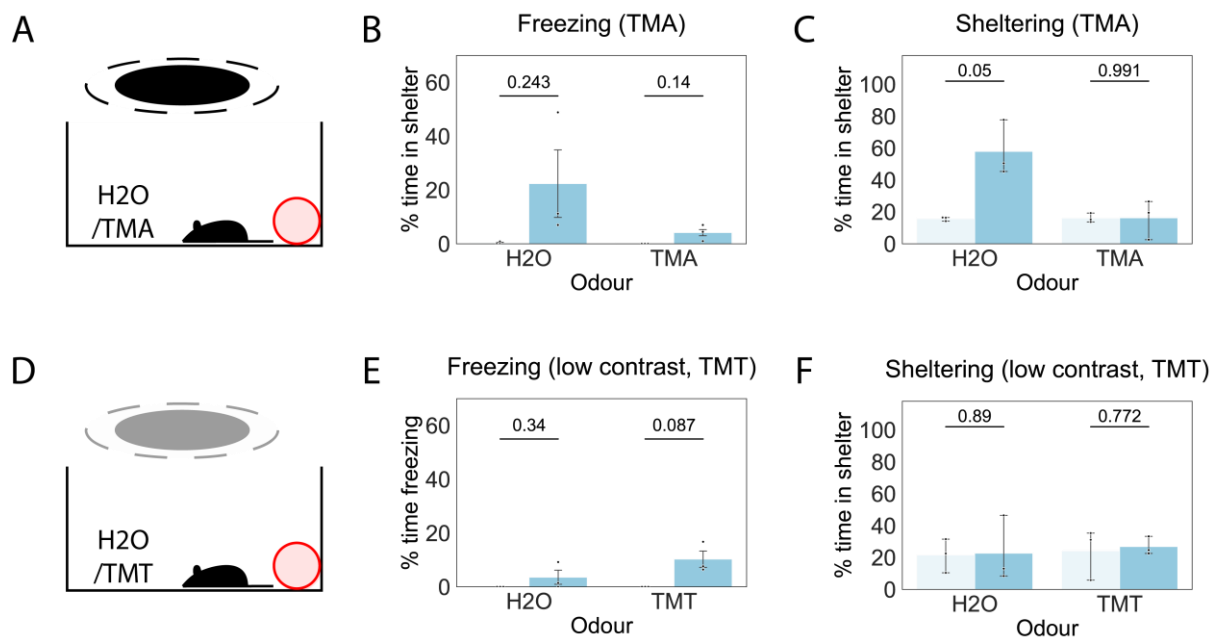


Figure 4.1.3 TMA and TMT olfaction influence threat responsiveness. (A) Behavioural paradigm for TMA effects on visual looming fear responses. (B) Freezing levels pre- and post-stimulus onset for H2O and TMA (Student's paired t test; H2O, $n = 3$; TMA, $n = 3$). (C) Sheltering pre- and post-stimulus onset for H2O and TMA (Student's paired t test; H2O, $n = 3$; TMA, $n = 3$). (D) Behavioural paradigm for TMT effects on low-contrast visual looming response. (E) Freezing levels pre- and post-stimulus onset for H2O and TMT (Student's paired t test; H2O, $n = 3$; TMT, $n = 3$). (F) Sheltering pre- and post-stimulus onset for H2O and TMT (Student's paired t test; H2O, $n = 3$; TMT, $n = 3$).

4.1.4 TMA olfaction induces different patterns of c-Fos expression in *Mus musculus* and *Mus caroli*

To determine how the brains of *Mus musculus* and *Mus caroli* respond to TMA, male *Mus musculus* and *Mus caroli* were exposed to air odorised with 184mM TMA or H₂O for 5min and perfused 60min later (figure 4.1.4A). Brains were sectioned and stained for c-Fos protein. Then, c-Fos expression was analysed throughout the olfactory cortex and the hippocampus (figure 4.1.4C-D). There was a statistically significant increase in c-Fos expression in the AON of both *Mus musculus* and *Mus caroli*. Meanwhile, the CoA and PAA showed a significant decrease in *Mus musculus*. The PAA showed no significant change in c-Fos levels in compared with H₂O in *Mus caroli*, however there was a significant increase CoA c-Fos during TMA olfaction in *Mus caroli*. In summary, there is an increase in c-Fos in the AON of both species during TMA olfaction, but divergent responses in the CoA across species. This highlights a potential site of divergence in the brain responses of the two species to TMA which may underly the divergent behavioural responses.

While the quantification of c-Fos in brain slices is a useful tool to characterise changes in activation of specific brain regions, the process is limited by it's need for extensive imaging time and manual counting. As such, it is less useful for exploratory investigations into the activity of many brain regions. To tackle this issue, a pipeline for the investigation of whole brain activity was established (figure 4.1.5A,B). Mice were exposed to either TMA or H₂O for 5min and perfused 60 min later. Brains were then cleared and stained as per the iDisco protocol (Renier et al., 2014) and imaged via light sheet microscopy. Raw images where then transformed into a common coordinate framework (CCF) based on the Allen Atlas using code developed by Dennis et al. (2023), with automated cell counting performed via ClearMap following manual parameter calibration to match cell detection rates with that of two human annotators (Kirst, 2020). The pipeline can successfully quantify c-Fos positive cells through Allen Atlas regions, as exemplified by a single H₂O and TMA-exposed brain (figure 4.1.5C-F). This pipeline lays the foundations for future research which will investigate brain-wide activity in both *Mus caroli* and *Mus musculus*.

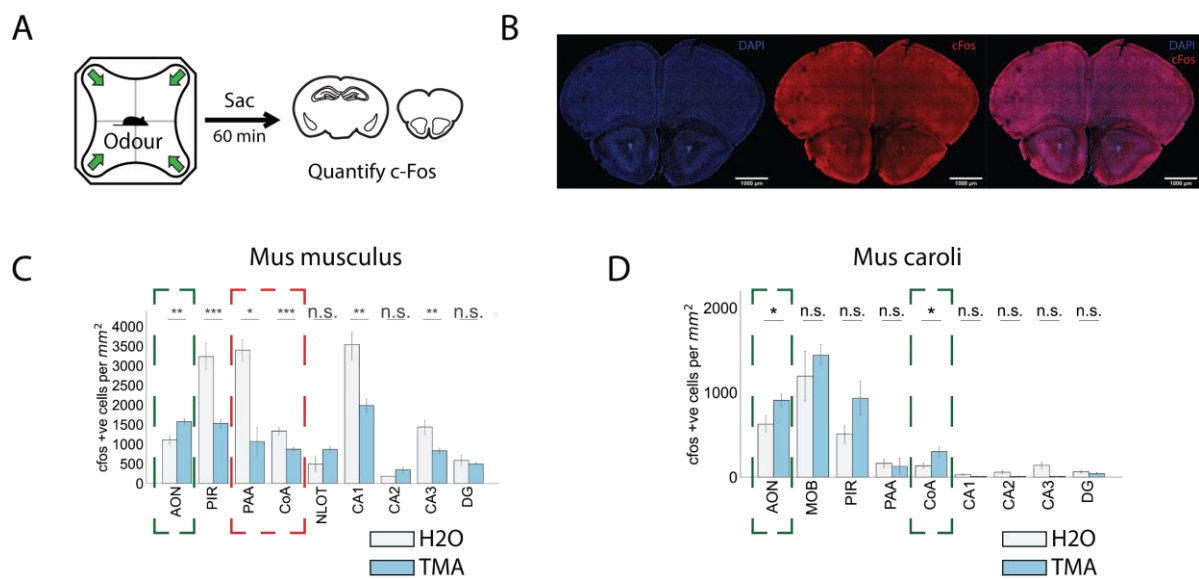


Figure 4.1.4 TMA olfaction leads to different patterns of activity in *Mus musculus* and *Mus caroli*. (A) Schematic indicating timeline for tissue harvesting following odour exposure. (B) Example slice including the AON stained for c-Fos. (C) Patterns of c-Fos expression on brain slices from *Mus musculus* (Student's unpaired t test with false detection rate correction; H₂O, n = 5; TMA, n = 6). (D) As in C, but for *Mus caroli* (H₂O, n=3; TMA, n=3). n.s. $p \geq 0.05$, # $p < 0.05$, * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$; where p is the Student's t test P value, and q is the false discovery rate-adjusted P value.

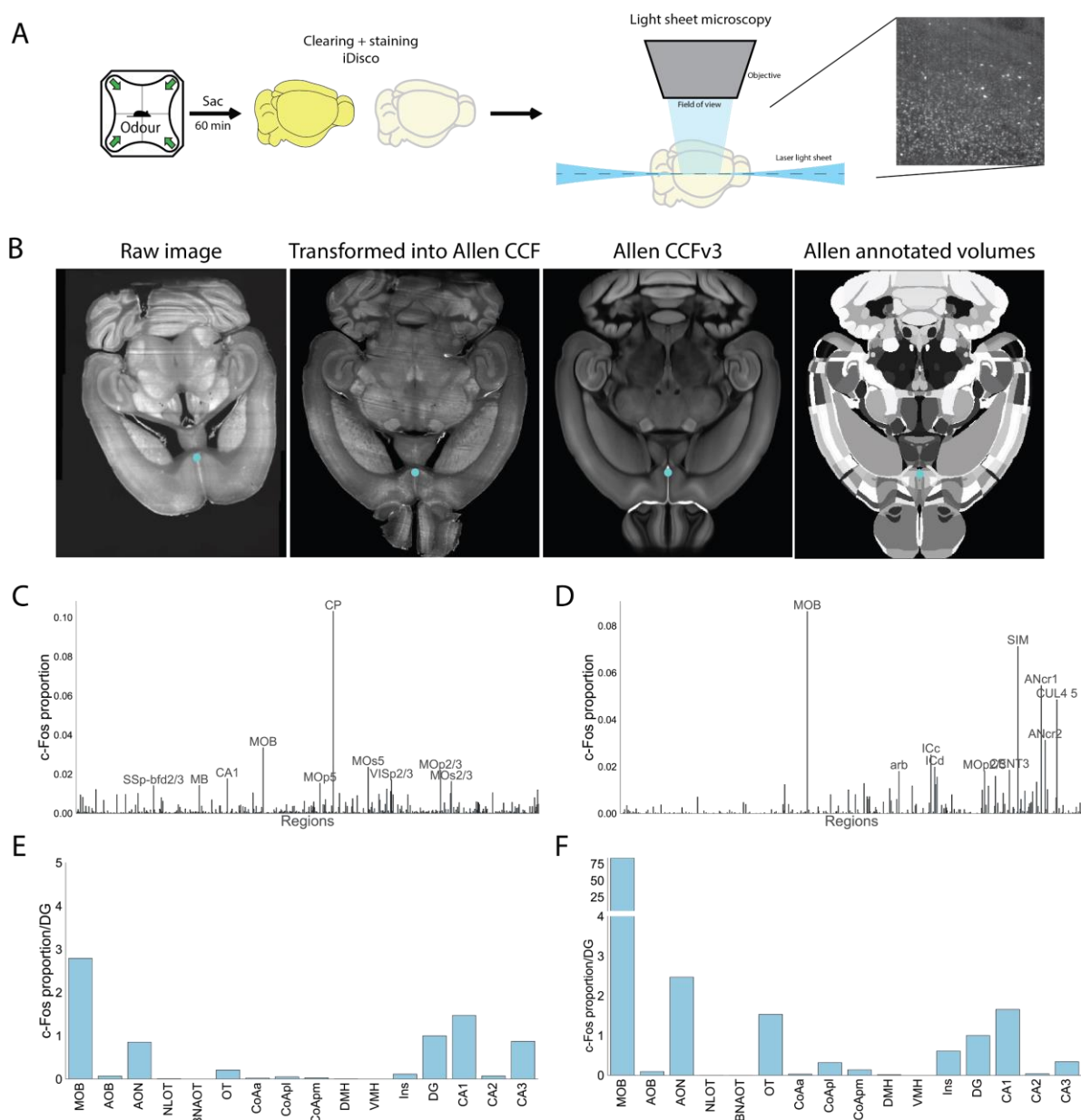


Figure 4.1.5 A pipeline for whole brain c-Fos analysis with light sheet microscopy. (A) Brains were cleared and stained according to the iDisco protocol and images on a light sheet microscope. (B) Raw images were transformed into a common coordinate framework and then fixed to the Allen Atlas template. Cells within each Allen Atlas region are quantified. (C) Relative c-Fos levels across the olfactory cortex and hippocampus in an example brain from a mouse exposed to H₂O. (D) Relative c-Fos levels across the olfactory cortex and hippocampus in the example brain from a mouse exposed to TMA. (E) Selected regions of interest from a H₂O exposed mouse, normalised to DG c-Fos. (F) Selected regions of interest from a TMA exposed mouse, normalised to DG c-Fos. ANcr1 = Crus 1, ANcr2 = Crus 2, CA1 = Field CA1, CENT3 = Lobule III, CP = Caudoputamen, CUL4 5 = Lobules IV-V, ICc = Inferior colliculus central nucleus, ICd = Inferior colliculus dorsal nucleus, MB = Midbrain, MOB = Main olfactory bulb,

MOp2/3 = Primary motor area Layer 2/3, MOp5 = Primary motor area Layer 5, MOs2/3 = Secondary motor area layer 2/3, MOs5 = Secondary motor area layer 5, SIM = Simple lobule, SSp-bfd2/3 = Primary somatosensory area barrel field layer 2/3, VISp2/3 = Primary visual area layer 2/3, arb = arbor vitae

4.1.5 Specific TMA-responsive ensembles in the AON, but not CoA, are sufficient to induce attraction in *Mus musculus*

To investigate whether TMA olfaction triggers global activity in the AON, or if there is heterogeneity in the responses of AON neurons to TMA, single-photon calcium imaging of the AON was performed on a single freely moving mouse while exploring a vacuum-powered olfactory assay (figure 4.1.6A,B). The activity of cells was recorded and normalised to activity throughout the session. Cells were then ranked based on the difference between their activity while in the TMA half of the arena versus the control odour benzaldehyde (BNZ) (figure 4.1.6C). The activity of these cells over space was then quantified and plotted as a heatmap (figure 4.1.6D,E). TMA responsive cells showed high spatial bias the TMA-side of the arena, and especially at the location of the odour inlet ports on the side wall (figure 4.1.6D). Meanwhile, non-TMA responsive cells showed less spatial bias overall (figure 4.1.6E). This indicates that only a subset of cells in the AON specifically respond to TMA.

To determine if TMA-responsive cells in the AON are capable of driving attraction, Male TRAP2 mice were injected with an AAV-DIO-ChR2 virus in the AON and implanted with optic fibres to allow c-Fos-dependent labelling of neurons during 4OHT injection and later used for optogenetic reactivation (figure 4.1.7A-B). Tagging of neurons was done following exposure to air odourised with either H₂O, TMA, or TMT.

Optogenetic stimulation was performed in a spatially controlled manner, with the laser being turned on while the mouse was in one half of the arena, and off in the other half. There were no other spatial cues in the symmetrical arena.

In the AON, optogenetic stimulation of neurons tagged during TMA olfaction, but not clean air or TMT, was sufficient to induce attraction to the laser-on half of the arena (figure 4.1.7C-F). In summary, this data suggests that the AON contains cells which respond specifically to TMA, and that the activation of these cells is sufficient to induce TMA attraction.

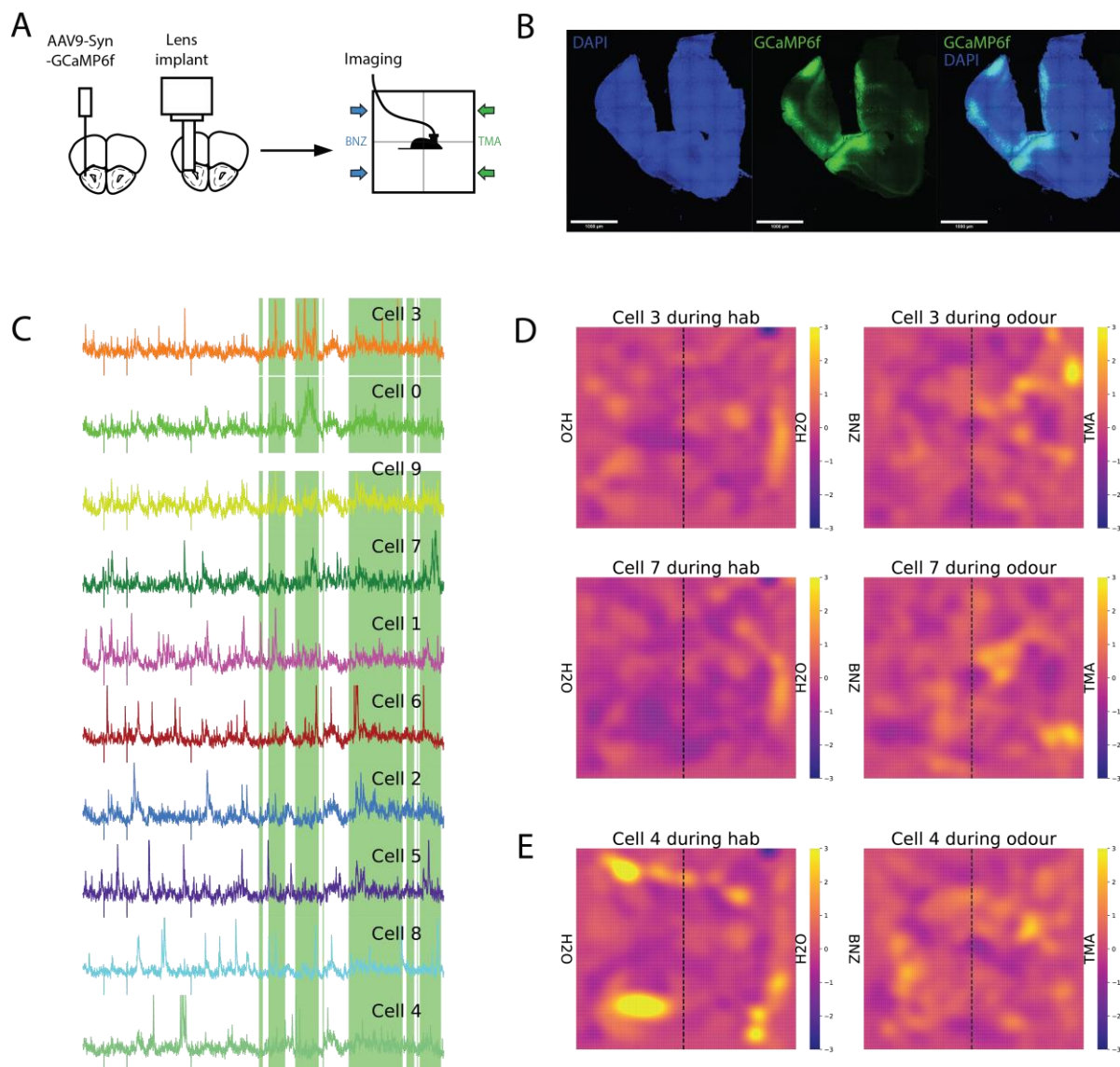


Figure 4.1.6 Calcium imaging TMA-responsive cells in the AON of a single mouse. (A) Schematic indicating timeline for calcium imaging surgery and behaviour (B) Example AON slice showing viral expression and implant track. (C) Timetraces of individual calcium transients of detected cells from an example mouse, ranked by TMA responsiveness compared with non-TMA responsiveness. (D) Example Heatmaps of TMA-responsive cells before odour presentation (left) and during odour presentation (right). (E) As in D, but for the least TMA-responsive cell.

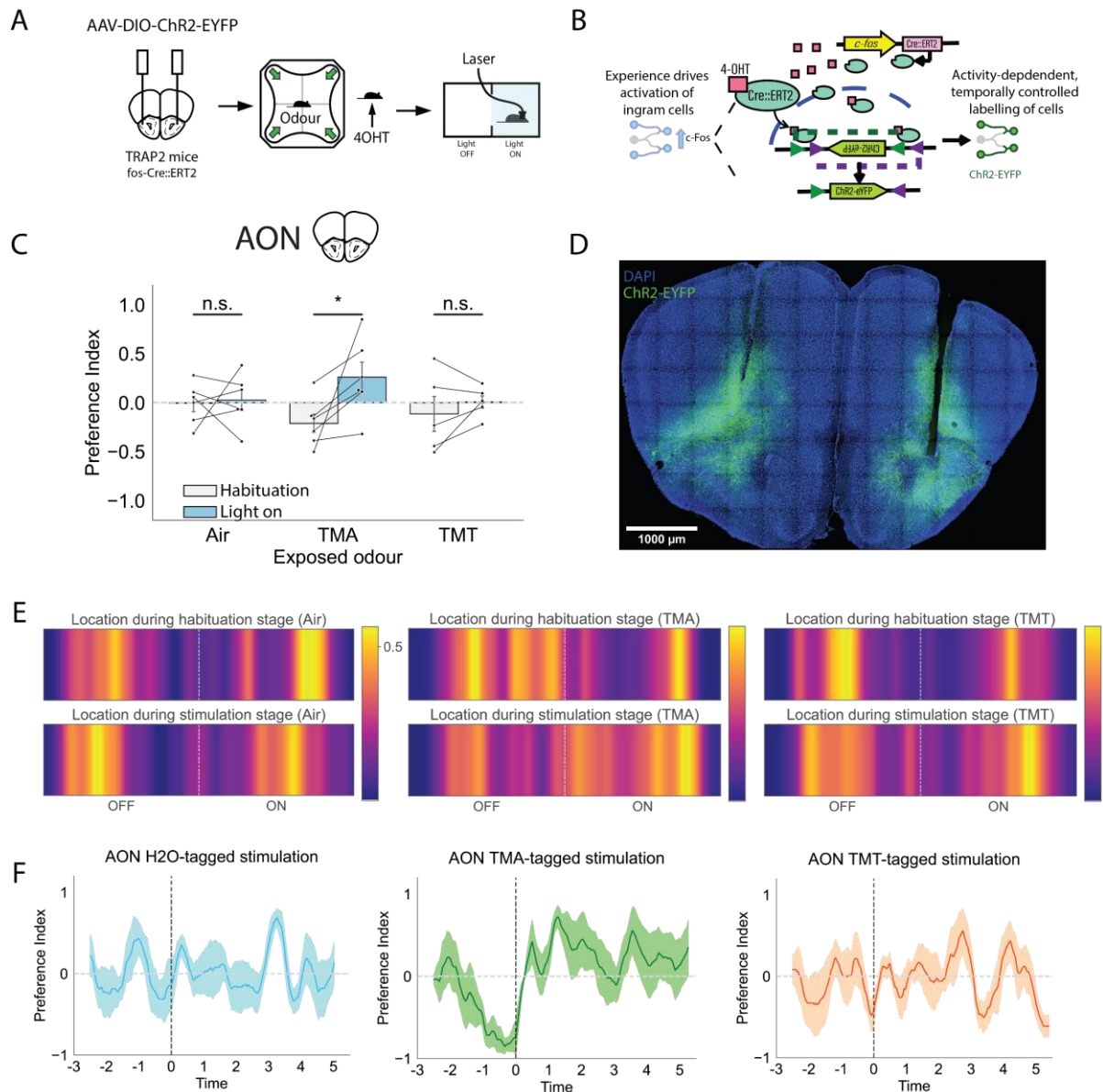


Figure 4.1.7 The activity of specific TMA-responsive cells in the AON are sufficient to induce attraction. (A) Schematic indicating behavioural timeline for engram labelling and spatially-controlled engram reactivation. The laser is activated when the mouse enters one half of the arena. (B) Engram labelling strategy. (C) Total preference index pre- and post-onset of laser stimulation for AON cells tagged during exposure to clean air, TMA or TMT (Student's *t* test; Air, *n* = 6; TMA, *n* = 6; TMT, *n* = 5). (D) Example AON slice showing viral expression and implant track. (E) 1-dimensional heatmap indicating time spent on each side of the chamber. (F) Timetrace indicating a 30s moving window of preference index. Laser onset indicated by dashed line. n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

As the CoA was identified as a region with divergent activity between *Mus musculus* and *Mus caroli*, the functional role of the CoA was investigated. TRAP2 mice were injected with AAV-DIO-ChR2 virus and optic fibers were implanted in the CoA (figure 4.1.8A,B). There was no significant attraction to the laser-on half of the arena during stimulation of cells tagged during clean air, TMA-odourised air, or TMT-odourised air relative to baseline (figure 4.1.10C-G). For TMT-tagged cells, there no significant difference change in preference. In summary, the activation of specific TMA-responsive cells of the AON, but not the CoA, is sufficient to induce attraction in *Mus musculus*. As such, the AON is capable of driving the TMA attraction behavioural response.

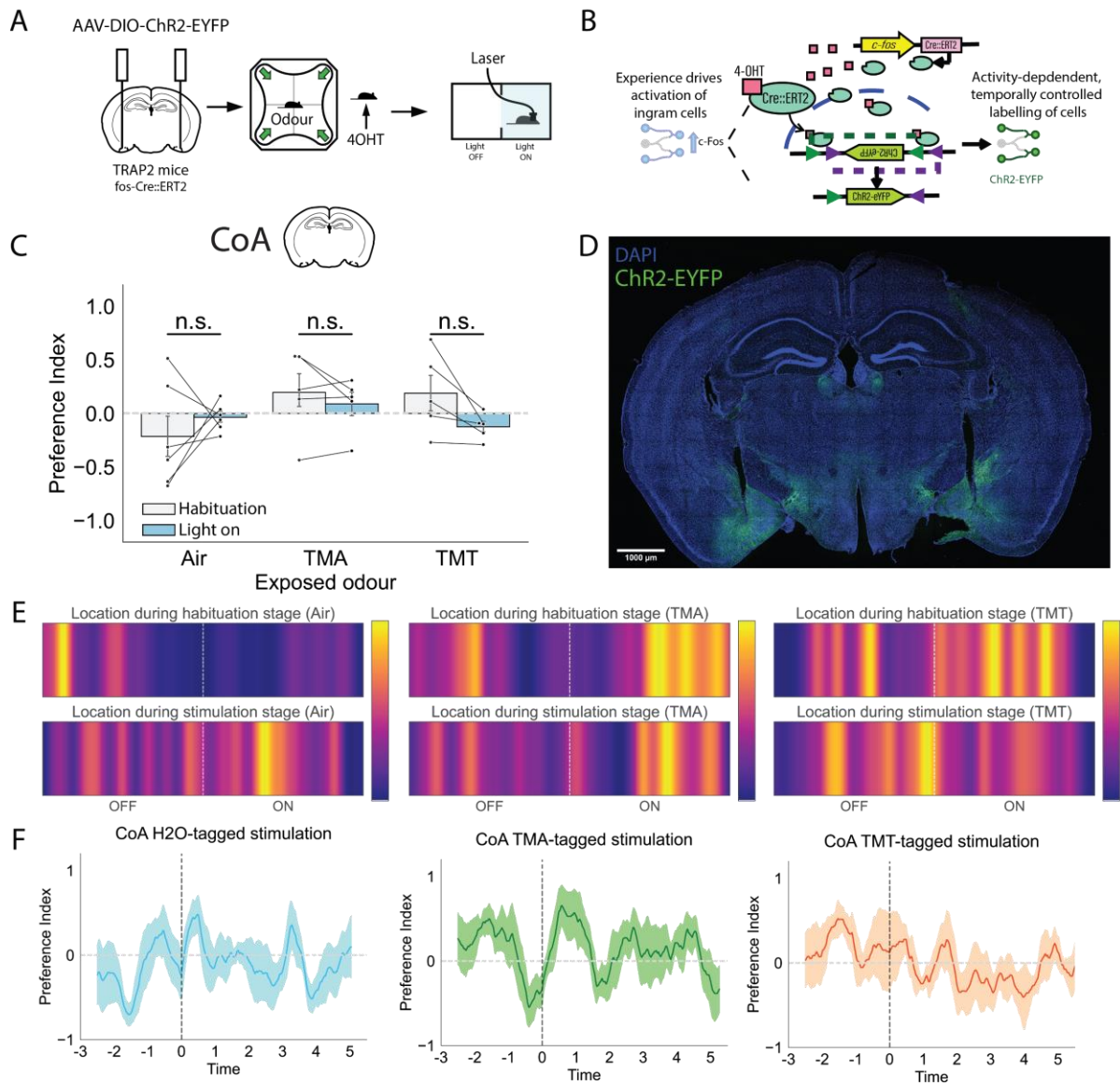


Figure 4.1.8 The activity of specific TMA-responsive cells in the CoA are not sufficient to induce attraction. (A) Schematic indicating behavioural timeline for engram labelling and spatially-controlled engram reactivation. The laser is activated when the mouse enters one half of the arena. (B) Engram labelling strategy. (C) Total preference index pre- and post-onset of laser stimulation for CoA cells tagged during exposure to clean air, TMA or TMT (Student's *t* test; Air, *n* = 6; TMA, *n* = 5; TMT, *n* = 5). (D) Example CoA slice showing viral expression and implant track. (E) 1-dimensional heatmap indicating time spent on each side of the chamber. (F) Timetrace indicating a 30s moving window of preference index. Laser onset indicated by dashed line. n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

4.1.6 Specific TMA-responsive ensembles in the MOB and AON, but not the CoA, are necessary for TMA attraction

It is clear that the AON is active during TMA attraction, and that TMA responsive cells in the AON are capable of driving attraction. However the question remains as to whether the activity of these ensembles are required to produce TMA attraction. Further, while the CoA does not appear to drive the TMA response, it could be possible that the activity of cells in this region is necessary for TMA attraction.

To test the necessity of TMA-responsive ensembles in producing TMA attraction, TRAP2 mice were injected with AAV-DIO-HM4D(Gi) virus in either the main olfactory bulb (MOB) (figure 3.2.7), AON (figure 3.2.8) or CoA (figure 3.2.9). MOB was included as a positive control for the inhibitory effect of DREADDs, and injections were specifically targeted into the dorsomedial region where the TMA-responsive TAAR5 glomeruli are present. As these glomeruli are the only direct recipient of TAAR5-expressing chemosensory neurons, inhibition of these cells should prevent detection of TMA. Following recovery, mice were exposed to TMA and injected with 4OHT to induce DREADD expression in c-Fos-expressing cells. Then, mice underwent two tests for TMA preference: one with injection of CNO to activate the inhibitory DREADD, and one with injection of saline. The order of these tests was randomised between mice.

Inhibition of the MOB neurons resulted in a negative preference index towards TMA compared with the control odour BNZ, while saline injection permitted TMA attraction (figure 4.1.9C). However, TMA attraction following saline injection in MOB-DREADD mice was not statistically significant, possibly due to low level constitutive activity of the DREADD (Guettier et al., 2009, Aldrin-Kirk et al., 2016). Overall, this indicates that TMA-responsive cells in the dorsomedial region of the MOB are necessary for TMA attraction. Further, the negative preference suggests that in the absence of MOB activity, TMA is less attractive than the neutral control odour BNZ. This could suggest that in the absence of these cells, TMA becomes aversive.

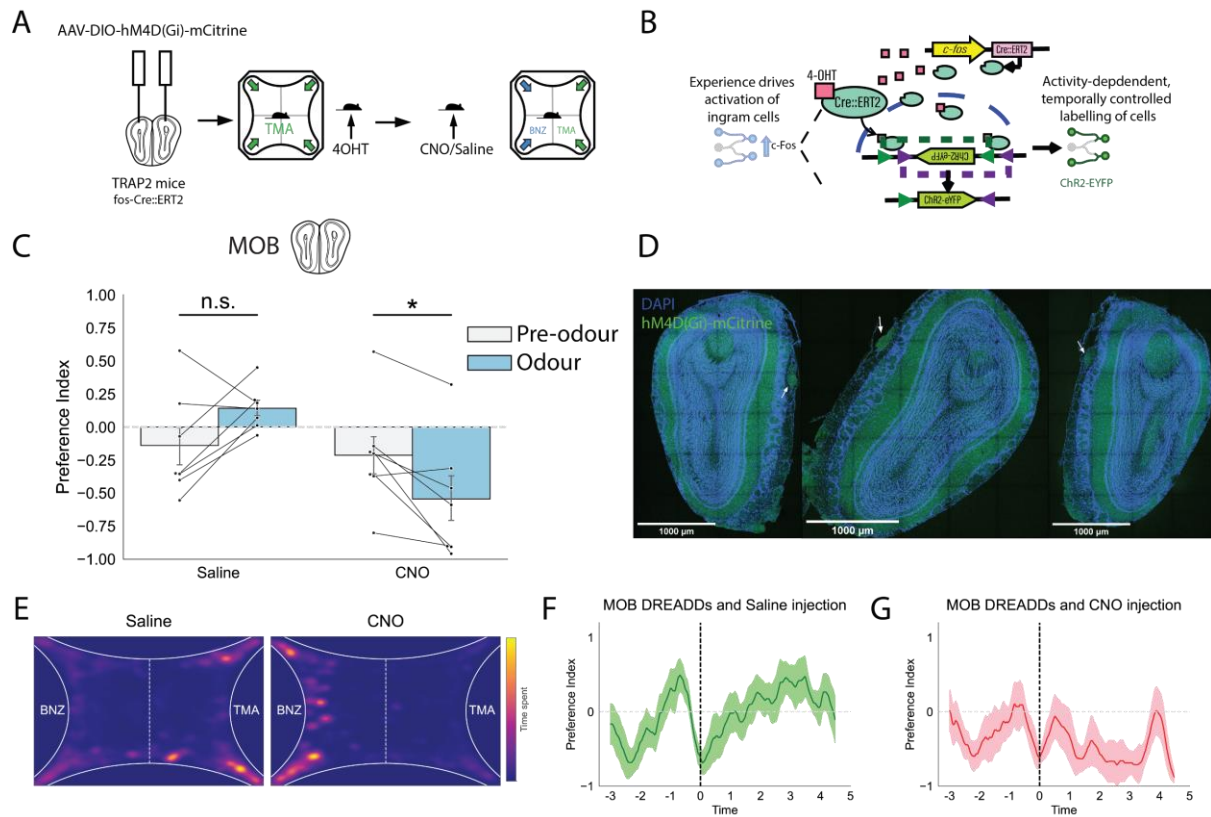


Figure 4.1.9 The activity of TMA-responsive cells in the Main Olfactory Bulb (MOB) are necessary to induce TMA attraction. (A) Schematic indicating behavioural timeline for engram labelling and engram inhibition during TMA olfaction. (B) Engram labelling strategy. (C) Effect of no inhibition (saline) versus inhibition (CNO) of MOB cells tagged during exposure to TMA (Student's *t* test; saline, *n* = 7; CNO, *n* = 7). (D) Example MOB slice showing viral expression. mCitrine positive glomeruli are annotated with white arrows. (E) Heatmap showing location of mice during odour presentation during inhibition (CNO) or no inhibition (Saline) of TMA-responsive cells in the MOB. (F) Timetrace indicating a 30s moving window of preference index. Odour onset indicated by dashed line. *n.s.* $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Next, the necessity of TMA-responsive ensembles in the AON was investigated (figure 4.1.10A,B). Saline injected mice maintained statistically significant attraction to TMA relative to baseline, while inhibition via CNO injection resulted in a statistically significant negative preference index for TMA (figure 3.2.8C-G). As such, TMA-responsive cells in the AON are necessary for TMA attraction. Similar to what was observed with the MOB, inhibition of these cells resulted in a negative preference for TMA relative to the neutral control odour BNZ. As such, TMA also become aversive in when AON cells are inhibited. However, this was to a lesser magnitude than what was observed for the MOB.

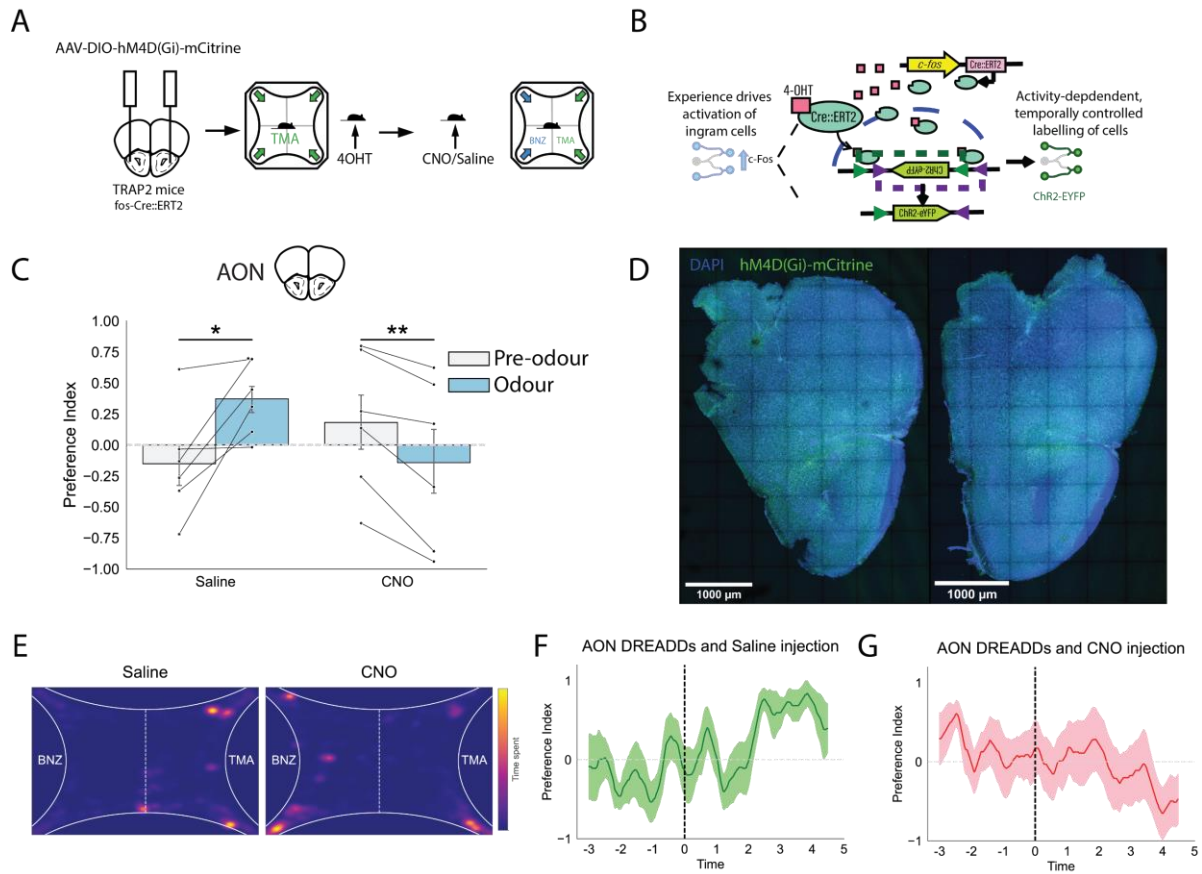


Figure 4.1.10 The activity of TMA-responsive cells in the AON are necessary to induce TMA attraction. (A) Schematic indicating behavioural timeline for engram labelling and engram inhibition during TMA olfaction. (B) Engram labelling strategy. (C) Effect of no inhibition (saline) versus inhibition (CNO) of AON cells tagged during exposure to TMA (Student's *t* test; saline, *n* = 6; CNO, *n* = 6). (D) Example AON slice showing viral expression. (E) Heatmap showing location of mice during odour presentation during inhibition (CNO) or no inhibition (Saline) of TMA-responsive cells in the AON. (F) Timetrace indicating a 30s moving window of preference index. Odour onset indicated by dashed line. n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

The necessity of TMA-responsive cells in the CoA was also investigated (figure 4.1.11A,B). Saline injection maintained a positive preference for TMA, indicating attraction. Inhibition of CoA ensembles via CNO injection resulted in no change in preference index relative to baseline, indicating a neutral preference (figure 4.1.11C-G). This result indicates that TMA was neutral relative to the control odour BNZ. As such it may be interpreted that inhibition of TMA-responsive neurons in the CoA reduces TMA attraction, but does not ablate it entirely.

In summary, TMA-responsive cells are necessary for TMA attraction, and inhibition of these cells results in a strong negative preference towards TMA, potentially indicating aversion. The AON also contains TMA-responsive cells, and these cells are both sufficient to induce attraction when optogenetically stimulated, and their activity is necessary for attraction to TMA. Inhibition of these cells also results in a slight negative preference, indicating mild aversion. Finally, TMA-responsive cells in the CoA are not sufficient to induce TMA attraction when stimulated optogenetically, but inhibition of these cells does result in a reduction in TMA attraction.

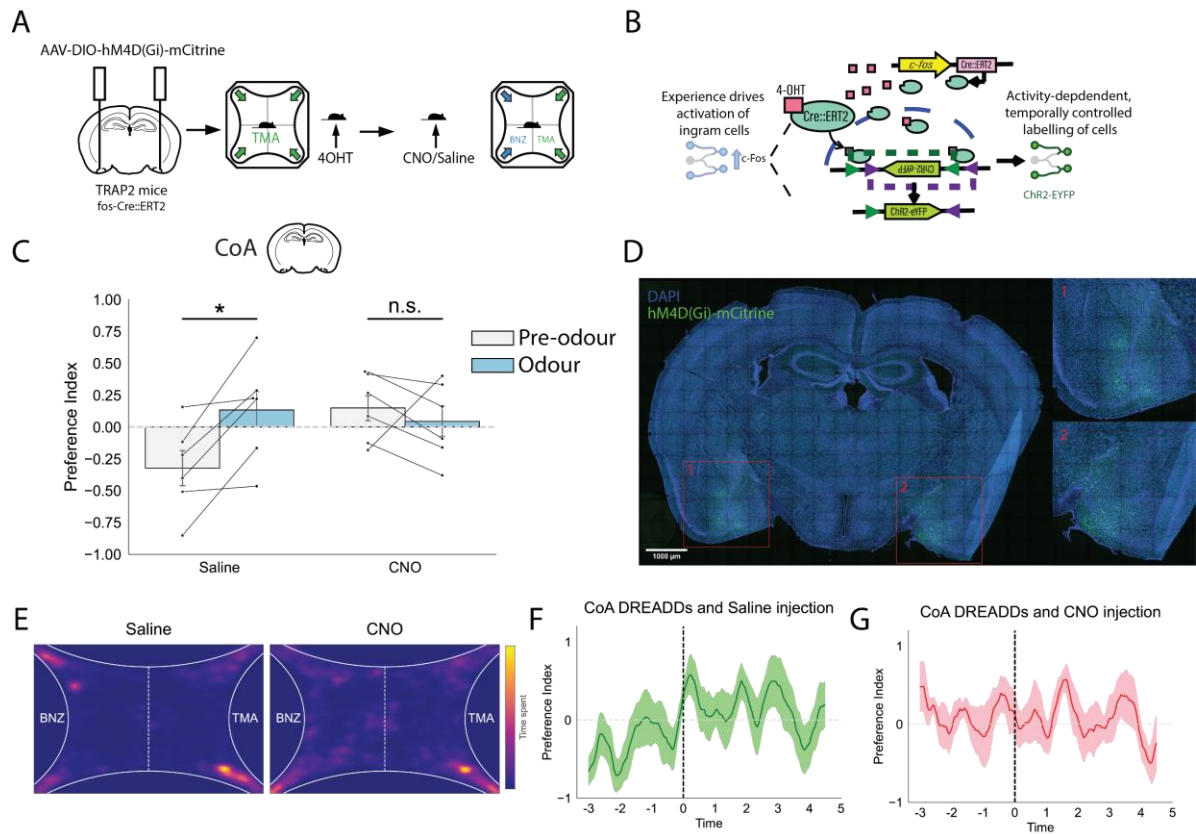


Figure 4.1.11 The inhibition of TMA-responsive cells in the CoA has a limited effect on TMA attraction. (A) Schematic indicating behavioural timeline for engram labelling and engram inhibition during TMA olfaction. (B) Engram labelling strategy. (C) Effect of no inhibition (saline) versus inhibition (CNO) of CoA cells tagged during exposure to TMA (Student's *t* test; saline, *n* = 612120, *n* = 6). (D) Example CoA slice showing viral expression. (E) Heatmap showing location of mice during odour presentation during inhibition (CNO) or no inhibition (Saline) of TMA-responsive cells in the CoA. (F) Timetrace indicating a 30s moving window of preference index. Odour onset indicated by dashed line. n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

4.2 Discussion

Summary

This project establishes the *Mus* clade olfactory behavioural responses to TMA as a cross-species model for the investigation of innate and learned olfactory behaviours and how they may change over the course of evolution. The TMA olfactory response of *Mus musculus* has been shown to rely on experience in infancy, in contrast to the totally innate aversion of *Mus caroli*. It identifies the AON as both necessary and sufficient for the learned TMA attraction in *Mus musculus*, while showing the CoA is neither necessary nor sufficient. Future studies will investigate the role of both of these regions in *Mus caroli*, with the hypothesis that innate TMA aversion will be driven by the CoA. Furthermore, this study highlights how a recently evolved innate behaviour may rely on both instinct and memory to enable adaptive behaviour.

***Mus musculus* evolved to suppress TMA-aversion and form attraction during a critical period**

The data presented here demonstrates that *Mus caroli* retain the ancestral state of TMA aversion, while *Mus musculus* form an attraction to TMA via exposure to the odour in the urine of conspecifics during a critical period (figure 4.2.1). In the absence of this exposure, *Mus musculus* become neutral to TMA, indicating that *Mus musculus* have evolved to lose the innate TMA aversion of their ancestors and sister species, and rely on critical period learning to develop learning. Experience in infancy has been shown to have long-term effects on olfactory behaviour in mice. For example, recognition of lactating dam odour by infants is dependent on an early critical period (Logan et al., 2012) and presentation of predator odour in infancy has been shown to prevent aversion later in life (Qiu et al., 2021a). In fact, it has also been shown that exposure to additional TMA in infancy can prevent attraction later in life (Qiu et al., 2021a), suggesting that this critical period may even be innately tuned into ethologically relevant concentrations.

Why would the TMA olfactory response in *Mus musculus* evolve to have such a phenotype? One possible explanation is that, given that TMA is produced by the infants' parents, there is adequate learning opportunity to learn the role of TMA, hence removing the selection pressure to develop innate TMA attraction. In fact, the behaviour response being plastic may even be beneficial, as the ethologically relevant concentration of TMA can vary

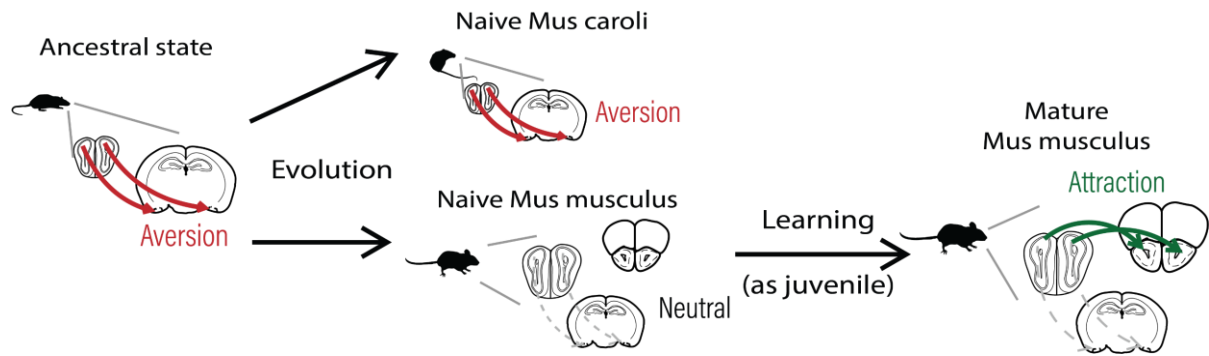


Figure 4.2.1 Evolution of the *Mus* clade TMA responses. Common ancestors of *Mus musculus* and *Mus caroli* were repelled by TMA, which may have been driven by the cortical amygdala. *Mus musculus* have lost this innate aversion, and instead rely on learning as juveniles to form AON-driven attraction.

between individuals (figure 3.2.2C). Meanwhile, the mice needed to lose the innate aversion to TMA over the course of evolution, as the presence of this circuitry innately may compete with the learned TMA attraction, wasting resources and possibly leading to non-adaptive behavioural responses. As such, the TMA attraction in *Mus musculus* likely evolved via two concurrent processes: the loss of innate TMA aversion, and the development of a critical period-dependent, learned TMA attraction.

TMA is a sexually dimorphic social signal

The NMR-H1 spectra replicate the findings of previous work (Li et al., 2013b) indicating that TMA is produced in *Mus musculus* but not *Mus caroli*, and that within *Mus musculus* there is significant sexual dimorphism in the amount of TMA produced in the urine. In complement with the sexually dimorphic phenotype of TMA production, the behavioural response to TMA appears to be sex-specific as well. In general, male mice show attraction to TMA. The response of female mice depends on their stage of the estrus cycle, with sexually receptive females being attracted to TMA and non-receptive females being repelled by TMA. This information indicates that the sex-specific component of TMA is recognised by *Mus musculus* males and females. These findings are in line with the reported findings on same-sex and opposite-sex social interactions in both male and female *Mus musculus*. For example, male *Mus musculus* show behavioural attraction to novel same-sex conspecifics (Yang et al., 2011), while females have been shown to engage in sexual behaviour only while in estrus stages (Kimchi et al., 2007,

Moncho-Bogani et al., 2004). Interestingly, the female aversion to TMA while not in estrus seems to be a unique property of TMA as opposed to male mice in general, as female mice have been shown to approach male cages in a three-chamber social interaction test while not in estrus, just without engaging in proceptive behaviour (Moncho-Bogani et al., 2004). The social role of TMA is further backed up by the novel finding that chronically isolation, which induces social aversion (Lee et al., 2021a, Ma et al., 2011, Zelikowsky et al., 2018), also causes aversion to TMA. In summary, TMA is recognised as a male-specific social signal.

As a social signalling molecule, the effect of TMA on the brain and behaviour is likely complex and multi-faceted. TMA is reported to have an anti-stress effect in various stress paradigms (Lee et al., 2022). This is corroborated by the finding in this project that TMA presence during visual looming stimulus presentation results in reduced freezing and sheltering behaviour. This may be a consequence of the social signalling role of TMA, as the presence of conspecifics during nociception is reported to have similar effects (Langford et al., 2006, Langford et al., 2010, D'Amato, 1998). Another factor in this includes the reported finding that applying TMA to juvenile males placed in a breeding males cage in a resident-intruder paradigm results in increased aggression by the resident mouse towards the juvenile (Cichy et al., 2021). This could be because the presence of TMA signals sexual maturity, and therefore competition for the older male. In line with this interpretation, earlier studies on the role of TAAR5 demonstrate that the TAAR5 receptor is only responsive to male mice urine once they reach puberty (29days) (Liberles and Buck, 2006) but not before. This observation is again in corroboration with the fact that female mice are attracted to TMA while sexually receptive, as it will signal the presence of sexually mature males.

In summary, TMA likely serves as a social signal indicating sexually mature males. The effects of this include behavioural attraction for males, estrus cycle-dependent attraction and aversion in females and anti-stress effects during stressful paradigms.

TMA olfaction leads to divergent activity across *Mus musculus* and *Mus caroli*

In complement with the divergent behavioural responses between *Mus musculus* and *Mus caroli*, c-Fos counts have revealed divergent activity between the two species in different regions of the olfactory cortex. While both species show an upregulation of c-Fos in the AON during TMA olfaction relative to H₂O, *Mus musculus* show a decrease in c-Fos in the CoA while *Mus caroli* report an increase. Further, this work highlights that the activity of specific TMA-

responsive cells in the AON is both necessary and sufficient to induce behavioural attraction, while the CoA is not. While the functional significance of these regions has not yet been assessed in *Mus caroli*, the upregulation of the CoA, alongside the well-established role of the CoA in innately aversive olfactory behaviour (Howe et al., 2024, Root et al., 2014), suggests that the CoA may be implicated in innate olfactory aversion in *Mus caroli*.

Mus musculus raised on doxycycline (and hence have no learning experience towards TMA) are neutral to TMA. This fact suggests that the potentially CoA-driven aversion towards TMA has been removed over the course of evolution. Mice which are exposed to TMA develop an AON-driven attraction to TMA, and hence it stands to reason that an AON engram may form during infancy which associates the odorant with mature male mice. These AON cells may then be involved in orchestrating downstream responses, such as inhibition of the CoA or activation of inhibitory neurons in the VMH (Lee et al., 2022), either via direct or indirect projections.

Instinct and memory are tightly intertwined in *Mus musculus* TMA olfaction

The *Mus musculus* TMA olfactory response has both innate and learned components, evolving from ancestors with an opposite behavioural response. While learning is clearly necessary to develop TMA attraction, it must occur during a defined time period and appears to depend on an expected concentration being presented. As such one may consider this behaviour to be at the intersection of instinct and memory. What are the selective pressures that result in the evolution of a phenotype such as this?

Firstly, TMA is aversive to competitors and predators, so the production of TMA in urine may have provided a selective advantage by acting as an allomone and deterring competitors and predators from the nest sites of *Mus musculus* where TMA would be most abundant (Li et al., 2013b). However, in order for the production of TMA to not be isolating for TMA-producing individuals, concurrent evolution of a change in the behavioural response would have been necessary. Once TMA production was established in the population, a phenotype of TMA-aversion would have been strongly selected against. Further, the offspring of TMA-producing individuals would have been exposed to TMA, potentially forming memories associating the odour with social signals (although to what extent this step may have been refined by evolution is unknown). Given that this learning experience is non-costly and easy to learn (it occurs in the safe home environment or during interactions with

conspecifics), there is limited selection pressure to canalise this behaviour as an instinct. In fact, there may even be advantages to not canalising the behaviour entirely, such as allowing the ability to fine tune to certain concentrations. As such, evolution has replaced innate aversion of TMA in the ancestors of *Mus musculus* with innate “neutrality” in *Mus musculus* which is overwritten by a TMA engram implicated in social behaviour under normal circumstances.

Remaining questions and future directions

The present study has focused on TMA-responsive ensembles in the AON and CoA in *Mus musculus*. While this has been illuminating in revealing the functional role of these regions in *Mus musculus*, the *Mus caroli* response can only be speculated upon by extrapolation from the c-Fos data and the known role of these regions in *Mus musculus*. Future studies should perform functional manipulations on the AON and CoA in *Mus caroli* to demonstrate what role they may have. For example, we may hypothesise that the CoA drives innate aversion in *Mus caroli*, but what of the AON – a region activated in both species? It could be the case that the AON contributes to innate TMA aversion as well. Alternatively, the AON may play a role in guiding learned responses to TMA, perhaps comparable to the role of the vCA1 in the visual looming response discussed in the previous chapter. Further, in both species, the AON and CoA are likely a component of a greater response across the whole brain which will trigger not just odour attraction or aversion, but include social information, have context-dependent effects on aggression, sexual behaviour and stress levels. Ongoing investigation will contribute to addressing these questions by identifying differences in how the brain responds to TMA between the two species, as well as across varying behavioural conditions in *Mus musculus*. Nonetheless, any regions identified within this project will then need to be further investigated in more detail.

Beyond this, this model presents a unique opportunity to reverse engineer an instinct via learning. *Mus caroli* retain the ancestral state of TMA aversion, but what would happen if *Mus caroli* were raised in an environment in the presence of TMA? This could be achieved by cross fostering *Mus caroli* pups with *Mus musculus* parents. Alternatively, TMA production could be artificially induced in *Mus caroli* by genetically induced knockdown of the *fmo3* gene. How would *Mus caroli* raised in such an environment respond to TMA, and which circuitry would this behaviour depend on? By comparing this response with the response in *Mus*

musculus, we may observe how evolution can change a brain from a totally learned to a partially innate behavioural response.

Another remaining question pertains to how the social role of TMA specifically is learned. Until the mouse TMA response is tested, mice have never been presented with the TMA odour in isolation. This is an important consideration, as combined odour identities are not a simple sum of their parts, often resulting in combined odour effects that may seem non-intuitive (Saraiva et al., 2016). For example, combining the neutral odour eugenol with the attractive TMA causes aversion, despite neither odour on its own causing aversion (Qiu et al., 2021b). So, how is the specific TMA component of mouse urine separately recognised as a social signal? It could be the case that TMA plays an important role in distinguishing the sexes for example, and hence the mouse is particularly sensitised to the detection of TMA. Further, perhaps the contrasting odours of male and female urine allows the developing mouse brain to recognise components such as TMA as specific indicators.

In addition to these questions, the precise role of development in TMA attraction remains to be clearly dissected. Within what time range is TMA exposure required to induce TMA attraction? Does altering the exposure change the response to TMA broadly, or does it simply shift the concentration at which mice find TMA attractive? Further, what are the precise circuitry changes that occur during TMA olfaction, and how does this interact with social information to produce a recognisable social signal? This final question in particular may implicate other brain regions which have not been investigated in this study. For example, the piriform cortex, which is involved forming more abstract associations with odours (Bouret and Sara, 2002, Haberly, 2001, Miyamichi et al., 2011), may receive social information from other brain regions, and update the AON ensemble with this information.

5. A computational model on the impact of learning on evolution

The previous two chapters have provided insight into how instinct and memory interact in the brain, revealing how learning can facilitate adaptive plasticity of innate behaviour and how instinct can depend on learning experience to form. Furthermore, by taking a cross-species approach, we have been provided a perspective as to how a new behaviour can evolve under the influence of learning. However, to understand how memory, instinct and evolution interact with each other over generations, one would need to directly observe and manipulate each of these factors in real-time. Doing this presents quite a logistical challenge. Even with rapidly reproducing organisms like *Drosophila melanogaster*, it may take many generations to observe a change in behaviour. Further, an experimenter will wish to test specific factors which they hypothesise affects how a behaviour evolves, but currently there is a limited understanding of what these factors may be.

This chapter will aim to address some of these concerns. Here I propose to use the olfactory behavioural response of *Drosophila melanogaster* as a model for future artificial selection experiments, by verifying variation across species and demonstrating plasticity of the behavioural response in *Drosophila melanogaster*. Next, I design a basic computational model to generate predictions as to how instinct and memory may interact with evolution. I further build on this model to create a simulation with a population of polygenic organism, with genes affecting both innate behaviour and plasticity, undergoing an artificial selection paradigm with or without the costly experience of learning.

5.1 Results

5.1.1 Species-specific and plastic behavioural responses to methyl hexanoate in the *Drosophila melanogaster* subgroup

To first establish whether the *Drosophila melanogaster* subgroup is an appropriate model for cross-species innate behavioural variation, *Drosophila melanogaster* and *Drosophila sechellia* were assessed for their olfactory behavioural response to methyl

hexanoate, a volatile odour from noni fruit which is attractive to *D. sec* but aversive to *D. mel* (Lachaise et al., 1988, Dekker et al., 2006, Auer et al., 2020) (figure 3.3.1A,B). A vacuum powered olfactory arena was used to deliver odour in a spatially and temporally controlled manner into specific quadrants of a circular arena, while a group of 20 flies freely explored the arena (figure 3.3.1C-D). A preference index is calculated as the difference in the number of flies in the odour quadrants divided by the total number of flies. *D. sec* were attracted to methyl hexanoate and had a significantly higher preference index than *D. mel* who were repelled by methyl hexanoate (figure 3.3.1E-G).

As future artificial selection experiments would investigate a role for behavioural plasticity, the plasticity of *D. mel*'s response to methyl hexanoate was investigated. Starved flies were exposed to 5 x 1min pairings of methyl hexanoate, or the odourless solvent paraffin oil, with sucrose to perform appetitive conditioning towards the odour (figure 3.3.1H). Flies which were appetitively trained towards methyl hexanoate showed a significantly higher preference index towards methyl hexanoate than flies trained with paraffin oil (figure 3.3.1I-J). Notably, while the preference index did increase, it remained negative indicating aversion towards methyl hexanoate.

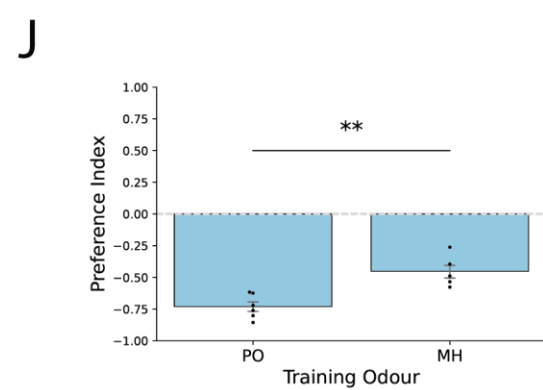
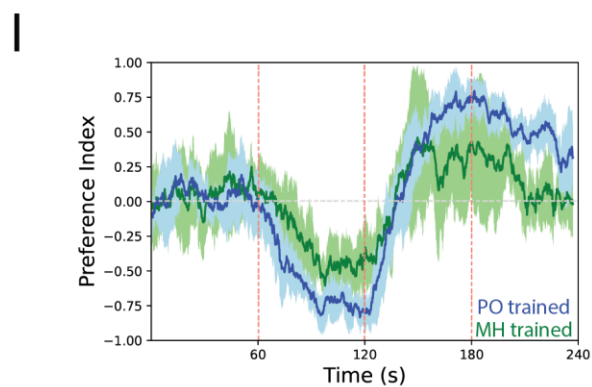
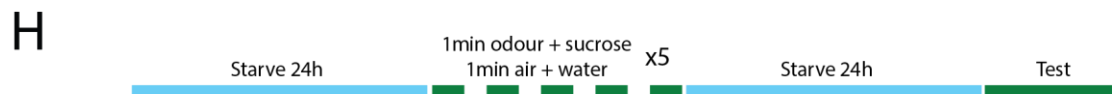
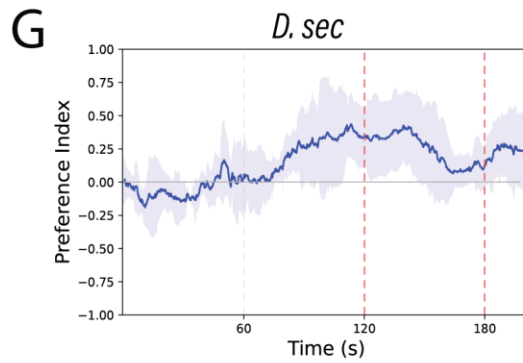
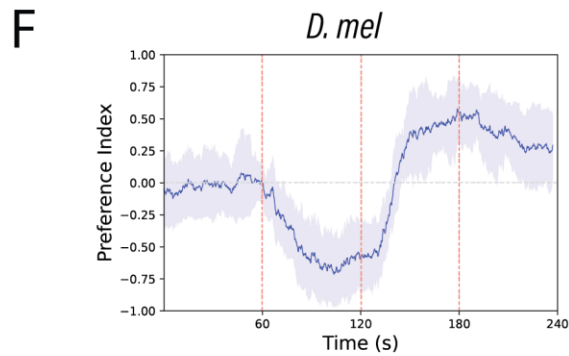
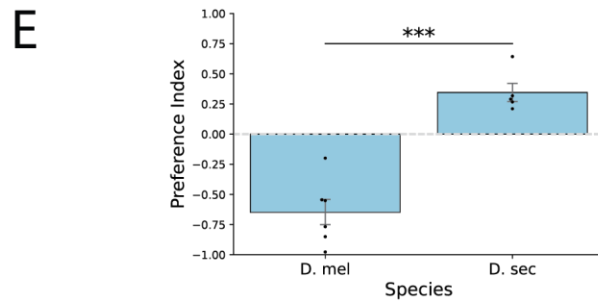
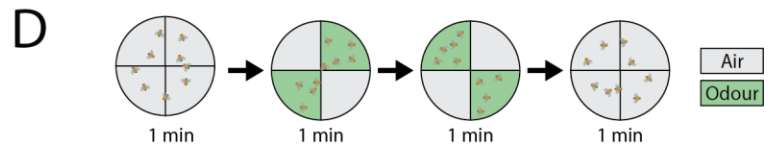
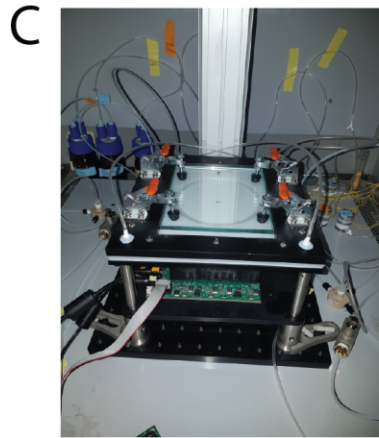
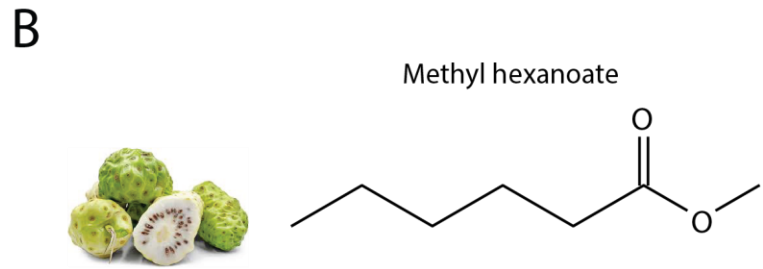
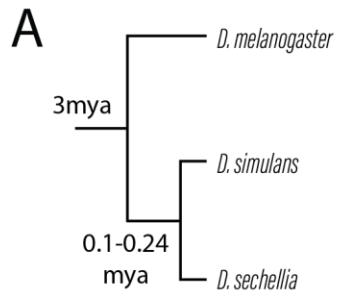


Figure 5.1.1 *Drosophila melanogaster* are repelled by methyl hexanoate and show behavioural plasticity. (A) Phylogenetic tree of the *Drosophila melanogaster* subgroup. (B) Noni fruit, toxic to *D. melanogaster* but upon which *Drosophila sechellia* specialises, releases the volatile methyl hexanoate. (C) Vacuum powered olfactory arena to test odour preference in *Drosophila*. (D) Timeline of odour presentation. (E) Average preference index for the final 30s of odour stimulation (Student's T test). (F) Timetrace indicating preference index of *Drosophila* over time when no odour is presented. (G) Preference index of *Drosophila* over time when methyl hexanoate is presented. (H) Behavioural schedule for appetitive conditioning to methyl hexanoate. (I) Timetrace indicating behavioural response of *Drosophila* following appetitive conditioning towards methyl hexanoate (MH) or the odourless solvent paraffin oil (PO). (J) Average preference index for the final 30s of odour stimulation (Student's T test). n.s. $p \geq 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

5.1.2 A simple model of artificial selection for olfactory guided behaviour

Inspired by the olfactory behavioural response of *Drosophila melanogaster* to methyl hexanoate, a computational simulation for the artificial selection of odour-guided behaviour was constructed (figure 3.3.2A). This simulation consisted of a population of “flies”, each of which is randomly assigned an innate “preference” from a normal distribution centred around -0.5. This innate preference is used to define a probability of a fly making a “choice” greater than or less than 0, which is randomly selected as a normal distribution centred around their innate preference with a standard deviation of 0.2. Depending on the choice each individual fly makes, they are assigned a probability of being selected for survival. The differing survival rates of the two choices are used to compute a selection pressure, “SP”, as the difference between two survival rates divided by the rate of the “correct” choice. Surviving flies are then randomly selected for cloning (with some normally distributed noise) to repopulate to the original number of flies which form the next generation of the simulation. A second group of flies, starting from the exact same baseline, received a simulation of training before making a “choice” (figure 3.3.2B). The training is a boost to their innate preference, sampled from a normal distribution with mean “x”. Each lineage runs for 100 generations and is repeated 50 times to monitor how the average innate preference index evolves over generations.

In this model an interaction of selection pressure (SP) with training strength (x) resulted in different outcomes between the two populations (figure 3.3.2C-D). For example, at a selection pressure of 0.6 and training strength of 0.3, the trained group evolved a higher innate preference more rapidly than the untrained group (figure 3.3.2C). One may calculate the average difference between the two populations over 50 generations to obtain a measure

of the effect of training vs no training on the evolution of innate preference. Doing this over a range of values for selection pressure and training strength reveals a pattern for the interaction of these two values (figure 3.3.2D). At low values of selection pressure, or extreme values of training strength, the trained group evolves lower innate preference than the untrained group over the course of 50 generations. At increasing levels of selection pressure, and moderate training strength, there an increase in the innate preference of trained values over 50 generations compared with the untrained population.

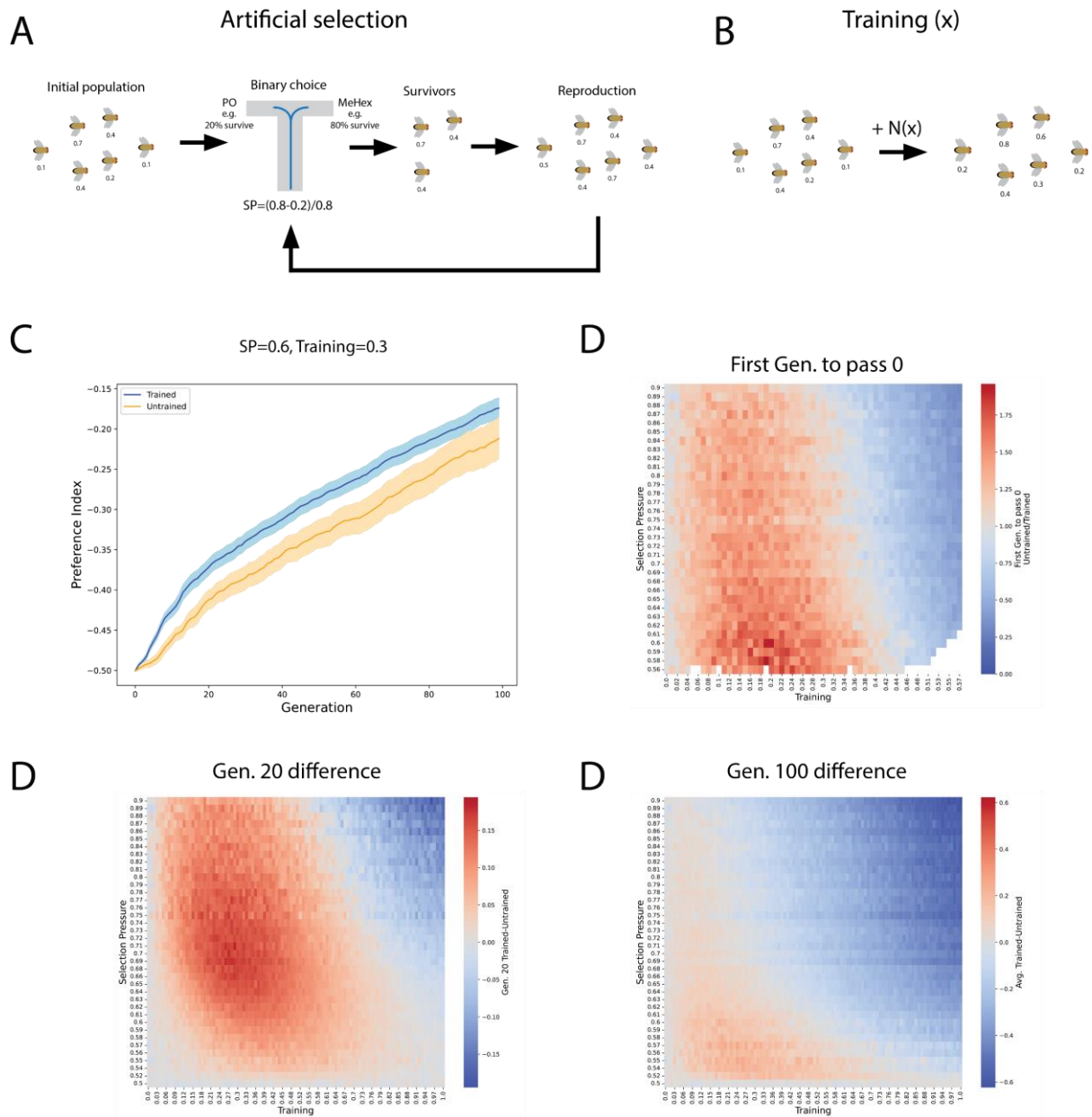


Figure 5.1.2 A computational model of artificial selection highlights varying effects of learning on the evolution of innate behaviour depending on selection pressure and training strength. (A) Illustration of the computational model for artificial selection. A group of “flies” are randomly assigned a value for their probability of approaching an odour (probabilistic selection of 1 or 0) in a model T maze paradigm. Based on their choice, individuals have a probability of being assigned as surviving. Successive populations are produced via random selection from surviving individuals for cloning until original population size is achieved. For details, see method. (B) One lineage will receive a boost to their score (normal distribution centred around training strength x). (C) Evolution of innate preference over generations in the trained and untrained lineages (averaged over 50 repeats each). (D) Heatmap indicating the summed difference between trained and untrained groups over 50 generations as a function of selection pressure and training strength. More positive differences are indicated in red, with more negative differences indicated in blue.

5.1.3 A polygenic, costly-learning, sexually reproducing model for olfactory guided behaviour

The simple model (figure 3.3.2) was developed further to establish a model where the innate preference of each fly was constructed based on the value of 5 genes, where each gene consists of 5 variants (figure 3.3.3A). Each variant was assigned a relative frequency, with negative variants being more frequent than positive variants (see methods for exact values). In addition to this, a single value randomly selected from a uniform distribution between 0 and 1 was assigned as a “plasticity” gene, which acts as a multiplier on training strength. In this model, a cost to training is applied, where the chance of survival = $(1-p*LR)$, where p is the value of the plasticity gene for the individual, and LR is the general learning cost applied. Flies then make a choice based on their trained preference in the trained group, or innate preference in the untrained group, and the choice determines probability of selection for survival (figure 3.3.3B). Surviving flies then repopulate via the random selection of pairs of flies for modelled sexual reproduction, where the value of each innate preference gene is randomly selected from each parent, and the value of the plasticity gene is taken as the average value from both parents (figure 3.3.3C). Each gene is also subject to a *de novo* mutation rate of 0.005. This is repeated over generations, and the value of innate preference and plasticity gene is recorded.

Varying the value of LR for fixed values of selection pressure (SP) and training strength resulted in differing outcomes for the trained population relative to the untrained population (figure 3.3.3 D-G). With low LR , the trained group achieved faster acceleration in innate preference for the first 20 generations than the untrained group, with high values for plasticity being selected for across all generations (figure 3.3.3 D,E). This suggests that at these parameters, training results in greater acceleration of innate preference and that the selective advantage of increasing preference via plasticity is greater than the cost of learning.

When LR was increased to 0.25, the trained group still maintained faster acceleration of innate preference for approximately 20 generations, before being overtaken by the untrained group (figure 3.3.3F). In addition to this, while high values for plasticity were selected for initially in the trained group, the plasticity value started decreasing at approximately generation 30 (figure 3.3.3F). This suggests that at $LR=0.25$, high plasticity was

initially advantageous while innate preference was still negative, but once innate preference was positive then the benefit of learning was outweighed by the cost of learning.

When LR was further increased to 0.5, the trained group still maintained faster acceleration of innate preference for approximately 20 generations before being overtaken by the untrained group (figure 3.3.3G). Meanwhile, plasticity values started decreasing immediately in the trained group, suggesting that the benefit of training was less than the cost of learning even at the beginning of the simulation (figure 3.3.3G). Together, these simulations illustrate that, at $SP=0.6$ and $training=0.2$, the benefit of learning to the population gradually decreases as the risk of learning increases.

To determine how varying values of SP and training interact with LR, the simulation was iterated through minimal to maximal values of SP and training over LR values of 0, 0.25 and 0.5 (figure 3.3.3H-J). Each simulation was repeated 50 times, and the following values were recorded: ratio of first generation to pass zero in each group, innate preference difference at generation 20, innate preference difference at generation 100, plasticity value at generation 20, and plasticity value at generation 100. This information provides a perspective as to the relative acceleration of each group throughout the simulation, and whether plasticity is selected for or against throughout the simulation.

At $LR=0$ (figure 3.3.3H), the trained group passes an innate preference of zero faster than the untrained group only for low values of training or SP. For higher values of training and SP, the untrained group passes zero more quickly. This is also reflected in the differences of innate preference between the two populations at generation 20 and 100. The trained group had higher innate preference at low values of SP or training at generation 20, but were overtaken in most cases by generation 100. At both generation 20 and generation 100, plasticity values remain high across all values of SP and training, indicating that plasticity is continually selected for. For low training and SP, the effect of training is to accelerate the evolution of higher innate preference. For higher values of training and SP, training creates a buffering effect. Further, with no cost of learning, plasticity is continually selected for.

At $LR=0.25$, a similar trend exists but with a reduced advantage for the training group (figure 3.3.3I). The trained group passes zero more quickly than the untrained group for low values of training and SP, but with a lesser magnitude. At generation 20, low training and SP values show a higher innate preference for the trained group, and this effect is mostly gone by generation 100. Further, at generation 20 plasticity is selected for in general, especially for

higher SP and training. At generation 100, plasticity is selected against, with plasticity persisting mostly at high SP and training. At $LR=0.25$, low values of training and SP result in a faster evolution of innate preference, but to a lesser extent than at $LR=0$. At higher values of SP and training, a buffering effect emerges. This aligns with the records of plasticity, where at generation 100 plasticity is selected against for low values of SP and training, and selected for at higher values of SP and training. As such, plasticity is only selected for when the benefit of learning is greater than its cost.

At $LR=0.5$, the cost of learning becomes more apparent (figure 3.3.3J). The higher innate preference of the trained group at low SP and training is minimised, as reflected by the reduced ratio at which the groups pass zero. The higher innate preference at generation 20 for low SP and training is still present, but to a lesser extent, and is gone by generation 100. Plasticity is selected for when training and SP are high at generation 20, with significantly reduced plasticity by generation 100. This data illustrates again that plasticity allows faster evolution at low SP and training, while buffering the change in innate preference at higher values for SP and training.

Together, this simulation highlights that plasticity can enable rapid changes in innate preference for lower values of SP and training. However, as SP and training increase, training instead creates a buffer effect, by allowing survival of individuals with lower preference. When the cost of learning is low, higher innate preference and plasticity increase together. As learning cost increases, plasticity continues to provide an advantage to early generations, but is selected against as generations proceed. The greater the cost of learning, the faster that plasticity is selected against.

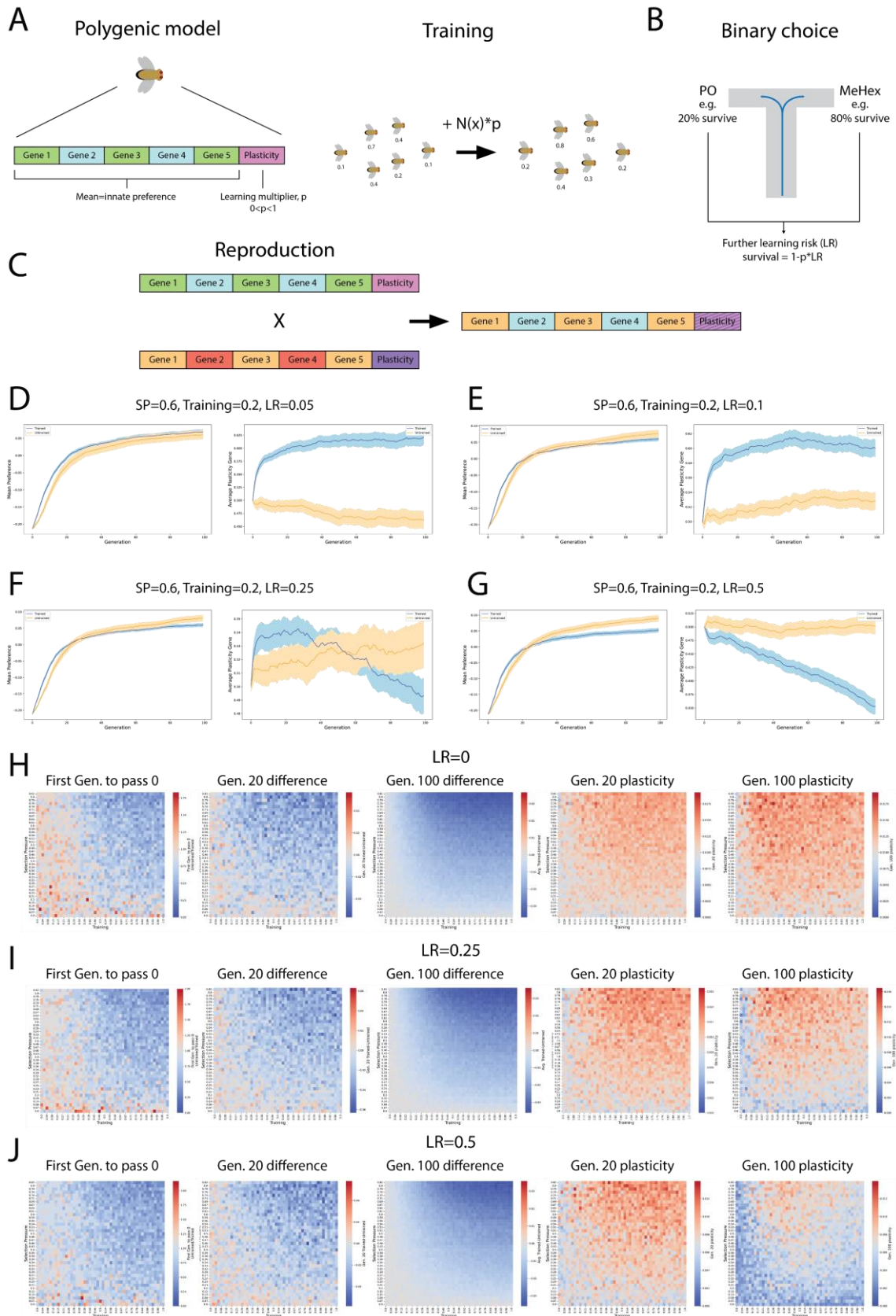


Figure 5.1.3 A polygenic model of artificial selection which includes genes for innate behaviour, plasticity, and applies a learning cost. (A) Polygenic model of behaviour consisting of 5 genes constructed from defined genetic variants to determine innate preference. In addition to this, a de novo mutation rate occurs randomly in at a probability of 0.001 per individual. A single but continuously varying (0-1) gene determines sensitivity to plasticity, which is used as a multiplier on training. For details, see methods. (B) “Flies” make a binary choice, before survivors are randomly culled as a factor of their plasticity gene (in the trained group only) multiplied by a general learning risk, LR. (C) Reproduction to full population size is modelled as sexual reproduction by pairwise selection of survivors, which each gene being randomly selected from each parent. The plasticity gene is calculated as an average of both parents. (D-G) Example trends of innate preference (left) and plasticity gene value (right) over generations for varying values of the general learning risk, LR. (D) Low learning risk models evolve greater innate behaviour faster and select for high values of plasticity. (E) An increased LR still promotes faster evolution of innate preference in the trained group initially before being overtaken. High plasticity is still selected for. (F) Further increased LR maintains faster evolution of innate preference in the trained group on the short term before being overtaken. Initially, plasticity is selected for but suppressed at later generations. (G) At higher learning risk, innate preference still evolves faster in the trained group, but plasticity is selected against immediately.

5.2 Discussion

This study builds evidence for a role of learning in the evolution of innate behaviour. Specifically, it highlights how the interaction of selection pressure and training strength can create different outcomes in the evolution of innate behaviour. In addition to this, it separates innate behaviour and a generalised innate plasticity, to determine how genetic control over these two components of behaviour may vary over evolution under the influence of varying selection pressure, training strength, and risk-associated learning. Depending on the specific interaction of these parameters, plasticity may increase the rate at which innate behaviour evolves, or prevent innate behaviour from evolving in a buffer effect manner. In addition to this, the selection for plasticity may vary over time as innate behaviour evolves.

Implications for visual looming and TMA olfaction responses

The previous two chapters investigated the interaction of instinct and memory in specific ethologically relevant behaviours. How can this chapter provide context for their findings?

Firstly, considering looming. As discussed previously, the visual looming response is a very innate and likely ancient behaviour which is important for survival. As such, it would stand to reason that the behaviour has a high selection pressure, and has been under the forces of evolution for many generations. On top of this, attempts to learn the behaviour would be very risky, as real-life scenarios which evoke visual looming stimuli include rapidly approaching predators or objects which are likely to cause injury or death. Given this information, this model would predict that plasticity of behaviour would be selected against. This insight may explain the apparent separation between the innate visual looming response which is driven by the SC, and the re-learned visual looming response in the vCA1. The brain may have evolved to limit plasticity on the innate looming-response circuitry in the SC, and hence relies on other brain regions to facilitate plasticity of behaviour.

Regarding the TMA olfactory response, the context in which this behaviour evolved is quite different. The selective advantage of recognising and appropriately responding to a species-specific and sex-specific chemical signal has very clear advantages for the individual in terms of social and sexual behaviour. However, the consequences of not recognising and responding to this sensory may be less immediate than failing to respond to a visual looming

stimulus. Further, the cost of learning for this behaviour may be considered very low, as exposure to the odorant during the juvenile stage is almost guaranteed due to the presence of conspecifics pre-weaning. Estimating a “training strength” is challenging. On one hand, the regular re-exposure to the odour and conspecifics may be considered a strong training, however the complexity of social behaviour and the various context-dependent behaviours mean that not all appropriate social behaviours can be readily “trained”. As such, the “training strength” may be considered as within the range of moderate to high. Given this information, the model would predict and increase in innate behaviour alongside a concurrent selection of plasticity of behaviour. This would explain the change in the behaviour from aversion in the ancestral state, to neutrality in the case of dox-fed mice which are never exposed to TMA. Meanwhile, plasticity of behaviour allows the learning of attraction, potentially in addition to other relevant social behaviours.

Informing an artificial selection experiment

One of the main ambitions of this chapter was to generate hypotheses which could be empirically tested in an artificial selection experiment. The rapid reproduction of *Drosophila* positions them as a potential candidate for this experiment. Specifically, the *Drosophila melanogaster* subgroup’s olfactory behavioural response to methyl hexanoate was selected, as it contains the recently evolved appetitive response in *Drosophila sechellia*. This would enable experimenters to perform artificial selection on *Drosophila melanogaster* as a proxy for the ancestral state of methyl hexanoate aversion, and compare the effect of artificial selection in this line with the naturally selected response of *Drosophila sechellia*. Further, existing studies on the behavioural response of the *Drosophila melanogaster* subgroup can inform as to what kind of changes may be expected. For example, the or22a receptor which detects methyl hexanoate, is overexpressed in *Drosophila sechellia* compared with *Drosophila melanogaster* (Auer et al., 2020), and has morphological changes leading to higher sensitivity to methyl hexanoate (Shaw et al., 2019). In addition to this, the projection from the DM2 glomerulus which detects methyl hexanoate shows distinct projection patterns in *Drosophila sechellia* compared with *Drosophila melanogaster*, including a reduced number of learning-associated mushroom body calyx branches and modified branching morphology in the lateral horn (Auer et al., 2020). In this chapter, the olfactory response of *Drosophila melanogaster* was shown to be plastic, although the training was insufficient to reverse the behavioural

response from aversive to appetitive. Further, previous studies have demonstrated that it is possible to achieve an appetitive response to methyl hexanoate in *Drosophila melanogaster* via artificial selection in as little as 30 generations (Brown et al., 2017). As such, this establishes *Drosophila melanogaster* as an appropriate candidate for an artificial selection experiment investigating the impact of learning over generations.

What are the implications of this modelling data on the proposed experiment? Firstly, the model predicts that varying factors such as training strength and selection pressure will alter the speed at which a population will develop an innate attractive behavioural response to methyl hexanoate. Further, it predicts that these factors will also influence the extent to which plasticity of behaviour will be selected for. As such, an experimental model should aim to have several lineages which receive varying degrees of training in the form of repeated sessions of associative conditioning. Over time, these lineages should develop unique strategies to adapt to the selection for methyl hexanoate attraction, ranging from totally innate behaviour to totally learned behaviour. Furthermore, these unique behavioural adaptations may be underwritten by unique circuit adaptations, such as varying numbers of synapses of the DM2 projection neurons to brain areas driving learned behavioural responses, such as the mushroom body calyx (Auer et al., 2019).

Model Limitations

While the model is helpful in providing predictions for an artificial selection experiment, it remains limited in its broader predictive capacity due to some specific limitations. For example, generations in this model are discrete and maintained at a constant population level. While this may be appropriate for an artificial selection experiment in the lab, where a certain number of flies are selected to seed successive generations, and each generation is restricted from breeding with one another, in the wild no such clear distinction between generations exist. In addition to this, the polygenic model consists of 5 genes, with 5 variants each. In reality, it is possible that many genes may be involved in behaviour, and the existing genetic variants may range from very few to very many. Meanwhile, the plasticity component of behaviour was modelled as a single continuously varying gene for simplicity. Again, this is unlikely to reflect the how genetic component of plasticity arises in nature.

6. Conclusion

The relationship between memory and instinct

This thesis investigated the relationship between memory information stored in the brain as memory and information stored as instinct. In particular, this was investigated from three perspectives; (1) habituation and reinstatement of the visual looming response in *Mus musculus*, (2) the recently evolved behavioural response of TMA attraction in *Mus musculus*, and (3) a computational model of how behavioural plasticity can interact with evolution.

The data presented here highlights that instinct and memory are complementary and overlapping systems. The extent to which innate and learned components of circuitry are separated in the brain may depend on the context in which the behaviour evolved. The visual looming response is an ancient behaviour, preserved across many species and very important for survival. As such, separation of innate circuitry in the SC and plastic circuitry in the vCA1 may have evolved as a means of enabling reliable innate behavioural responses while still enabling behavioural plasticity. In the case of TMA olfaction, innate and learned components of circuitry are much more intertwined. The behavioural response of TMA attraction is unique to *Mus musculus*, indicating a component of innateness, but also requires exposure in infancy, indicating a component of learning. This is also reflected in the activation of the AON in both *Mus musculus* and *Mus caroli*, even though the behaviour is entirely innate in *Mus caroli*.

These two behavioural models, alongside insights from computational modelling, highlight that instinct and memory exist along a continuum of behaviours from mostly innate to mostly learned. Exactly where on this spectrum a particular behaviour will fall will depend on the evolutionary history of this behaviour.

Updating an instinct

There is growing evidence that memory loss often reflects deficits in accessibility rather than a total loss of stored information (Autore et al., 2023, O’Leary et al., 2023, Guskjolen and Cembrowski, 2023). It may be possible that a similar retrievability deficit could affect instinct, depending on when learning occurs relative to development of the instinct.

In the case of looming habituation and reinstatement, the original loom responsive cells in the vCA1 become necessary for producing fear responses to the looming stimulus.

Furthermore, there is a partial recovery of responses in the SC, albeit to a much lesser degree than in initial baseline behavioural responses. As such, the original looming information does seem to persist across the paradigm in the vCA1 particularly, and perhaps also to some extent in the SC. In contrast, the olfactory system presents a different dynamic. If mice do not receive exposure to TMA in infancy, they fail to develop TMA attraction, even if they are thereafter exposed to TMA in the urine of their cohabiting conspecifics. In this case, whatever TMA-responsive circuitry which does develop appears to be resistant to updating later in life, indicating a critical window in which the behaviour must develop.

This indicates that the brain employs multiple strategies for balancing the rigidity and adaptability of instinctive behaviours. Learning can reshape some instincts over the lifespan, while others have their plasticity constrained by early-life experiences.

Concluding remarks

This work advances our understanding on how instinct and memory are organised in the brain, how they interact with one another, and what implications this has for the evolution of behaviour. This was achieved by, firstly, observing how habituation-reinstatement of visual looming fear responses results in a transition of fear behaviour from SC-dependency to vCA1-dependency. Secondly, a cross-species approach investigated how a behaviour can evolve in the context of learning. Specifically, the olfactory response of *Mus musculus* to TMA was revealed to rely on a critical period of exposure to generate a complex social signal which is driven by the AON and suppresses the CoA, in contrast to the aversive response of *Mus caroli* which includes both AON and CoA activation. Finally, a computational model was used to investigate how varying the extent to which selection pressure and plasticity are present on a population can result in varying outcomes for the evolution of behaviour.

Together, these findings shed light on the dynamic interplay between instinct and memory, emphasizing alternate approaches to balancing innate and plastic behaviour that may evolve. This understanding only deepens our knowledge of how behaviour is shaped and how information is organised in the brain.

7. Bibliography

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