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An investigation of the cognitive processes necessary for a complex innate behaviour

Experimental and Theoretical Systems Neuroscience
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Faculty of Life Sciences
University College London

April, 2021

Matthew G. Phillips: *Navigational strategies and the role of the hippocampus in mouse escape behaviour,* An investigation of the cognitive processes necessary for a complex innate behaviour Experimental and Theoretical Systems Neuroscience

With what sense is it that the chicken shuns the ravenous hawk?
With what sense does the tame pigeon measure out the expanse?
With what sense does the bee form cells? Have not the mouse and frog
Eyes and ears and sense of touch? Yet are their habitations
And their pursuits as different as their forms and as their joys.
Ask the wild ass why he refuses burdens and the meet camel
Why he loves man: is it because of eye, ear, mouth, or skin,
Or breathing nostrils? No, for these the wolf and tyger have.
Ask the blind worm the secrets of the grave, and why her spires
Love to curl round the bones of death; and ask the rav'nous snake
Where she gets poison, and the wing'd eagle why he loves the sun;
And then tell me the thoughts of man, that have been hid of old.

William Blake

The difference in mind between man and the higher animals, great as it is, certainly is one of degree and not of kind.

Charles Darwin.

Nothing in biology makes sense except in the light of evolution.

Theodosius Dobzhansky

ABSTRACT

Executing appropriate defensive actions is vital for survival. In mice, imminent threat elicits fast and accurate escape behaviour that relies on a rapidly formed spatial memory to reach shelter locations. I investigated the navigational strategies used by mice to navigate to safety upon imminent threat, and the role of the hippocampal formation – classically associated with spatial representations – in guiding escape navigation. Through a series of behavioural experiments designed to distinguish between navigational strategies guiding escape, I found that while flight was consistent during the the first 800ms across light and dark conditions, visual cues enabled faster, more efficient escape trajectories later on in flight, suggesting escape has two phases: orienting and accelerating towards the shelter, relying on a memorised vector; and a second phase using vision to refine escape trajectories. Accordingly, I found that path integration was necessary for navigation in the dark, but not in the light. I next investigated the dependency of escape on brain structures associated with spatial representation in the hippocampal formation. An abrupt lesion targeted to the hippocampus using ibotenic acid disrupted escape navigation. A more targeted lesion of the primary hippocampal output - an infusion of muscimol into the subiculum – also led to a disruption of escape navigation and an increased propensity to freeze in response to looming visual stimuli. Finally, while disrupting neural activity in the subiculum by stimulating with channelrhodopsin reduced acceleration, this effect was present with optogenetic stimulation alone, precluding any firm conclusions from these experiments with respect to escape navigation. Together, these data further our knowledge of defensive behaviours in mice by implicating high-level spatial representations of the environment in guiding escape navigation, identifying behavioural signatures of navigational strategies requiring these representations, and showing the dependency of escape navigation on brain regions associated with spatial representations.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my supervisor, *Tiago Branco*, whose insights and guidance made any progress presented here possible. On numerous occasions his suggestions for experiments, analysis, and visualisation unlocked a new way forward. I hope to have learned from him and take forward these qualities into my future work.

The present work would also not have been possible without the support and training from members of the *Branco Lab*. All have contributed in some way to this project, whether directly, through discussions, or through creating such a supportive lab culture. I owe a significant debt of gratitude to *Ruben Vale*, who invested much time and effort into my training, whose prior work I am building on, and who was directly involved in guiding this project. Particularly during the early stages of my PhD, I was trained by two exceptional scientists, *Vanessa Stempel* and *Dominic Evans*, to who I am very grateful. *Kostas Betsios* programmed the software used for data acquisition and helped me debug issues on multiple occasions. Other members of the lab contributed to particular aspects of the project, including *Philip Shamash* whose code was used to process videos into a common reference framework, *Federico Claudi* who helped me set up DeepLabCut tracking, and *Panagiota Iordanidou* who provided expertise and guidance on the use of viruses and histology throughout. I'm further grateful to *Dario Campanger* and *Oriol Pavon* who contributed guidance and help when needed.

Many people from the Sainsbury Wellcome Centre have contributed through this project. The animal husbandry unit were always diligent and helpful in caring for the animals used in this project. Special thanks are owed to those associated with the PhD program, including my colleagues *Stephen Lenzi* and *Jesse Geerts* who I shared many late nights building microscopes and coding with in my first year, *Adam Kampff* and the members of his lab who were so engaged and helpful during our first year training, and *Klara Olofsdotter Otis*, *Karen Fergus*, and *Alexandra Boss* who helped deal with many different issues I ran into through my PhD. The FabLabs team helped to design and build many aspects of the experimental arenas used in this study, and were exceptionally professional, helpful and efficient.

More generally, I am grateful to too many people within the Sainsbury Wellcome Centre to name individually, but who contributed to a happy and collegiate atmosphere that supported me through my studies. In particular, *Daniel Regester*, *Peter Vincent*, *Sarah Olesen*, and

Spencer Wilson were there for many lunches, drinks and nights not thinking about work.

The Wellcome Trust and Gatsby Charitable Foundation funded me through this PhD and are responsible for creating a research centre and PhD program as interesting and innovative as that at the Sainsbury Wellcome Centre. I also thank UCL for their support through this PhD.

I am grateful to my secondary supervisors *Neil Burgess* and *Matteo Carandini*, who provided assessment on where I was up to in my project throughout, and guidance on where to focus next.

My friends and family have supported me through all highs and lows and I could not have done this without them. *John Mitchell*, *James Rowland*, *John Cousins* and *Gabriel Gavin* all listened to my problems in our *Neuroscience Researchers Club*, which was essential to completing this project. Most of all, I would never have reached this point without my wonderful family, *Iain*, *Jacqueline*, *James* and *Katherine*, who provide the foundation for everything else.

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ACRONYMS

BVC: Boundary vector cell
CA: Cornu amonis
cm: Centimetre
dlPAG: Dorsolateral Periaqueductal gray
DMM: Dry Morris Maze
fMRI: Functional magnetic resonanace imaging
FPS: Frames per second
GPS: Global positioning system
HDC: Head direction cell
IQR: Interquartile range
khz: Kilohertz
m: Metre
mm: Millimetre
mSC: Medial superior colliculus
n: Number in sample
nL: Nanolitres
nm: Nanometres
PAG: Periaqueductal gray
PFA: Paraformaldehyde
s: Seconds
SC: Superior colliculus
SEM: Standard error of the mean
STD: Standard deviation
t: Time
TMT: 2,3,5- trimethyl-3-thiazoline
vlPAG: Ventrolateral Periaqueductal gray
VMHdm: Dorsomedial division of the ventromedial hypothalamic nucleus
VNO: Vomeronasal organ

Part I

INTRODUCTION

How do our brains transform basic drives into complex plans and actions? This question underpins how our behavior is orchestrated; it's answer being of paramount importance to many domains, including artificial intelligence, understanding human choices, how these choices can go wrong in illness and disease, and on the most fundamental level our understanding of free will. Nested within it are questions ranging how organisms form models of the world distantly abstracted from the senses, to how environmental stimuli are associated - either innately or through a learning - with an increase in the drive for a particular goal. The following is my attempt to make progress towards understanding how our brains achieve this, utilizing behaviours that are built into mice for survival.

1

BACKGROUND

1.1 THE ORIGIN AND EVOLUTION OF BRAINS

The principle function of the nervous system is to control behaviour. Nervous systems have evolved to produce a remarkable repertoire of behaviours, ranging from rapid reflexive responses designed for immediate protection from harm to mastering chess and appreciating literature. Each of these behaviours is produced by some combination of genetic information and its interaction with the environment. Explaining how the repertoire of behaviours displayed by animals is produced by the nervous system is therefore a crucial goal for neuroscientific research.

To achieve this purpose, evolution has produced highly complex lifeforms. However, in seeming contradiction with this complexity, the second law of thermodynamics states that, within a closed system, entropy always increases. How, then, to explain the panoply of ever more intricate, ordered and intelligent lifeforms on earth? How are they stable over such extended timescales?

In his book 'What is Life?', Schrodinger's answer was that life is a local reversal of entropy, where a decrease in entropy in a physically restricted space could be bought at the cost of increased entropy elsewhere (Schrodinger, 1945). How could this local decrease in entropy be achieved? In a classic thought experiment, Maxwell suggested entropy could be decreased if an all-knowing demon could apply a filter selecting for high-energy particles between two chambers (Maxwell and Niven, 2011). The solution to the problem posed by Maxwell - that an all knowing demon could defy the second law of thermodynamics - is resolved by the introduction of information theory, but the analogy is useful when thinking about the relationship between entropy and the evolution of complex lifeforms (Jeffery, Pollack, and Rovelli, 2019; Jeffery and Rovelli, 2020). In nature, one can think of evolution as using a kind of Darwinian version of Maxwell's demon in the form of natural selection, where instead of selecting particles the demon selects organisms most fit to the environment (Krakauer, 2011). Viewed through this framework, life is a self-reproducing process that exploits its environment to increase complexity through natural selection.

To understand our own nervous systems, it is necessary to delve into this evolutionary history and trace the origin of modern brains.

Around 2.6 billion years ago, early cellular life forms developed flagella to enable translocation through space. Cells could therefore position themselves so as to maximise the probability of reproduction and survival. The resulting increase in order in spatial arrangements was a prelude to multi-cellular life, eventually with non-homogeneous, spatially specialised components evolving. With this increased complexity, new communication strategies to coordinate between the multifarious cells were required, as the diffusion of molecules became too slow, short-range, and imprecise to convey the messages required. It was for this reason that the earliest prelude of current day neurons evolved.

Moving through space was therefore a vital driver of the development of complex life forms. However, until 560 million years ago, this ability was primarily the responsibility of flagella - small appendages to the exterior of individual cells that produced movement by 'wafting' the environment around them. Multicellular organisms only developed the components to allow self-propulsion around 560 millions years ago, originally in the form of small wormlike organisms. Studies of contemporary organisms suggest that even after the shift from unicellular to mutlicellular life, coordinated navigational strategies are possible. In the colony-forming choanoflagellate *Salpingoeca rosetta*, which is one of the closest ancestors of animals, the organism is capable of moving in a coordinated manner towards oxygen - known as 'aerotaxis' - in either its uni- or multicellular form (Kirkegaard et al., 2016), suggesting that in the transformation between unicellular and multicellular life basic navigational abilities can be maintained.

As movement increased, so did competition. Moving towards the most favorable position inevitably means competing with others for that position. As multi-cellular life flourished, so did a range of new behavioural strategies for survival. Rather than move to the position in space that best provides for their needs, organisms could now hijack the hard work of others by eating them as prey. An evolutionary arms race began, with predator-prey dynamics exacting new selective pressures. New, ever more specialised niches developed. Foraging, hunting and evading predation now required sophisticated sensing of the environment, and evolution produced the structures necessary to do so.

As a result of the versatility of DNA and biological structures such as proteins and cells, the capacity of the genome to adapt is immense. As the world is consistently correlated through time, organisms that use this regularity to predict the world by building a model of it have a substantial advantage in achieving goals and avoiding threats. Vital to forming a world model is the ability to store aspects of past states. The changing of the strength of connections between neurons, a process known as synaptic plasticity, enables such storage to occur on much shorter timescales, and to exploit correlations detected

across much larger distances, than is possible through gene regulatory change. One particularly important consistency in the world is its spatial organisation, with particular spatial locations conferring greater survival and reproductive value. As organisms with spatial memory have substantial evolutionary advantages, various forms of spatial representation and memory have arisen through evolutionary history. Through the evolutionary framework, we see that predator-prey dynamics and spatial memory are intrinsically linked.

Representing the environment, whether spatially or in other ways, enables greater flexibility. This provided a crucial step in the evolution of nervous systems and the behaviours enabled by them. Behaving adaptively in a wide range of challenging environments to achieve goals is so central to brain function that it has been used as a definition of intelligence (Legg and Hutter, 2007). Using this definition, intelligence requires use of previous experience and the construction of representations of the world that can be leveraged rapidly based on recent sensory stimuli.

Evolution thus gives us a route into understanding biological intelligence. Evolution is the most successful theory in all of biology, providing an explanation for the diversity and complexity of nature (Ayala, 1977). Resultant from the variety of the planet, a range of organisms evolved, some highly specialized to particular environments, and some built to be adaptable and survive in a range of diverse environments. Mammalian brains, and particularly human brains, fall into the latter category. Due to the range of environments inhabited by these organisms it is much harder to 'hard-wire' a general solution of how to function. These nervous systems must therefore learn to make sense of a bewildering array of sensory information, extract its key features at a variety of levels of abstraction, and select and execute an appropriate action in the current situation.

The resulting behaviours can be rapidly, flexibly and intelligently combined to form hierarchical structures such as plans appropriate to the structure of the environment, and yet are often motivated by basic drives such as hunger and sex. How this occurs is largely unknown, but it's clear that an explanation of intelligence must include how learned representations guide the selection of actions to fulfill instinctive drives. Rather than simply responding to the set of sensory features immediately present like, say, a bacteria might (though even very simple organisms have forms of memory), we store, process and leverage past experience to improve future actions. Our ability to extrapolate, integrate, translate and abstract from immediate sensory cues is fundamental to our intelligence and survival.

In addition to flexibility, intelligent organisms also display behaviors specific for survival in their ecological niche. How do these evolutionary drives and more flexible learning rules interact? The aim of the present thesis is to further our understanding in this field by studying the intersection of defensive behaviours and navigation. I begin by ex-

ploring the literature of instinctive behaviour. I then overview spatial navigation. The final background section introduces with more focus the subject of investigation: defensive navigation, how cognition guides it, and the relevant neural correlates.

1.2 INSTINCTIVE BEHAVIOURS

Instinctive behaviours are characterised as a set of actions that can be taken without learning. Taking Donald Hebb's definition, these are behaviours where "the motor pattern is variable but with an end result that is predictable from acknowledgment of the species, without knowing the history of the individual animal" (Hebb, 1949). They can be driven by internally generated triggers, such as the desire for food, or external triggers, as is more common of defensive behaviours (Tinbergen, 1963). These behaviours - and in particular defensive behaviours - are essential for animals to survive long enough to reproduce, and are therefore under intense evolutionary selective pressure. The terms instinctive and instinct are often used interchangeably and the do refer to similar concepts. However, the term instinctive has a wider range of usages, and so for precision we will primarily use the term instinctive behaviour here.

Early studies of instinctive behaviour arose from a range of species. Pre-scientific observation of instinctive behaviours can be found in Aristotle's 'History of Animals', including hawk predatory behaviour and reproduction in various fish species (Thompson, 2014). Similar observations later played a role in the debates around the theory of evolution: Lamarck's theory of instinctive behaviours - that they are those learned by previous generations - couldn't explain once in a lifetime behaviours like butterflies emerging from cocoons; rather, this was successfully explained by Darwin's theory of natural selection in the 'Origin of Species' (Darwin, 1859), in which he cited behaviours such as the ability of bees to make hexagonal honeycomb as being attributed to heritable instinct evolved across generations rather than being learned. Darwin argued that *'it will be universally admitted that instincts are as important as corporeal structure for the welfare of each species, under its present condition of life. Under changed conditions of life, it is at least possible that slight modifications of instinct might be profitable to a species; and if it can be shown that instincts do vary ever so little, then i can see no difficult in natural selection preserving and continually accumulating variations of instinct to any extent that may be profitable. It is thus, I believe, that all the most complex and wonderful instincts have originated*'.

However, as the 20th century began, the scientific study of animal behaviour was divided into different schools of thought, and one of them rejected the idea of innateness. A prominent, primarily American school of thought held that behaviour should primarily be understood in terms of associations between cues - a school known

as behaviourism. Behaviorism focused on the individual history of the animal and the associations formed through their lives. A useful summary of this view can be found in John Watson's seminal work in 1913, 'Psychology as the Behaviorist Views it' (Watson, 1994). Watson lays out the fundamental principles of behaviorism, arguing for a focus on learning associations in the environment rather than any preconditions of the animal. In this sense, Behaviorism endorses a 'tabulua rasa', or blank slate, view of the mind (Pinker, 2005). Behaviourism is further characterised by the rejection of the study of internal cognition and emotion, regarding such questions as unscientific, believing they should be explained purely in terms of associations formed through learning, studied only through the observable behavior of animals. This view drew upon evidence from Ivan Pavlov and his famous experiments training dogs to associate a bell to food (Jones and Skinner, 1939; Pavlov, 2010), and gained momentum with the success of researchers like Skinner in explaining the ability of pigeons to associate particular cues to rewards (Skinner, 1958). Skinner would go on to criticise the idea of instinct as 'explanatory fictions' rather than objects of scientific enquiry.

However, there were crucial features of psychology that could not be explained by behaviourism. For example, Chomsky's observations that humans learn vast numbers of words with very limited input is hard to square with a view that each stimulus must be associated with a reward for learning to occur (Lees and Chomsky, 1957). These limitations therefore led to a counter-movement known as the 'cognitive revolution'. Incorporating insights from fields ranging from psychology, linguistics and computer science to anthropology, neuroscience and philosophy, the cognitive revolution sought to provide an interdisciplinary, scientific study of mind (Mandler, 2002). As opposed to the behaviourist school, the cognitive revolution aimed to provide explanations in terms of the mental systems used to process sensory information, was open to innateness, and viewed the mind as being modular (Newell, Shaw, and Simon, 1958). Seminal works argued against the simple stimulus-response association as the foundation of behaviour, arguing instead for planning and behavioural sequences based upon sophisticated models of the world (Miller, Galanter, and Pribram, 2017). Today, the field of cognitive science is highly influential in neuroscience, artificial intelligence, and beyond, owing its origins to the cognitive revolution.

In parallel, observations of instinctive behaviours were being formulated into a field of study known as Ethology, pioneered by Konrad Lorenz, Nicolaas Tinbergen, and Karl Von Frisch. In modern terms, Ethology can be described as the study of behaviour from a biology perspective, where behaviour is defined following Tinbergen's view that it's the 'total of movements made by the intact animal' (Tinbergen, 1990). Ethology began as a primarily descriptive field, aiming to discover interesting behaviours that were not dependent on learning. One of the earliest studies can be found in von Frisch's 1927 book

'The Dancing Bees', in which he details the 'waggle dance' performed to communicate the location important locations such as new nest sites, pollen, or water to other bees. Heinroth and Lorenz further extended the field with their description of imprinting in greyleg geese, in which they showed that young chicks form a bond with the first moving object they see during a critical period 13-16 hours after birth (Lorenz, 1937). While this is usually their mother, any moving object can be substituted in it's place. Tinbergen introduced the idea of a triggering stimulus for instinctive behaviours through his paper 'The curious behaviour of the Stickleback', in which he showed that complex behaviours such as aggression can be triggered by relatively simple stimuli such as the color red (Russell and Tinbergen, 1973; Tinbergen, 1942). Similar observations had been made a few years earlier by Lorenz in his famous book *'King Solomon's Ring'*, in which he describes his experiences of a life studying animal behaviour through living with them. Lorenz describes the mating rituals of fighting-fish and its triggering by their ability to *'recognize the sex of a member of their own species not simply by seeing it but by watching the way in which it responds to the severely ritualized, inherited, instinctive movements of the dancer. The meeting of two previously unacquainted fighting-fish begins with a mutual "showing-off", a swaggering act of self-display in which every luminous color-spot and every iridescent ray of the wonderful fins is brought into maximum play'*. Similarly complex dances are displayed when two males come across each other, only in this case they are more menacing than enticing. That these magnificent behaviours are so stereotyped across individuals and occur without previous exposure strongly indicates they are not learned. Together, these efforts discovered and described crucial examples of instinctive behaviours and began to unpick how they work.

Even in the comparatively simple organism *Drosophilia Melanogaster*, at least relative to mammals, a range of sophisticated instinctive behaviours have been uncovered. Analogous mating dances to those seen in fighting-fish have now been studied in depth, detailing a series of actions going from orienting, tapping, singing, licking and finally copulation (Bastock and Manning, 1955; Greenspan and Ferveur, 2000; Spieth, 1974; Yamamoto and Koganezawa, 2013); the circadian clock has been unpicked and its influence on behaviour uncovered (Gilestro, 2012; Huber et al., 2004; Liu et al., 2015; Sakai and Ishida, 2001; Tononi, 2000; Williams and Sehgal, 2001); aggressive behaviours have been similarly identified and dissected down to the genetic and neural level (Kravitz and Fernandez, 2015; Wang et al., 2008; Zwarts, Versteven, and Callaerts, 2012). These behaviours are now understood in sufficient depth that genetic lines exist which allow 60 different behaviours to be elicited by undergraduate students through a simple thermogenetic activation assay (McKellar and Wyttenbach, 2017).

The modern day study of behaviour is thus extending its focus beyond description. Rather, modern techniques and computing power enable the study of behaviour in new ways, such as attempting to

discern its underlying structure and define the behaviours in algorithmic and neural terms. Tinbergen provided a useful framework through which to understand a given behaviour by proposing complementary explanations along two axes. On one axis, we could consider the behaviour either statically - that is, as it currently presents itself - or dynamically by studying its history, usually through evolution. On the other axis, we can address the question proximately by understanding how an organism's structure contributes to the functioning of the behaviour, or ultimately by viewing it through the lens of evolution. This yields 4 'categories of explanation': an ontological (or developmental) view, which details the dynamic development of the relevant structures within the organism for executing the behaviour; a phylogenic view, comprising the history of evolutionary changes over generations leading to the present behaviour; a mechanistic explanation, involving how structural features contribute to the functioning behaviour; and finally a functional view, detailing how the behaviour contributes to a reproductive or survival function.

In the present study, my focus is on the mechanistic and functional features of defensive behaviours: how are they produced by the structures of the brain, and how do the distinct cognitive processes that guide them contribute to survival. This study therefore lies at the intersection two fields: their selection and execution by nervous systems requires neuroscientific understanding; and the behaviour of the organism and its relationship to its evolutionary history resides in ethology.

1.2.1 *Instinctive behaviour as an insight into cognition*

Instinctive behaviours are particularly useful for experimenters attempting to understand cognition. Instinctive behaviours are readily elicited under laboratory settings without training. A further property of instinctive behaviours that facilitates their study is the evolutionary pressure they are subject to. As they are essential to survival and not learned from experience, the neural circuits underlying their implementation are likely to have genetic correlates that enable the constituent neurons to be accessed experimentally.

As instinctive behaviours are not learned, it is often assumed that they are not cognitively sophisticated. A false dichotomy is often drawn between instinctive and learned behaviours. However, while not requiring learning, instinctive behaviours can be adapted and adjusted by experience. To be maximally effective, instinctive behaviours should incorporate learned knowledge. Instinctive behaviours therefore have non-learned components embedded within them. Rather than viewing instinctive behaviours and learned behaviours has dichotomous, we should view them as complementary elements in a spectrum of behaviour.

There are many examples of impressive cognitive feats being performed in response to instinctive cues. We can decompose the factors relevant to computing an instinctive behaviours into three groups: information about a triggering stimulus, the internal state of the animal, and the animals broader model of the external world.

For instinctive behaviours that are triggered by external stimuli, there have been many studies of how varying the stimulus changes the behaviour elicited. One such behaviour in mice is parental care, which is elicited by the presence of a pup. These behaviours include nest building, grooming, and increased aggression towards intruders. However, a sophisticated, plastic mechanism underlies its initiation. While virgin females, mothers and fathers will instinctively enact parental care towards pups, virgin males will instead behave aggressively (Isogai et al., 2018). Pup recognition further requires multi-sensory combinations of cues, with morphological and chemosensory features of the pup interacting to determine whether parental care is initiated. Parental behaviours in mice are thus changed significantly depending on animal history and can be dependent on combinations of stimulus features.

1.3 INSTINCTIVE DEFENSIVE BEHAVIOURS

1.3.1 *Initiating instinctive defensive behaviours*

While some instinctive behaviours are specific to their species - such as the bees waggle dance - others are widely shared across species, reflecting a common selection pressure. One such set of behaviours are defensive behaviours. All animals must respond to imminent threats in order to avoid damage or predation. Not only are instinctive defensive behaviours widely shared across species, but so are the stimuli that trigger them.

LOOMING VISUAL STIMULI Perhaps the best example of a shared triggering stimulus for defensive behaviours are responses to looming visual stimuli. Many species are subject to predators in the form of birds swooping from above (Peek and Card, 2016). Accordingly, many species display instinctive defensive responses to dark visual stimuli that rapidly expand overhead. Moreover, while usually associated with the looming stimulus being overhead, looming stimuli can indicate an impending object collision when approaching from any angle, and therefore often elicit responses from other angles. Responses to looming visual threats tend to be fast and robust, but can be surprisingly complex, even in relatively simple insect species (Card, 2012). Looming visual stimuli are therefore useful tools for studying the initiation of defensive behaviours.

In laboratory settings, the influence of various properties of visual looming stimuli on inducing escape have been studied in great detail across species. These parameters are based upon the properties of the contrast and area covered by the looming stimulus on visual field. As predators approach, the angle subtended by the shadow they cast, known as the angular size, increases non-linearly. The response time of animals to these various parameters can be used to read out the features of the stimulus that are detected by the nervous system when initiating defensive actions (Evans et al., 2018). For example, by varying the rate at which the visual angle changes, two distinct models of defense initiation can be distinguished: initiating escape when the stimulus exceeds a certain size, or initiating escape at a given time-to-collision. Escape from looming stimuli in most organisms can be classified into one of these two groups.

Other defensive responses can be elicited depending on the properties of the visual stimulus displayed. For instance, freezing rather than flight is induced if a black spot moves in a linear path over the head of a mouse, with mice attempting to avoid being detected by a predator searching for prey rather than immediately attempting to reach shelter (De Franceschi et al., 2016).

LOUD, UNEXPECTED SOUNDS Cues can also signify an impending threat through other modalities, including auditory. For instance, predators often make noise when approaching, and conspecifics raise alert calls when in danger. Accordingly, robust instinctive defensive responses such as escape or freezing are initiated in mice upon the presentation of ultrasound sweeps between 17-22 kHz (Mongeau et al., 2003). Further evidence from within our laboratory has found that loud, unexpected sounds induce similar defensive responses.

PREDATOR ODORS Another cue that can signal imminent threat is the odor produced by predators. The rodent olfactory system has dedicated channels for the processing of kariomones, the class of chemical emitted by other organisms that can be detected by members of another species. For example, in mice, the olfactory system detects 2,3,5-trimethyl-3-thiazoline (TMT), which is component of fox secretions and thus signals threat (Root et al., 2014). In rodents, the receptors for kariomones in the vomeronasal organ activate the defensive system in the hypothalamus, by way of the amygdala (Pérez-Gómez et al., 2015). Analogous odours can be found in a range of species, but as they are not used in the present study, they are introduced here for completeness and to illustrate the range of threatening stimuli that animals have instinctive defensive responses to.

MODELS OF DEFENSIVE ACTION INITIATION If the only aim of behaviour was to avoid predation, defensive behaviours could be relatively simple: hide in safe locations, and if for reason you are exposed,

run quickly. However, animals have multiple aims that sometimes conflict, and the animal must select the most adaptive action from these competing choices. For instance, while exploring for food, exposure to threat introduces a trade-off between sacrificing potential food and avoiding threat.

How should these trade-offs be computed? Ydenberg and Dill propose that defensive behaviours should be initiated at the point at which the cost of fleeing is lower than the cost of not fleeing (Ydenberg and Dill, 1986). Viewed through this economic framework, the decision to escape must incorporate an estimation of the threat provided by the stimulus, it's likely cost, and the value lost to opportunity and energy costs. A clear corollary is that, if the cost of fleeing increases (energetically and in terms of opportunity cost), the initiation time should be later.

1.3.2 *Defensive action selection*

That instinctive behaviours in response to looming stimuli are found in a wide range of species makes such responses particularly useful for gaining insights into how behaviour works across species (Peek and Card, 2016). In particular, through cross species comparisons of defensive behaviours we can probe the cognitive capabilities of different species, and as we will see, arrive at a model system for combining high level cognition and instinctive defensive behaviour.

In organisms with more limited capacity for high level cognition, defensive behaviours tend to be relatively simple, often involving primarily moving away from an impending threat rather than a goal-directed behaviour (Card, 2012). Their defensive responses are therefore much more stereotyped and have been studied in depth in a few model organisms. For example, fish display defensive responses to rapidly approaching threat by initiating a 'c-start' response, a rapid reflex-like turning and swimming away from the threat similar to the response of crayfish known as the 'jack-knife'. Similarly, the defensive responses of a range of insect species have been studied in depth, and are typically relatively simple (Card, 2012). However, while some forms of escape rely less on cognition than those in other species, even these simple forms of escape are modulated by experience and rely on information about the environment. Repeated exposure to the stimulus with no negative consequence leads to habituation, a relatively simple form of learning in which the animal learns not to escape to the stimulus. Habituation has been observed in a range of species with comparatively simple escape responses - including drosophila (Duerr and Quinn, 1982), betta splenden fish (Rhoad, Kalat, and Klopfer, 1975), and stickleback fish (Peeke and Peeke, 1973), and mosquito larvae (Baglan, Lazzari, and Guerrieri, 2017) - and in species with more

sophisticated responses such as rats (Davis, 1970) and humans (Geer, 1966).

Mammals, and especially humans, are considered to have highly sophisticated cognition. Their responses to instinctively threatening stimuli are thus of substantial interest. Mice have become one of the most studied group of mammals in neuroscientific research. In nature, their defensive tactics depend on their micro-habitat and circumstance. In a comparative study of locomotion modes of rodents in the Argentinian Monte desert, Taraborelli and colleagues found differences in the mode of escape that are dependent on the habitat of the rodent (Taraborelli, Corbalán, and Giannoni, 2003). Bipedal escape is more efficient than quadrupedal escape, enabling better monitoring of overhead threats, higher speeds, faster reactions, and more rapid changes in direction. Accordingly, being bipedal is associated with animals living in arid regions in which escape from predators must be rapid and agile; in regions with ample plant cover to hide in, quadrupedal locomotion is more prevalent (Mares, 1983). For example, Taraborelli and colleagues found a close relationship between habitat and running mode among three species of rodents - the South American grass mouse (*Akodon molinae*), gerbil mice (*Eligmodontia typus*) and leaf eared mice (*Graomys griseoflavu*). While the South American grass mouse primarily uses quadrupedal running during escape, gerbil mice and leaf-eared mice use either quadrupedal or bipedal running. Moreover, gerbil mice were found to adapt their running mode depending on the plant cover, using bipedal hopping more when plant cover was more sparse (Taraborelli, Corbalán, and Giannoni, 2003). This indicates that in the wild, mouse escape is tuned the their ecological niche and is flexibly adapted in differing environments.

PRIOR LABORATORY STUDIES OF INSTINCTIVE ESCAPE NAVIGATION These findings have been translated into the laboratory setting. A recent growth in interest in mouse escape behaviour was sparked by Yilmaz and Meister showing that escape to shelter is readily elicited in mice by displaying a looming visual stimulus over the mouse's head (Yilmaz and Meister, 2013). This behaviour was specifically elicited by dark looming stimuli, and not by other stimuli such as bright flashes or light looming stimuli, indicating dark looming stimuli act as a specific trigger for mouse defensive behaviours. However, different behavioral patterns can be elicited by different stimuli. De Franceschi and colleagues found that by instead displaying a spot sweeping over a screen overhead, they could elicit an alternate defensive strategy in the form of a freezing response, designed to avoid detection rather than escape danger (De Franceschi et al., 2016). Furthermore, combining these two stimuli in sequence elicits the expected sequence of behaviour: an initial freezing, followed by an escape when the stimulus changes from a sweep to a loom. Similar responses have been reported in response to instinctively threatening ultrasound stimuli in mice (Mongeau et al., 2003). The behavioural

response is also tuned to the magnitude of the threat displayed. Recent work from our laboratory has shown that escape vigor and reaction time is dependent on the contrast of the visual stimulus (Evans et al., 2018). This indicates that the nature of the stimulus matters enormously for the behavioural response evoked, with the behaviour being adaptive to the specific threat posed.

Laboratory settings allow effect of the environment to be explored with greater control than through observations in the wild. A series of experiments from our laboratory recently investigated the role the environment plays in controlling the expression of defensive behaviour (Vale, Evans, and Branco, 2017). Strikingly, Vale and colleagues found that in the absence of shelter, mice respond to looming stimuli by freezing rather than escaping. Importantly, escaping to shelter was not dependent on vision of either the shelter or of visual cues, being maintained in the dark and after rotation of proximal visual cues. This indicates that a memory of the shelter and its location guides the selection of defensive action.

These findings have analogues in humans. Instinctive responses to looming stimuli have been demonstrated in human infants as young as 2 weeks old (Ball and Tronick, 1971), who startle, withdraw their head, and raise their arms. However, response timing strategy seems to shift through development, with 5-6 month olds prioritising stimulus velocity, and 11-12 month olds prioritising the more efficient measure of stimulus collision time (Weel and Meer, 2011). As using an estimate of stimulus collision time requires more extrapolation and prediction, this shift in strategy could be due to learning through locomotion experience.

Defensive behaviours in mice are therefore highly sophisticated, incorporating high level cognitive representations of the environment and adapting appropriately to the ethological threat posed by the stimulus. We will now see how, across a range of species, specialised neural structures have evolved to underlie to detection of threatening stimuli and the execution of defensive behaviours.

1.3.3 *Anatomy of defensive behaviours in mammals*

Across species, highly specialised circuits have evolved to detect predators. For example, in drosophila, highly sensitive looming detection neurons have been found and characterised (Klapoetke et al., 2017). However, as focus of the present thesis is in mammalian brains, I will focus here on the mammalian circuitry underlying the detection of instinctively threatening stimuli and the initiation of defensive actions.

SUPERIOR COLLICULUS The superior colliculus is classically associated with directing sensory structures towards regions of space of

interest, such as towards salient features of the environment (Gandhi and Katnani, 2011; Harsay et al., 2011; Krauzlis, Liston, and Carello, 2004; Krauzlis, Lovejoy, and Zénon, 2013). Early studies in awake, head-fixed monkeys found superior colliculus neurons that fire in relation to saccade eye movements. It is strategically positioned for this function, laying across the midline above the brainstem, receiving inputs from multiple sensory modalities (Redgrave, Westby, and Dean, 1993). These features also mean the superior colliculus is able to act as an 'event detector', filtering the visual sensory scene for important events (Dean, Redgrave, and Westby, 1989), including impending threats.

Accordingly, the superior colliculus and its analogue in other species (the optic tectum) seem to be the primary brain region responsible for detecting threatening looming stimuli (Westby et al., 1990; Wu et al., 2005). The superficial layers of the superior colliculus receive direct input from the OFF-channel retinal ganglion cells, which are highly sensitive to looming visual stimuli (Yilmaz and Meister, 2013). In turn, the neurons of the superior colliculus inherit this sensitivity to looming stimuli (Evans et al., 2018; Westby et al., 1990; Zhao, Liu, and Cang, 2014). The neurons in the deep superior colliculus project to brainstem structures involved with premotor activity and the initiation of movements, such as the periaquectal gray, providing a rapid route from detection of a threat to action.

A crucial feature of the function of the superior colliuclus is its topographic organisation. Across the surface of the SC, the sensory-motor space is mapped in egocentric coordinates. Accordingly, the superior colliculus can be divided anatomically into medial and lateral subdivisions corresponding to different points in the azimuth plane. As the natural predators of rodents typically descend from above, the medial SC, which corresponds to this part of visual space, is particularly related to escape and stimulating the medial SC invokes escape behaviour in a stimulation strength dependent manner, suggesting this stimulation mimics the accumulation of threat evidence in the medial SC (Evans et al., 2018). The escapes are extremely rapid, with latecies as low as 250ms in mice (Evans et al., 2018; Yilmaz and Meister, 2013).

In accordance with this model, neurons in the superior colliculus increase their activity in response to looming stimuli (Liu, Wang, and Li, 2011). This role for the superior colliculus was tested by Evans and colleagues, who provided evidence that the mSC accumulates threat evidence evoked by looming visual stimuli by recording and manipulating neural activity in the medial SC (Evans et al., 2018). Neural activity accumulated in mSC after stimulus onset but prior to escape onset, suggesting a role in detecting and accumulating evidence of threat. Mimicking this increase in mSC neural activity with optogenetics evoked escape in a manner that scaled with the strength of stimulation.

Together this data provide strong evidence that direct input from the retina allows the mSC to act as a detector of visual threat. However, under this schema, it remains unclear how defensive actions are actually initiated. One output of the deeper layers of the superior colliculus is the periaqueductal gray, whose anatomical position, connectivity, and role in initiating a range of behaviours makes it a prime candidate to initiate defensive behaviours.

PERIAQUEDUCAL GRAY The periaqueducal gray is situated in the midbrain and is involved in a wide range of behavioural functions, particularly autonomic and motivated behaviours and responses to threat. Its patterns of connectivity suggest it is well situated to participate in defensive behaviours in particular, receiving inputs from limbic and sensory areas and projecting to areas that control motor output.

In addition to it's connectivity patterns, the dorsolateral periaqueductal gray (dlPAG) in particular has long been associated with the initiation of defensive actions through behavioural experiments. Early indications of its involvement in initiating defensive actions came from stimulation studies, in which electrical or chemical means were used to study the role of the various sub-areas within PAG (Bandler and Carrive, 1988; Bandler, Prineas, and McCulloch, 1985; Bittencourt et al., 2004; Fanselow, 1994; Vargas, De Azevedo Marques, and Schenberg, 2000). These studies revealed a functional topology in which active defensive responses such as running were elicited by stimulating the dlPAG, while immobility and freezing were elicited by stimulating the ventrolateral PAG (vlPAG) (Tovote et al., 2016). Accordingly, lesioning dlPAG prevents escape responses, clearly demonstrating a crucial role for the dlPAG in initiating escape (Blanchard et al., 1981; Halpern, 1968). Moreover, the dlPAG shows increased c-Fos activity in response to predator associated odors (Canteras and Goto, 1999; Cezario et al., 2008; Comoli, Ribeiro-Barbosa, and Canteras, 2003).

Both recordings and more precise manipulations of neural activity have provided further evidence for the role of of PAG in defensive behaviours. Calcium imaging of excitatory neurons in the dlPAG showed they reliably increase their activity during escape, with the onset of activity being tightly aligned to the onset of escape (Evans et al., 2018). These neurons seem to purely encode escape: they show no change in activity during instinctively threatening stimuli that do not lead to escape. Moreover, manipulating these neurons optogenetically to replicate this activity induces escape, indicating a causal relationship between the marked increase in activity and escape (Evans et al., 2018). Together this strongly indicates the dlPAG encodes the variable of whether to escape that is then used to drive escape behaviour.

How does the PAG compute this variable? The PAG sits one synapse downstream of the SC, receiving direct synaptic input. As shown previously, the SC accumulates evidence of threatening stimulus. The

PAG therefore receives direct input about accumulated threat. This suggests a model in which the PAG thresholds this accumulated evidence in order to compute the escape decision (Evans et al., 2018). In accordance with this model, the synaptic connection from SC-PAG is weak, excitatory and unreliable, meaning activity must accumulate in SC before it can trigger PAG to initiate escape. This circuit structure ensures that when threat is imminent animals escape to shelter while minimizing the opportunity and energy costs due to false escapes.

The PAG seems to play a conserved role in humans. Typical studies of threat perception in humans involve participants engaging in a computer task escaping from a virtual predator, with the aim being to minimize the loss of reward incurred if the participant gets caught (Qi et al., 2018). In this study, rapidly approaching predators elicited responses in PAG, but not slowly approaching predators. Similarly, PAG activity was seen if the participants were shocked instead of losing reward, with activity peaking when the shock was imminent (Mobbs et al., 2009, 2007; Wendt et al., 2017). These features of the human fMRI response to imminent threat indicate the PAG encodes threat in a similar way to that observed in rodents.

The periaqueductal gray therefore seems to have similar roles in rodents and humans. This conserved role is now increasingly well understood at the circuit level with respect to instinctive defensive behaviours: PAG thresholds evidence accumulated in the superior colliculus to initiate escape. However, PAG also plays an important role in initiating other behaviours, including other forms of defensive behaviour, owing to the wide range of inputs it receives.

AMYGDALA AND HYPOTHALAMIC SYSTEMS Perhaps the most studied mammalian brain region with respect to defensive behaviours is the amygdala. Importantly, while the superior colliculus is primarily associated with instinctive defense, the amygdala has also been shown to be involved in processing learned fear responses. An extensive literature explores the role of the amygdala in learned defense which has detailed the distinct roles of each of the partitions of the amygdala - basolateral, lateral, medial, and central - in fear learning. While a detailed review of the literature regarding learned fear is beyond the scope of the present thesis and is covered extensively elsewhere (see LeDoux, 2003; LeDoux, 2000), there are important lessons to take from these studies. Perhaps most importantly, early studies showed that the expression of fear responses evoked by electrical stimulation of the amygdala is suppressed by lesioning the dPAG. This suggests that the amygdala exerts its influence on behaviour via the PAG (Hunsberger, 1956; Molina and Hunsperger, 1962).

While many studies of the amygdala have focused on its role in learning fear associations, the amygdala also receives input from the vomeronasal organ (VNO), a sensory area dedicated to detecting non-volatile pheromones and kariomones, which signal the presence of in-

stinctively relevant con-specifics or predators respectively (Root et al., 2014). Importantly these circuits seem to be distinct from those underlying learned fear, suggesting the presence of parallel circuits underlying the different behavioural outputs associated with the amygdala. For example, it is primarily the cortical amygdala that is active in response to instinctively threatening kariomones, whereas the lateral amygdala is necessary for associative fear learning.

Traditionally seen as downstream of the amygdala, the hypothalamus plays a similarly important role in defensive behaviours. In the classical view of defensive circuitry, the hypothalamus acts as a kind of relay, taking the processing occurring in the amygdala and linking it to behavioural output. More recent evidence suggests that this is an overly simple view, and that the hypothalamus plays a more direct role in computing defensive responses. For instance, in mice, optogenetic stimulation of a sub-population of the dosromedial division of the ventromedial hypothalamic nucleus (VMHdm) induces defensive behaviours whose expression as either escape or freezing depends on the stimulus intensity (Kunwar et al., 2015); inhibiting these neurons led to a lack of response to predators, but not to overhead looming stimuli (Silva et al., 2013). Moreover, a reduction in place aversion after exposure to these predators is observed (Silva et al., 2016). It is therefore clear that the hypothalamus, and particularly the VMHdm, plays a vital role in inducing defensive behaviours.

The VMHdh seems to enact this role particularly with defensive behaviours induced by olfactory stimuli. In fact, it may be its primary role with respect to defensive behaviours, as other modalities seem to bypass the hypothalamus entirely. Downstream of the hypothalamus, its effect on behaviour seems to be mediated via collaterals projecting to the dorsolateral periaqueductal gray (dlPAG) and the anterior hypothalamic nucleus, the dlPAG primarily mediating freezing responses, the anterior hypothalamic nucleus underlying escape (Wang, Chen, and Lin, 2015). Together, these data indicate that amydala-hypothalamic system predominantly underlie defensive responses to olfactory stimuli, with the amygdala also involved in learned associations.

1.3.4 *Summary of instinctive defensive behaviours*

Defensive behaviours range from learned to instinctive. So far we have seen that these behaviours are readily elicited in the laboratory and in the case of instinctively driven defensive behaviours do not require training. Moreover, they involve high level cognition, particularly of the spatial environment. They are ethologically relevant and have clearly defined circuits underlying their initiation. It is for these reasons that defensive behaviours provide a particularly useful tool to study cognition.

These features of defensive behaviours, particularly in rodents such as mice, provide an ideal model to study cognition. Through this model it becomes possible to being to resolve a long standing aim of ethologists and some neuroscientists alike, as best described by Tinbergen when he wrote that "The "no-man's land" between Ethology and Neurophysiology is being invaded from both sides. While ethologists are making progress with the "descending" breakdown of complex phenomena, neurophysiologists are "ascending", extending their research to phenomena of greater complexity than was usual 20 years ago". It is the aim of this study to contribute towards the merging of ethology and neurophysiology by investigating escape navigation and it's neural correlates. Having explored the first aspect of this - instinctive escape to shelter - I now overview spatial navigation and its underlying algorithms and neural underpinnings.

1.4 SPATIAL NAVIGATION AND REPRESENTATION

Spatial navigation and its underlying representations in the brain is one of the best studied models of cognition. Movement is arguably the most fundamental component of behaviour, and to moving adaptively requires a form of guided navigation. In this section, I overview three questions in turn:

- What forms of navigation are possible for animals?
- What representations are required for their use?
- How does the brain implement them?

1.4.1 *Algorithms for navigation*

How is this route to shelter computed? There are several possible algorithms that could be implemented, with combinations therein also possible (O'Keefe and Nadel, 1980).

TAXON NAVIGATION At the point at which navigation must be enacted, one strategy is to simply use a sensory feature as a guide, be it sight, sound, or odour (Hamilton, Rosenfelt, and Whishaw, 2004). This strategy is known as taxon navigation, and it involves identifying a feature of the goal and performing gradient descent until it is reached (Redhead et al., 1997; Whishaw and Kolb, 1984). This approach suffers limitations when clear sensory cues are not available, and requires a time delay after stimulus presentation for sensory processing to occur. It is therefore relatively rudimentary. In some organisms, such as insects, it is hardwired, but it can also be learned from experience. As a broad strategy it is flexible and not cognitively

demanding. For example, it can be extremely useful - the only available strategy, in fact - when first introduced into an environment, and therefore with no memory or habits to rely on.

PRAXIC NAVIGATION If the mouse has undertaken the route previously, a habitual form of navigation is possible where the learned route is simply recited (Redish, 1999). In general this requires associating particular stimuli with actions, though in theory this kind of route navigation can be done purely from memory. We experience this form of navigation when, for instance, going to and from work: we do not work out our route each time, but rather rely on a habit of where to go. It's shortcomings are that you have to have taken the route before, and it requires significant memory. Moreover, this strategy has little capacity for shortcuts or flexibility. This means that should the environment change or if the environment were incompletely explored originally, there may be a shorter route to the goal that is not being exploited.

PATH INTEGRATION This form of navigation involves adding up your changes in position since you last visited the goal and summing them to calculate the vector to it (Etienne and Jeffery, 2004; Mittelstaedt and Mittelstaedt, 1980). It's very computationally efficient and simple to represent, but degrades with time since visiting the goal and can fail with a lot of movement or disorientation without a means to reorient within the environment.

LOCALE NAVIGATION A form of map based navigation using localisation through inference via the positions of the available cues (O'Keefe and Conway, 1978). The cues allow animals to work out where they are on some map like representation, which can then be used to calculate where to go. While highly cognitively demanding, it enables great flexibility of action.

Each of these strategies for navigation has been documented in living systems. Before providing an overview of how the brain achieves these feats, I will first discuss the organisms that use each and under which circumstances they are used.

DISTINGUISHING BETWEEN NAVIGATIONAL ALGORITHMS How do we know which of these algorithms is used in a given situation? In the wild, animals are likely to employ combinations of these different strategies. This provokes a question of paramount importance to experimentalists, especially neuroscientists, as different processes in the brain are likely to underpin each. If there is redundancy between their capabilities, how can we be sure we are observing the cognitive process that we think we are?

Experiments have to be carefully designed to address this issue. Taxon navigation can be assessed by removing the cues that signal the goal location and seeing if navigation is still successful, though it is challenging to be sure all relevant cues have been sufficiently controlled for. In the case of praxic navigation, one can assess the ability of animals to navigate in novel environments, and track their exploration patterns to test whether navigation depends on having explored that part of the environment before. Distinguishing between path integration and cognitive map based navigation is even more demanding: typically animals must be removed from the environment and disoriented to ensure path integration is not used, while detecting the use of path integration is often done by slowly rotating animals below the vestibular threshold in cases where no other cues are available to navigate.

1.4.2 *Representations for navigation*

Each algorithm for navigation requires a distinct set of spatial representations. For instance, for taxon navigation to be achieved, a representation of the sensory feature being navigated towards must be formed and then used to guide navigation, while for path integration the information required in a representation is a constantly updated vector. To narrow down the possible algorithms that could guide escape navigation, I will explore the spatial representations that are known to be present in the brain.

1.4.2.1 *Behavioral studies of navigation*

The animal kingdom has an array of different solutions to navigational challenges. Behavioural studies have revealed remarkable navigational feats across a range of species, some of which have been richly studied. This range of navigational abilities and their clear, observable behavioural readout has led to navigation being perhaps the most closely studied aspect of cognition.

Navigational abilities range from the relatively simple to the remarkable. On the most simple end of the scale, most organisms, ranging from bacteria to highly complex mammals, have some form of taxon navigation, wherein an important sensory feature is identified and gradient descent performed. But on the more sophisticated end of the scale, some of the most impressive documented navigational feats involve large scale migratory patterns. For example, the bogong moth (*Agrotis infusa*) travels up to around 600 miles across Australia - a remarkable distance for such a small organism (Warrant et al., 2016).

However, while these migratory patterns are no doubt impressive, it has been suggested they rely on sensory cues observed along the jour-

ney - including olfactory, visual and possibly magnetic cues - rather than environmental representations, as the can perform the navigation having never visited the destination before (Warrant et al., 2016). Path integration requires a different form of representation. In this case, a very basic form of environmental representation is required in the form of a vector in space. While relatively simple, navigation based on path integration can be highly accurate, as has been characterised in a range of insect species, particularly the desert ant and honeybee. Owing to its arid habitat, the desert ant has to navigate significant distances while foraging, and must maintain a representation of the vector back to its nest, often in the absence of significant landmarks (Andel and Wehner, 2004; Muller and Wehner, 1988). The honeybee faces the opposite problem: it must navigate back to its hive based solely on visual cues. Most path integration strategies fall somewhere between these two extremes and use a combination of visual flow and self-motion information to compute the vector (Collett, Chittka, and Collett, 2013; Collett and Collett, 2002; Heinze, Narendra, and Cheung, 2018; Hoinville and Wehner, 2018; Lambrinos et al., 2000). However, a directional fix is required to orient the path integration system, otherwise errors in the estimation of distance and direction in each step accumulate (Valerio and Taube, 2012).

Impressive progress has been made in how path integration might be implemented, to the extent that biologically plausible neural level models have been implemented involving a combination of attractor networks which are reset by visual information and shifted with self-motion cues derived from head-direction signals (Stone et al., 2017).

There is significant behavioural evidence for the use of path integration in mammals. For example, in gerbils, escape to shelter is possible even when the shelter is not visually observable, indicating a form of spatial memory is present, and when an obstacle is present their trajectory is often corrected before the shelter is visible from behind the obstacle (Ellard and Eller, 2009). Moreover, when there are different available paths to the shelter, the gerbils would typically take the shortest path (Ellard and Eller, 2009). Similar findings have been observed in a range of species, including mice (Vale, Evans, and Branco, 2017; Yilmaz and Meister, 2013), an extensive discussion of which can be found in (Domenici, Blagburn, and Bacon, 2011). Similarly, ethologists have studied path integration in the wild by observing the ability of animals to return to their nest after foraging for food, most famously in the case of the golden hamster (Etienne and Jeffery, 2004).

In experimental settings, to study path integration in mammals there are three main approaches. First, you can eliminate all other cues to the best of your ability (visual, audio, odour). The second is to make the cues that are available give conflicting information about the goal location. More invasively, you can alter the path integration input. This strategy of disruption has, for example, included slowly rotating

animals so that the internal signal provided by path integration is no longer accurate (Mittelstaedt and Mittelstaedt, 1980). More recently, other techniques such as virtual reality have been employed to investigate path integration with respect to linear distance in the form of step counting (Tennant et al., 2018). Others have used techniques such testing whether mice can navigate directly to a goal location after they have been trained to navigate down a corridor with a turn in, allowing direction-distance coding to be investigated. (Gil et al., 2018). Each of these behavioural investigatoin techniques enables a distinct component of path integration to be probed.

Despite the remarkable efficiency of path integration, more sophisticated strategies exist which involve forming a map of the environment. Laboratory studies of these abilities often take the form of behavioural studies of animals navigating mazes. Such studies have provided clear evidence of sophisticated forms of navigation under tightly controlled conditions. Seminal work in this field was performed by Tolman, who designed mazes that had unexplored shortcuts. When opened, animals could use their knowledge of the structure of the environment to take the shortcut, implying they had a form of map based spatial knowledge (Tolman, 1948). This type of navigation, Locale navigation, has become very widely studied, and has had many behavioural tasks designed to specifically require it, such as the Morris water maze (Morris et al., 1982). While there has long been a debate about whether animals truly possess maps of the environment due to a lack of evidence from natural habitats, recent field studies have shown that animals use such cognitive maps to navigate their natural environments. In particular, bats have emerged as a model organism in such field studies due to their impressive long-range navigational abilities, performing complex 3-dimensional movement patterns, and having navigation strategies likely to be guided by sensory cues rather than self-motion cues (Finkelstein et al., 2014; Genzel, Yovel, and Yartsev, 2018; Yartsev, Witter, and Ulanovsky, 2011). For example, bats have been shown to be able to navigate over scales approaching 100 km when displaced by an experimenter (Tsoar et al., 2011). These abilities have been tracked since the bats first exposure to the wild. Using light-weight GPS trackers, Toledo and colleagues tracked bats in their natural habitat, finding the regular use of shortcuts (Harten et al., 2020; Toledo et al., 2020). When trans-located to places never visited by the bat, shortcuts to their regular foraging route were readily enacted, implying they rely on a form of map. The use of bats as a model organism has therefore helped resolve a longstanding debate over whether animals truly use cognitive maps in the wild.

We therefore see that navigational abilities take many forms, the most sophisticated of which is the cognitive map. In many ways these different forms of navigational ability are complementary and add redundancy to the strategies available to an organism. Moreover, some provide the basis for others: path integration being a crucial contrib-

utor to the formation and maintenance of the cognitive map. The integration of these different sources of navigation abilities have been particularly important with respect to neurophysiological studies attempting to decipher the neural correlates of navigation. In the next section, I explore how the mammalian brain in particular represents the spatial environment.

1.4.2.2 *Neuroscientific study of spatial representations*

Tolman's studies provided evidence for internal representation of the spatial world. Where did this representation reside? Initial evidence came from early neurophysiological studies in which single neurons were recorded in the hippocampal CA3 region (O'Keefe and Dostrovsky, 1971). Using single unit recordings in freely moving mice, O'Keefe and Dostrovksy provided evidence that neurons in the hippocampus are tuned to fire when the animal is at a particular location within an environment. Further evidence for the role of the hippocampus in spatial navigation was provided with the advent of the Morris water maze (Morris et al., 1982). Prior to this, there were not reliable, convenient ways of studying spatial navigation that were certain to be dependent on a form of a spatial map, with other methods confounded in various ways. The task involves training an animal that a pool of clouded water has a platform in, and then reintroducing it at random points and testing its ability to swim to it. Variations exist wherein the position of the platform changes either within or between days in order to test various strategies of spatial cognition. Thus, the Morris maze enabled the ability of the mouse to navigate dependent on a map to be isolated. A decline in performance on this task after lesioning the hippocampus provided convincing evidence for a role of the hippocampus in providing a form of map of the spatial environment.

SPATIALLY RESPONSIVE NEURONS As previously shown, sophisticated navigational abilities are present in a wide range of species and comprise a core component of high-level cognition. These clear neurophysiological and behavioural correlates led the hippocampus to became a crucial focus of neuroscientific investigation. Place cells have subsequently been found in the hippocampus of a wide range of species including mice, bats (Finkelstein et al., 2014; Genzel, Yovel, and Yartsev, 2018; Tsoar et al., 2011), humans (Ekstrom et al., 2003), and other primates (Ludvig et al., 2004). In addition to providing an estimate of the animals position within an environment, place cells maintain their firing fields across days, allowing a form of memory to be maintained. While place cells had previously been reported to have been recorded over periods as long as 153 days, with the firing field of a cell being consistent throughout (Thompson and Best, 1990), more recent data suggests that place cell fields are not consistent across a weeks long timescale (Ziv et al., 2013). Place cells do

however conserve a form of representation related to navigational abilities and spatial memory across a range of species.

However, place cells do not form place fields in every environment. What is the statistical structure of the spatial representation formed by place cells on a population level? Rich and colleagues addressed this by studying spatial representations over a larger scale by constructing a 48m track along which recording apparatus could be carried in parallel (Rich, Liaw, and Lee, 2014). Over these spatial scales, some place cells were found with multiple place fields, with the recruitment of new place fields described by a two-parameter stochastic model, with the distribution described by a process in which each cell draws its poisson rate from an underlying gamma distribution. This gamma-poisson distribution of place field formation has been suggested to be advantageous in ensuring each environment is adequately represented, but that different environments can be represented uniquely. Together this evidence suggests that the place cell representation is tuned to give an efficient spatial mapping of the environment, scaling dependent on the size of the environment.

The cues that drive place cells have also been studied extensively. One of the earliest studies found that place cells are invariant to the removal of individual cues (O'Keefe and Conway, 1978). Moreover, place cells are maintained in total darkness (Quirk, Muller, and Kubie, 1990). However, while visual information alone doesn't control place cell firing, it is nevertheless a significant influence. Renaudineau and colleagues studied the influence of different visual cues by rotating proximal cues within the arena and distal cues on the external part of the arena in opposite directions (Renaudineau, Poucet, and Save, 2007), finding that some place cells tracked the proximal cues, some the distal cues, and some fired in a new place altogether. Together these studies suggest that while visual information influences place cell firing, place cells are not driven by purely visual information.

Environmental boundaries also play a crucial role in driving place cell firing. By stretching the experimental arena, O'Keefe and Burgess observed that some place cell firing fields stretched a corresponding amount, while others maintain their firing field at a fixed distance from walls (O' Keefe and Burgess, 1996). On the basis of this evidence, a type of cell was hypothesised that fired at a fixed distance from boundaries in a certain direction, a so called 'Boundary Vector Cell' (BVC) (Hartley et al., 2000), which were later verified experimentally (Lever et al., 2009). Boundary vector cells are found in different parts of the hippocampus. They are relatively sparse in the medial entorhinal cortex, making up less than 10% of the local cell population, but can be found in all layers of the medial entorhinal cortex as well as the adjacent parasubiculum, often intermingled with head-direction cells and grid cells (discussed later) (Solstad et al., 2008) (Lever et al., 2009).

After the place cell, chronologically the next cell class to be discovered was the head direction cell (HDC) (Taube, Muller, and Ranck, 1990). Originally discovered in the subiculum, HDCs have been found in the entorhinal cortex, retrosplenial cortex, anterior thalamus, dorsal tegmental nucleus, and lateral mammillary body. These neurons fire whenever the animal's head is pointing in a particular allocentric direction in the environment, acting as a kind of compass. Both vestibular and visual cues guide HDC firing preferences. Rotating the sole visual cue in an arena induces HDCs to rotate their firing fields in tandem, showing they are anchored to the external environment (Sharp, Blair, and Cho, 2001). In tandem, neurotoxic lesioning of the vestibular labyrinth leads to the loss of HDCs for as long as 3 months (Stackman and Taube, 1997). Despite HDCs continuing to maintain a firing field in the dark, indicating visual input is not necessary for HDC function, when visual and vestibular cues are in conflict visual cues take precedence (Goodridge and Taube, 1995). This suggests that HDCs provide the animal with an estimate of its bearing within the environment, using the vestibular system to update. Soon after their discovery, HDCs were found to rotate in unison with place cells after a cue in the environment was rotated (Knierim, Kudrimoti, and McNaughton, 1995), implying place cells are under the control of the head direction system. Moreover, lesioning the head direction system leads to a decrease in the specificity and stability of place cell firing fields, though does not totally degrade place cell function (Calton et al., 2003). Through the course of development, HDC's are the first spatially responsive cells to acquire appear (Wills and Cacucci, 2014). The early development of HDCs and their marked influence on other spatially responsive cells highlights the importance of HDCs to hippocampal function.

Perhaps the most beautiful of the spatially responsive neurons are the grid cells, which tile a hexagonal grid across the environment (Fyhn et al., 2008; Hafting et al., 2005; Sargolini et al., 2006). Grid cell firing patterns have been observed in a range of species including rats, human neurosurgical patients during a virtual reality task (Jacobs et al., 2013), and bats (Yartsev, Witter, and Ulanovsky, 2011). These grids are maintained in darkness and are quite robust to cue removal. Grid cells were originally found in the medial entorhinal cortex, and have subsequently been found in the presubiculum and parasubiculum. The grid of each cell is characterised by the spacing between vertices of the grid, which are maintained across environments (Stensola et al., 2012). Their remarkable firing properties and consistency across environments have led to grid cells being thought of as a 'metric' for the hippocampal cognitive map. The independence of grid cell firing properties from visual cues implies self-motion primarily drive grid cells. In order to compute this, current heading and speed of travel are required. While heading can be decoded from the well studied head direction system, more recent studies have founds neurons in the medial entorhinal cortex that explicitly encode speed (Kropff et al., 2015). Consistent with a role in path integration, rats with lesions

to the medial entorhinal cortex fail to navigate back to refuge when only self-motion can be used (Kim et al., 2013).

THE HIPPOCAMPAL COGNITIVE MAP After the discovery of place cells, a hypothesis for the role of the hippocampus in spatial navigation was provided by O'Keefe and Nadel in their book 'The Hippocampus as a Cognitive Map' (O'Keefe and Nadel, 1980). The hypothesis they laid out was that the hippocampus provides an allocentric spatial representation of the environment akin to a 'cognitive map'. Prior to this, the hippocampus had long been known to be associated with episodic memory, mainly through lesion studies and studies of humans with damage to the hippocampal formation (Scoville and B., 1957; Squire, 2009). Episodic memory and spatial representation are intrinsically linked: memory of episodes must necessarily include a representation of the spatial context (Eichenbaum, 2000, 2017). Whether other aspects of cognition are also represented within the hippocampal cognitive map is still an area of ongoing research. For example, a recent study found that after a mouse was trained on a task where they manipulate a continuously variable auditory stimulus, cells with firing fields in auditory space akin to place cells and grid cells could be found in the hippocampus (Aronov, Nevers, and Tank, 2017). This suggests that, rather than being specific to space, the hippocampus is capable of representing any behaviourally relevant continuous variable. Nevertheless, the idea that hippocampus represents space and that a form of a map exists is now, due to the weight of neurophysiological data, widely accepted. Behavioural evidence also supports this hypothesis, with the performance of hippocampus lesioned rats on the Morris water maze (Morris et al., 1982) and the eight arm maze (Olton, Collison, and Werz, 1977), while maintaining forms of navigation such as taxon navigation, providing evidence for a role of the hippocampus in spatial navigation of the kind only possible with a cognitive map.

PRAXIC NAVIGATION As described in section 1.4.1, animals are capable of navigating based upon other strategies than the cognitive map that has been so well studied. Other forms of navigation based upon habitual memory are possible. What brain structures underlie these forms of navigation?

Learning habitually requires linking the series of movement taken to the desirable end outcome. Under a reinforcement learning framework, this can be thought of as using a reward signal - in particular temporal difference error between expected and actual reward - to learn. Such signals have been found in the brain, most famously in the dopaminergic neurons of the substantia nigra, but also throughout the basal ganglia system (Schultz, Dayan, and Montague, 1997; Schultz, 2006). In reinforcement learning models, the 'actor-critic' architecture comprises a model with two interacting components: an

actor which represents the reward related outcomes of actions, and a critic, which predicts future reward. This 'actor-critic' model is thought to map onto distinct structures in the striatum: the dorsal striatum corresponding to the actor, and the ventral striatum to the critic (O'Doherty et al., 2004). While this is a simplification of the function of the striatum (Bornstein and Daw, 2011), according to this hypothesis, a lesion to the striatum should lead to a decline in the ability to learn praxic navigation. Such an effect was demonstrated by comparing silencing the hippocampus and the caudate nucleus, a sub-region of the dorsal striatum, in rats trained on a cross-maze (Packard and McGaugh, 1996). In this task, rats navigate to find a food reward either based on absolute allocentric place ('place navigation') or in response to cues that indicate they should turn in a particular egocentric direction ('response navigation'). While silencing the hippocampus reduced the tendency to use a place strategy, silencing the caudate reduced response navigation. This indicates that praxic navigation depends upon the dorsal striatum in a manner that aligns with the actor-critic model. The neural responses in the dorsal striatum further support this model, being tuned more to task stage and choice points than allocentric position (Barnes et al., 2005). Together this suggests a crucial role for the striatum in learning praxic navigational strategies.

EGOCENTRIC REPRESENTATIONS To move through an environment, it is also important to know how the environmental structure represented in allocentric form in the hippocampus relates to the animals body and its current view of the environment. This egocentric form of representation allows viewpoint independent representations to be integrated with the current view of the local sensory environment from the animals perspective to guide action. This transformation is thought to be mediated by two regions in particular: the medial parietal cortex, which represents egocentric scene elements such as boundaries and landmarks (Bisiach and Luzzatti, 1978; Nitz, 2009; Nitz, 2006; Save and Poucet, 2009; Wilber et al., 2014); and the retrosplenial cortex, which receives input from both the hippocampal formation and the parietal cortex, and is therefore well placed to transform between these two representations. Accordingly, retrosplenial cortex neurons have been shown to map the conjugation between these two spaces, encode sub-features of routes, and maintain information about the current head direction of the animal (Alexander and Nitz, 2015, 2017; Burgess et al., 2001; Byrne, Becker, and Burgess, 2007).

This function of the retrosplenial cortex in transforming between egocentric and allocentric reference frames places it in a unique position of importance for our purposes. Behavioural studies across a range of organisms - and particularly mammals - have shown that escape navigation requires transforming environmental knowledge into egocentric actions. Recent data from our laboratory has demonstrated the

pivotal role of a retrosplenial cortex to superior colliculus circuit in guiding escape navigation. Neural recordings during exploration of an arena with a shelter showed that neurons within the retrosplenial cortex continuously encode the shelter direction. Specifically silencing neurons in the retrosplenial cortex that project to the superior colliculus resulted in disrupted orienting to shelter upon presentation of an instinctively threatening stimulus, while the ability to orient towards a novel stimulus was maintained. That other types of orienting movement were maintained indicates that this RSC-SC circuit may be specific for memory guided orienting movements, explaining its vital importance in translating environmental knowledge into action for escape navigation.

The retrosplenial cortex is therefore a crucial node in the network of brain structures underlying escape navigation. According to the hypothesis outlined by Bicanski and Burgess (Bicanski and Burgess, 2018), retrosplenial cortex acts by translating between egocentric and allocentric reference frames. This implies that escape navigation may depend upon allocentric representations of space, but only under particular conditions. A crucial juncture in determining which representations guide escape navigation is clarifying the algorithms initiated upon predatory stimulus detection and the conditions under which they are used. Our present understanding suggests that the retrosplenial cortex allows either allocentric representations or egocentric representations to be integrated into an estimate of the shelter location. We do not know how these representations interact during escape navigation and how they are combined to drive adaptive defensive behaviour.

1.5 A HYPOTHESIS FOR ESCAPE NAVIGATION

We have seen that the brain maintains a rich set of spatial representations, and is tuned to detect threatening stimuli and rapidly enact defensive actions. However, several questions remain about the relationship between the spatial representations and defensive behaviours: How is the vector between animal position and shelter position computed from these representations? Given the short reaction time between stimulus presentation and initiation of a flight, is a representation of the vector to shelter constantly maintained, ready to drive action at any moment? Or is it computed from the spatial representations in this short time-frame?

The aim of the present study is to first clarify the algorithms initiated under conditions that aim to selectively require either egocentric or allocentric representations. This will be achieved through carefully designed behavioural experiments. These behavioural experiments then guide a series of inactivation experiments, guided by the foundation provided by Vale et al., 2020's study of retrosplenial cortex, in which

different brain regions are silenced pharmacologically and through lesions to assess their relative role in guiding escape.

Part II

METHODS

2

METHODS

2.1 ANIMALS

Mice were selected as the model organism to be used in the present study. This was due to the range of genetic manipulations and viral tools possible in mice, their similarities in brain structure to humans allowing a comparison and practical impact of this study, and the prior studies in this sub-field in this species.

Mice between the ages of 7-24 weeks were used in the behavioural experiments. All mice were male C57BL/6J's acquired from Charles River. All mice were housed in a holding room for at least 1 week before the experiment, and single-housed at least 24 hours before experimental onset. Mice were also single housed immediately after any surgical procedure until the experiment was undertaken. All experiments were undertaken during the light phase of the light cycle.

All animals were given ad-libitum food and water and were housed on a 12 hour light cycle. Within their cages, animals had various forms of enrichment, including a shelter, wooden toys and a wheel, which were changed regularly to keep animals stimulated. The shelters used in the homecage was different to those used in the behavioural experiment.

2.2 BEHAVIOR EXPERIMENTS

2.2.1 *Behavioral experimental setup*

To study flight trajectories, I built a large behavioural experimental setup. To perform behavioural experiments in a range of large environments, I constructed a 3 x 3 x 2m aluminum frame. A white screen attached across the top of the frame enabled presentation of visual stimuli. Several horizontal bars were fixed across the top of the frame to ensure the screen was taught and to enable an ultrasound speaker (Pettersson L60) and a near-infrared long-pass filtered (>700nm) camera to be attached at the centre. The camera, ultrasound speaker, and projector are all triggered by the same custom software, produced within the lab (by software engineer Kostas Bestios in LabVIEW).

Experiments were recorded with the near-infrared camera at either 24 or 30 frames per second (mostly 30, some early experiments used 24), with infrared lights (Abus TV6700) placed on the frame enabling experiments to also be conducted in the dark. When light conditions were used, a projector (BenQ MW843UST) was used to provide illumination to the arena and to project looming visual stimuli onto the white screen overhead. Ultrasound stimuli delivered through the speaker were triggered from the custom behavioural software, which was connected to the speaker through a soundcard (Xonar D2) and an amplifier (QTX PRO240).

Below this frame is therefore a flexible 3x3m space in which a range of different behavioural arenas can be placed, with varying lighting levels.

2.2.1.1 *Large open field*

I first determined the conditions under which previous studies could be replicated and trajectories observed over these scales. My first arena design was a sheet of perspex resting on the floor, with a large circular wall placed to form the arena and a small red perspex shelter. While mice adopted the shelter in this arena, they showed a spatial preference for positions close to the wall, indicating they perceive these positions as safe.

This preference for locations near the wall was manifest in escape trajectories. Rather than take a direct path towards the shelter, mice would first navigate towards the outer wall, and then follow a circular path towards the shelter. This indicates that rather than acting as a boundary of the arena, mice perceived the outer wall as safety in its own right, confounding the study of long-range escape trajectories. Due to this confound, I limited this study, and instead changed the design of my arena.

I next designed an arena with no objects that could provide cover other than the shelter. To do so, I rested a 2x2m perspex sheet 30cm off the floor to produce a large arena, with the sheer drop at the edge acting as the arena boundary. It was supported by two rings of flexible black plastic 30cm tall, leaving a flat, raised platform.

In this arena, mice readily adopted the shelter as a safe location and escape trajectories did not follow the arena boundaries. The shelter was located in different locations in the environment in each experiment, but tended to be placed close to a corner of the square arena in order to maximise navigational distance. When the mouse had travelled outside of the shelter and explored the arena, the stimulus was manually triggered by the experimenter.

This setup was used for several different experiments to study long-range escape navigation, including comparisons between escape in

light and dark condition, in the 'dry Morris maze' assay, in the 'shelter raise' assay', and the 'trap' assay. In light conditions, luminance was provided mainly by the projector in order to reduce the influence of time of day, weather or seasonal changes on lighting in the arena. Distal visual cues were visible around the arena. In the dark, an opaque curtain was drawn across between the computer and the arena, curtains were lowered, and further sources of light sealed as much as possible (luminance = 0.04 lux). While impossible to achieve total darkness, all lights in the room were turned off other than a computer monitor, which was on red light setting at lowest brightness, and was covered by two curtains. In the arena, it was impossible for humans to see anything, even after a period of adaptation.

In these experiments, trials were taken from a total of 57 mice. A total of 276 trials were taken, with a maximum of 15 trials taken from a single mouse, and a minimum of 1.

2.2.1.2 *Rotating Barnes*

To study how vestibular cues influence the computation of escape trajectories, I built a variant of the Barnes maze with a rotating centre 2.2 (Barnes, 1979).

The arena was constructed from perspex, metal tubing, and a small electronic motor 2.2. The arena had a diameter of 1.6m and was raised 0.6m off the ground. The inner, rotating portion of the maze was 1.2m in diameter; the external, non-rotating portion had a width of 0.2m. Rotations as slow as 1.5 degrees per second were possible with this experimental arena, and rotations typically fell into the range of 1.5-3.0 degrees per second, usually encompassing around 15-45 degrees.

The shelter was placed either on the external, stationary circular perspex platform, or the part of the internal, rotating portion furthest away from the centre. The holes used ordinarily were filled in and an overground shelter used to facilitate a comparison with the other experiments.

2.2.2 *General behaviour protocols*

To allow for comparison across experiments, several features of experimental protocols were kept constant. In accordance with previous experiments in the lab, 7 minutes after first entry into the shelter were allowed before any stimuli were delivered in order for the mouse to establish familiarity with the shelter and environment. If mice did not discover or adopt the shelter within 7 minutes of entry into the environment, some bedding from the mouse's home cage was added to the shelter. Control experiments were performed without bedding,

and as mice lay scents in new environments anyway, adding bedding is not anticipated to provide a new experimental confound.

Stimulus delivery was adjusted according to the experiment. For example, when comparing light and dark conditions, stimuli were delivered at times to maximise the range of responses, including covering a range of movement directions, head angles, distances to shelter, and distances to arena edges. In addition, only ultrasound stimuli were used to facilitate the comparison between light and dark conditions. In rotation experiments, stimuli were delivered when the mouse was close to the centre of the arena to minimize effects of translation during rotation.

Stimuli were delivered at intervals of at least two minutes, even if the mouse left its shelter straight after the end of a stimulus. This was to minimize the effect of the previous stimulus on the current trial and to reduce the likelihood of habituation to the stimulus.

The visual environment surrounding the arena was controlled with two types of blackout shields: curtains that could be pulled around the arena or attached to the external part of the frame, and blinds that were permanently attached to the frame on each side and could be lowered to cover almost all of the distal environment. In dark experiments, all blinds and curtains were fully drawn around the environment, all room lighting was turned off, the computer monitor turned to red light and reduced to minimal levels, and blinds drawn over room windows and doors. To reduce the influence of the experimenter, all experiments - even those where all other blinds and curtains were raised to provide ample distal cues - were performed with the side of the arena closest to the experimenter shielded with a blind.

All details of this protocol were maintained in all experiments unless otherwise stated.

2.2.3 *Sensory stimuli*

Two modalities of innately threatening stimuli were used in this study: ultrasound sweeps and visual looming stimuli.

ULTRASOUND STIMULI Ultrasound stimuli comprised an up-sweep lasting 3s in the 17-20kHz range between 70-80 dB, as recorded from the centre of the arena at arena height, repeated 3 times in succession.

VISUAL STIMULI Visual stimuli were presented by projecting a dark expanding circle onto the overhead screen above the arena (Weber contrast = -0.98, luminance = 7.0 lux). The circle was centred above

the moving animal, beginning at an angle of 3.0^{o} and expanding linearly to 41.8_{o} over 500ms. The stimulus then remained at this size for 250ms, before restarting for a total of 5 repeats.

2.2.4 *Instinctive escape assay*

Previous studies have shown that mice escape to shelter upon presentation of innately threatening stimuli. I adapted an assay used to study escape previously in the lab to my larger spatial environment (Evans et al., 2018; Vale, Evans, and Branco, 2018; Vale, Evans, and Branco, 2017).

In the 'Instinctive escape assay', a mouse is first introduced to the arena with a shelter present (figure 2.1). The mouse finds the shelter, and seven minutes are allowed for the mouse to fully adopt the shelter as it's 'home base' in the arena. A small amount of bedding is added to the shelter to make it more likely to be adopted by the mouse. The mouse will then voluntarily leave the shelter to explore the arena, during which at a random time point an innately threatening stimulus is delivered, and the response of the mouse observed. Several minutes were allowed to pass between stimuli to reduce the influence of subsequent stimuli, and to reduce the chance of habituation of the stimulus.

As these experiments are focused on navigation and not stimulus detection, if the mouse did not escape from the first two stimuli, this was taken as evidence that the mouse is not detecting the stimulus, and the experiment was aborted and trials discounted. Otherwise, the instinctive escape assay proceeded to collect as many trials as possible within a 120 minute session. Sessions were terminated if mice habituated to the stimulus, as judged by a lack of startle response, or if mice stopped exploring.

2.2.5 *Shelter raise assay*

I developed an assay to test whether mice use visual detection of the shelter itself to navigate. To do so, an experiment was performed as in the 'Instinctive escape assay', except that the shelter was held by a piece of string that allowed it to be move by the experimenter. Immediately prior to the stimulus, the shelter was raised up such that it was no longer in the line of sight of the mouse. This involved using a microphone stand to hold a piece of string connected to the box, which meant the box could be pulled upward by the experimenter sitting behind a curtain. These 'shelter raise' trials were interleaved with trials in which the shelter was stationary in order to provide control comparison trials.

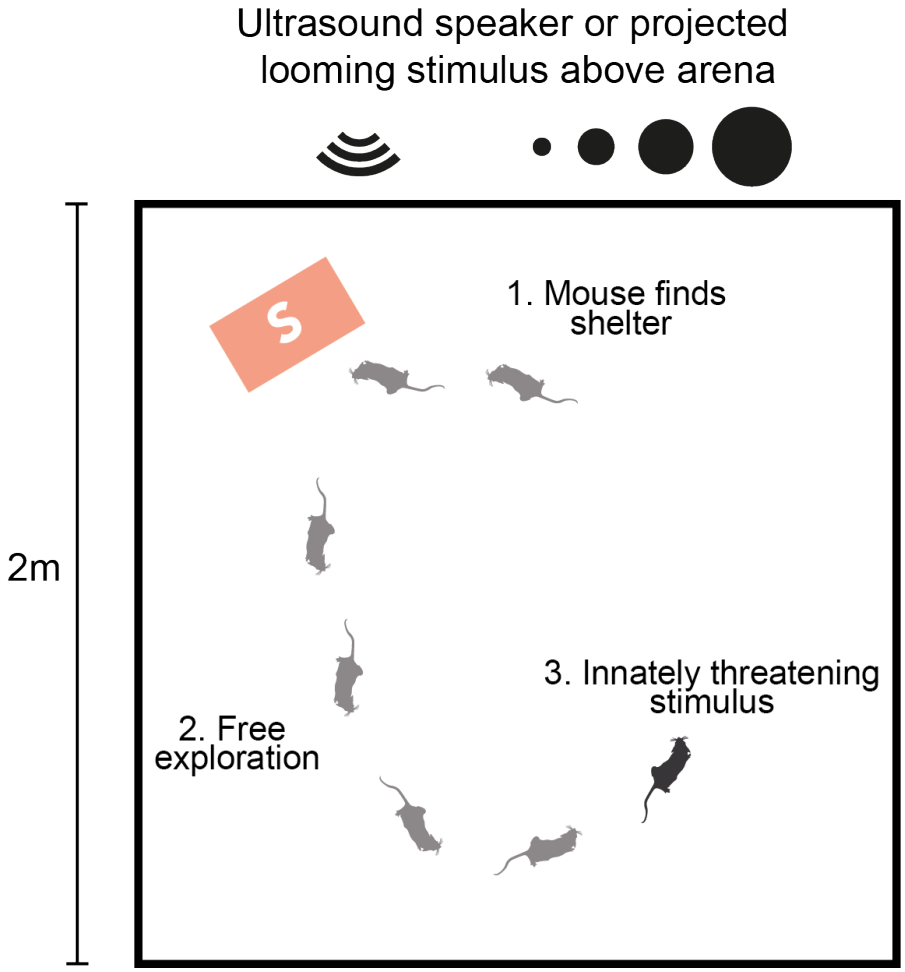

Figure 2.1: **Summary of the instinctive escape assay.** 1) The mouse finds and adopts the shelter. 2) The mouse then voluntarily leaves the shelter to explore the environment. 3) Finally, in step 3 an innately threatening stimulus is delivered and the mouse's response observed.

A total of 38 trials were taken, 19 of which were shelter raise trials. They were taken across 10 experimental sessions with 10 mice.

2.2.6 *Arena platform rotation*

To test the relative influence of self-motion/vestibular cues and visual cues, I built an arena based on the Barnes maze (Barnes, 1979) in which the central circle was able to rotate, while the external section was stationary (figure 2.2).

The rotation assays were performed by first taking 3 baselines in the circular Barnes arena, in accordance with the 'Instinctive escape assay'. If these escapes were reliable, a petri dish with a small amount of bedding was added to the centre of the arena, which the mouse tends to investigate. While the mouse is investigating the bedding,

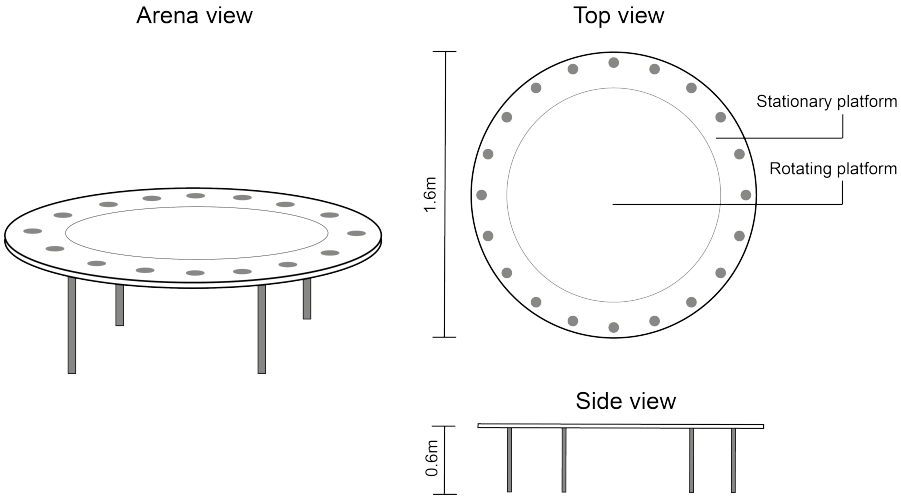

Figure 2.2: **Schematic depiction of the rotating arena.** Arena view shows a schematic of the arena in 3D. Top and side views show a schematic of the arena as viewed from above and side. The central portion of the arena is able to rotate independent of the external portion, which remains stationary.

the arena is slowly rotated, after which an ultrasound stimulus is delivered. Stimuli were then delivered at intervals of a minimum of two minutes until mice either adapted to the stimulus, stopped exploring, or would not be stationary long enough for rotation.

Two variants of these experiments were performed: with the shelter on the external, stationary part of the arena, such that only the mouse rotates; and with the shelter on the internal, rotating part of the arena, such that it rotates in tandem with the mouse. The location of the shelter was the only difference between these sets of experiments. Differences in lighting were as above, except in the dark broadband white noise was played from 4 speakers under the arena to prevent mice localising themselves using auditory cues.

A total of 49 experimental sessions were included, with 49 mice. 15 experiments were taken in light, inner shelter conditions; 10 were taken in dark conditions with the shelter on the inner portion; and 24 with the shelter on the outer portion in the dark. A total of 367 trials were taken.

2.2.7 *Dry Morris Maze*

The Dry Morris Maze (DMM) was designed to isolate the ability of mice to escape to shelter based only on visual cues (figure 2.3). In this assay, baselines were taken as in the 'Instinctive escape assay' in 4.1.4. After verifying that the mouse could escape accurately to shelter, the mouse's home cage was left by the side of the arena with a small platform for it to climb down into it. Mice voluntarily entered their home cage, which was then placed into a large opaque container. The

container was rotated to disorient the mouse. The arena was cleaned with 70% ethanol to remove odour cues, and the shelter removed (unless otherwise stated, as with some experimental controls in dark conditions). The mouse was then returned in a transparent bottomless box attached to a string. The mouse stays in the box for 5 minutes to allow it to reorient itself via the available cues. The box is then raised, and an innately threatening stimulus immediately played. The test is for the mouse to navigate to the former shelter location.

In these experiments 119 trials were collected, including 30 mice. 46 were DMM trials, 10 of which were in the dark as these were the most challenging trials to obtain.

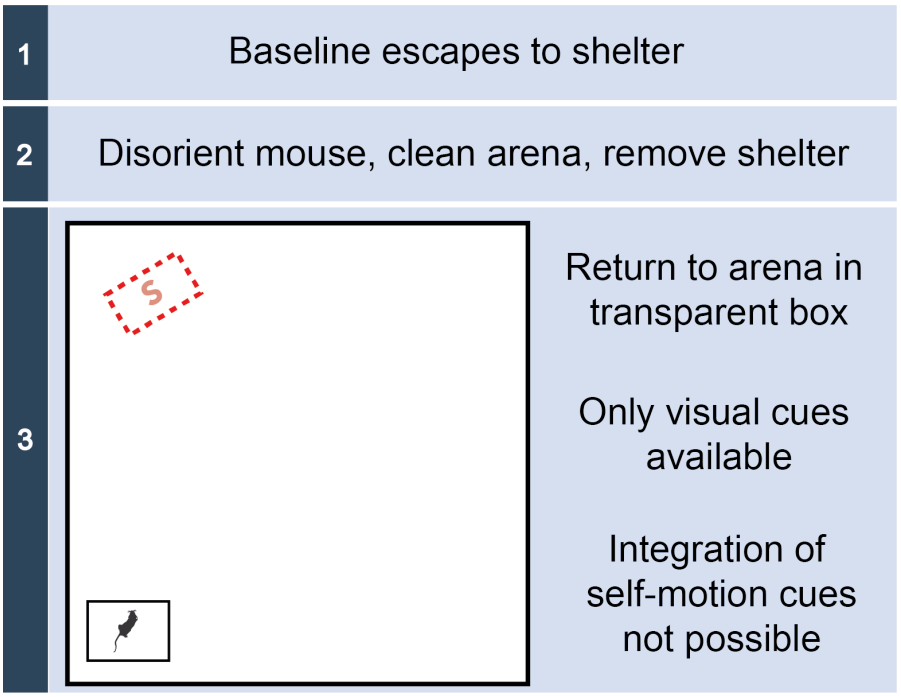

Figure 2.3: **Schematic depiction of the Dry Morris Maze assay.** 1) The mouse is introduced to the arena and adopts the shelter as it's base, and baselines are taken as in the instinctive escape assay. The mouse's homecage is added next to the arena, and the mouse voluntarily enters it. 2) The cage is then moved into an opaque box and disoriented. The arena is cleaned and the shelter removed. 3) The mouse is reintroduced to the arena in a transparent box, allowed to reorient using the available visual cues. The box is then lifted and an innately threatening stimulus delivered.

2.2.8 *Trap assay*

To investigate whether the loss of path integration was the driver of the slower reaction time in DMM trials, I developed an assay in which path integration is maintained, but the rest of the DMM protocol occurs. In this assay, rather than the mouse leaving the arena, it is trapped underneath the transparent container. The mouse waits there, with visual cues available for 5 minutes, replicating the DMM trials,

as the shelter is removed and arena cleaned, before they are released and an innately threatening stimulus delivered. This serves to control for the influence of experimental confounds such as being trapped in a box on the phenotype observed in the DMM trial. 7 mice were included in this experiment, yielding 7 trials for baseline escapes and trap stimuli.

2.3 SURGICAL PROCEDURES

All surgery was undertaken in a dedicated room while wearing sterile personal protective equipment. Surgery was performed on a stereotaxic frame under aseptic technique.

For all surgeries, animals were first anaesthetised with isoflurane. The scalp was then shaved, and the mouse attached the sterotactic frame (Kopf Instruments, models 1900 and 963). Analgesics were given subcutaneously. A heat pad under the mouse was used to maintain body temperature at 37^{o}C. Anaesthesia was maintained with isoflurane mixed with O_2 at between 0.5-1.5% through a mask attached to a stereotaxic frame. Moisture in the eyes was maintained by applying Lubrithal gel. The scalp was disinfected with a chlorhexidine-based solution, and a sagittal incision was made with surgical scissors.

Once the skull is exposed, the skull was levelled in the anterior-posterior axis and the stereotaxic frame used to align the equipment being used to bregma on the skull's surface. The surgery then proceeds as is required for that procedure, each of which is detailed below.

Post surgery, the scalp is resealed, and the mouse monitored for an hour to ensure it recovers from anaesthesia. The mouse is then monitored over the next 24 hours.

The purpose of these experiments was to disrupt neural activity and observe the effects. This was done in three ways: destroying neural tissue with iobotinic acid; silencing neural activity with muscimol; and disrupting neural activity with ChR2. Muscimol acts as a selective agonist of the $GABA_a$ receptor, meaning it tends to reduces neuronal activity in the neurons whose receptors it binds to. Conversely, ChR2 stimulation was intended to 'scramble' the neural code for space in the experiments attempted.

2.3.1 *Stereotactic Injections*

For viral injections, viral constructs were delivered through pulled glass pipettes (10 ul Wiretrol II pulled with a Sutter P-97). The virus was loaded prior to surgery. After the mouse has been aligned into

Injection	A/P	M/L	z	Volume (uL)
1	-1.50	±0.50	1.75	400
2	-1.50	±1.25	1.55	400
3	-2.00	±1.00	1.45	400
4	-2.00	±2.00	1.75	400
5	-2.50	±1.50	1.50	400
6	-2.50	±2.35	1.75	400
7	-3.10	±3.05	2.30	300
8	-3.10	±3.05	4.00	400
9	-3.65	±3.40	3.00	400
10	-3.65	±3.40	4.00	400

Table 2.1: **Injection coordinates for hippocampus lesion.** A/P = Anterior-posterior axis from bregma. M/L = Medial-lateral axis from bregma. z = depth post brain surface. Volume = volume of ibotenic acid injected at site.

the sterotaxic frame, the pipette is aligned to bregma on the mouse's skull. The pipette was then moved to the desired injection site, and a small craniotomy made over that site. The pipette was then lowered to the desired depth, and virus slowly delivered at around 50nL/minute. 10 minutes was allowed for the construct to be delivered into the brain before removal of the pipette.

IBOTENIC ACID INJECTION For the hippocampus lesion experiments, ibotenic acid was used to damage the hippocampal tissue. Ibotenic acid was injected at stereotactic coordinates. This surgery was performed differently to other injections and infusions as ibotenic acid can have detrimental effects. In particular, on guidance from other laboratories with experience using ibotenic acid, I split the bilateral surgery over two dates, separated by a week. On each surgery, a different hemisphere was injected with ibotenic acid. This allowed mice to recover in-between surgeries. Otherwise, the surgical protocol for these injections was the same as in the other stereotactic injections.

Ibotenic acid was injected at 10 injection sites in each hemisphere. Injection coordinates and volumes are found in table 2.1.

2.3.2 *Brain region specific infusions*

2.3.2.1 *Cannula implantation*

For cannula implantation, mice were first fixed in the stereotaxic frame and a calibration of the coordinate frame made to ensure ac-

curacy, as above. Guide cannula were then attached to a holder on the frame and used to identify the desired implant sites, allowing maintaining of accuracy of depth of implant. Implant sites were identified and marked on the skull, and a small craniotomy made. To ensure the cannula penetrated the dura, a small hole in the dura was made with a needle. The cannula was then lowered into the brain to the desired depth. After any bleeding was stemmed, the craniotomy was sealed with a small amount of veterinary glue (vetbond), and the cannula held in place with dental cement (3M RelyX Unicem 2). For bilateral cannula implants this processes was then replicated on the other hemisphere.

Once all cannula were implanted, they were fixed into position with more dental cement such that all revealed skull was covered and a seal with the skin formed. Dummy internal cannula were then attached to the guides to prevent debris entry. Mice were allowed 3 days to fully recover from surgery before infusion.

2.3.2.2 *Infusions*

To infuse drugs or saline through the cannula, syringes, tubing and internal cannula were first assembled and loaded with drug or saline. The mouse was then placed under isoflurane anaesthesia. The dummy cannula was removed, and the internal connected through the guide cannula and sealed into place with KwikSil. 0.8 - 1.2 nL of drug or saline was then infused via a syringe (Hamilton, 10ul Model 1701 syringe; in Kopf Instruments Model 5000) connected to the internal cannula. Post infusion, the tubing was cut and sealed with Kwik-Sil. 40 minutes was allowed for the drug to infuse through the tissue and the mouse to recover from anaesthesia before testing began.

2.3.3 *Optogenetic manipulations*

The surgical procedure for optogenetic manipulations took place in two stages: a injection of a viral construct, and subsequent implant of an optic fibre.

2.3.3.1 *Viral Injection*

As for cannula implants, mice were first fixed into a stereotaxic frame under isoflurane induced general anaesthesia. The scalp was disinfected, an incision made, and bregma identified on the skull surface, to which the pipette was aligned. The craniotomy site was identified and a craniotomy made of sufficient size for the fibre implant later. The viral injection then proceeded as outlined in section 2.3.1. The viral constructed injected was AAV-CamKIIa-ChR2-EGFP at the same

Injection	A/P	M/L	z	Volume (uL)
1	-3.00	±2.0	1.70	500

Table 2.2: **Targeting coordinates for subiculum cannula implants and injections.** A/P = Anterior-posterior axis. M/L = Medial-lateral axis. z = depth post brain surface. Volume = volume at site for injections

coordinates as the cannula implant. While ChR2 is a excitatory opsin, the purpose of these experiments was to disrupt, rather than silence, neural activity. Injection coordinates can be found in table 2.2.

2.3.3.2 *Fibre Implant*

The implantation of a fibre optic into the brain for the delivery of the laser pulses occurred in the same surgical session as the viral injection to reduce surgical procedures and increase the likelihood of the angle and position of the implant being similar to the injection, thus maximising the chances of an accurate implant. After the viral pipette had been removed from the brain, 3mm doric optic fibres were lowered 1mm into the brain from the brain surface. A small amount of veterinary glue (vetbond) was applied to seal the optic fibre in place and stem any bleeding, before dental cement (3M RelyX Unicem 2) was applied to the skull to hold the fibre firmly in place. After the cement had dried, the scalp was sealed to the cement with veterinary glue and the mouse was monitored for 1 hour post surgery.

2.3.3.3 *Optogenetic experimental procedure*

Optogenetic stimuli were delivered via a 473nm diode laser (Stradus 473, Vortran Laser Technology Inc.). A solid-state laser was used in conjunction to set the light intensity (NDC-50C-4M, Thorlabs). Stimuli were controlled through custom LABVIEW software. Stimuli were delivered at 10 light pulses of 10ms at 20Hz.

For the optogenetic experiments, fibre optic cables were attached to implanted mice via magnetic patchcords (Doric Lenses Inc) combined with a rotary joint (FRJ 1x1, Doric Lenses Inc), allowing unhindered movement. Once attached, mice were introduced to the experimental arena with a shelter present. Mice were allowed to find and adopt the shelter before any stimuli were delivered. Prior to any optogenetic stimuli, baseline stimuli were delivered to test whether mice are able to escape to shelter. Trials of optogenetic and innately threatening stimuli were then interleaved with innately threatening stimuli alone. Later as a control, optogenetic stimuli were delivered alone.

2.3.3.4 *Histological processing*

After the completion of experiments, the placement of viral injection and cannula implant was verified histologically. Brain tissue was fixed through transcardial perfusion of 30ml of phosphate buffered saline, followed by 20ml of 5% paraformaldehyde (PFA), and was left overnight in a 4^{o} fridge in PFA. Slices were then taken at a thickness of 100um in phosphate buffered saline, and stained with SlowFade Gold (S36938, Life Technologies, contains DAPI). These slices were subsequently imaged on a wide-field microscope (Nikon TE2000) at either 10 or 20x with an air objective.

2.4 ANALYSIS

Analysis was performed primarily in Python 3.7, using a combination of publicly available software, in house lab software, and custom scripts.

2.4.1 *Statistics and visualisations*

Statistical tests were performed using Python packages SciPy Stats and NumPy. Statistical significance was decided at a threshold of $p < 0.05$. Within figures, the value of statistical test performed is denoted with asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). Statistical tests used are stated in the text.

Overlays on plots are standard error of the mean (SEM) unless otherwise stated. When ± is used next to a mean value stated in a figure legend or in text, this refers to the standard deviation.

2.4.2 *Video Processing and Animal Tracking*

A behavioural analysis pipeline was set up allowing the conversion of difference experimental sessions to a common coordinate framework, in which the head, body and tail of the mouse was tracked throughout the session. Initially, the video was corrected for artefacts such as fish-eye effects on the camera, and then the environment external to the arena removed such that the frame consists of only the experimental arena. Animal tracking was then performed using DeepLabCut 2.0, leading to tracking data that can be compared across sessions.

Figure 2.4: **Processing pipeline from video to mouse tracking.** The original video is converted to a common reference frame by removing fish-eye artifacts from the camera and applying any necessary rotations and cropping to the image. This converted video was then passed into a DeepLabCut neural network trained on a range of behavioural videos from this and similar arenas.

2.4.3 *Trial analysis*

Data was stored in several formats: raw videos prior to the processing pipeline, videos after processing, videos clipped into trials, tracking data covering entire sessions, and then tracking data from all experimental trials. Videos were stored as either .mp4 or .avi files. Tracking data was stored in .pickle files. In exploratory data analysis, primarily the trial data was used, both in the form of video clips and tracking data. Observations were then more interrogated in Python using custom scripts.

To analyse trial data, trials were cut based upon the stimulus index.

2.4.3.1 *Manual labelling of trials*

When manually labelling reaction time, three data points were recorded by sifting through frames per trial: the first visible startle response after the stimulus, orienting towards the shelter, and the movement of the body. Additionally, a manual score of whether the response constituted an escape was recorded, based upon the classic signs of escape and defensive behaviours: the initial startle reaction, an orienting of the head towards safety, and an acceleration of the body.

STARTLE The startle response was labelled based on either a lowering of the body and spreading of the legs or a straightening of the tail.

ORIENTING Orienting movements were defined as the first significant post-stimulus change of head direction towards the shelter after the startle response.

MOVEMENT Movement onset was defined as the first movement of the body in the direction the head was pointing after the orienting response.

2.4.3.2 *Definition of variables*

FLIGHT Flights were defined as an orienting of the head towards the shelter, followed by an acceleration. In cases where the mouse was already facing the shelter the flight was defined purely by acceleration towards the shelter. Two methods were used to verify a flight after a stimulus: manual labelling blind to experimental condition based upon the above criteria, and a secondary, data driven approach that was used to classify ambiguous cases based upon head-orientation and body tracking.

PHASES OF FLIGHT Based on viewing trial clips, I observed that there seemed to be two phases in flight. Initially, the mouse orients towards the shelter and accelerates, as observed in Vale and colleagues (Vale, Evans, and Branco, 2017). However, a distinction can be made between light and dark in longer range flights during the latter half. In the light, mice accurately maintain their heading towards the shelter. In the dark, mice 'search' for the shelter and adjust their heading direction more regularly. To distinguish these two phases, I developed a criteria for a 'phase threshold index'. This index was the time at which the mouses body had started accelerating post stimulus plus 1 second. This threshold served to capture the initial phase of the flight, involving orientation and initial acceleration, and segregated it from the later phase.

HEAD ORIENTATION VARIABLES Two measures of head orientation were used in this study. First, the absolute head angle of the animal was computed by taking the body and the head co-ordinates and computing the vector between them, and comparing this to the vertical vector (0, 1). This provided a measure of the heading angle. Second, this angle was compared instead to the shelter position, rather than (0, 1), to give a measure of the head-angle to shelter. For experiments in which the shelter moved, the angle was computed relative to the original shelter location unless otherwise stated.

Part III

RESULTS

3

ALGORITHMS FOR DEFENSIVE DECISIONS AND ACTION

What cognitive processes underlie the computation of the route to shelter after imminent threat? As we saw in the introduction to the present thesis, a limitation of previous work in studies of escape is the relatively short spatial scale over which mice have navigated. This small spatial scale increases the likelihood that sensory features of the shelter are used to navigate via taxon navigation, decreasing the difficulty of the navigational task, reducing the need for abstract representations of the environment, and precluding the study of how flight trajectories change through escape. To address these deficiencies in our understanding of escape behaviours, I undertook a series of behavioral experiments investigating the properties of trajectories in larger environments, up to 2 x 2 meters.

3.1 VISUAL CUES ARE USED TO GUIDE ESCAPE NAVIGATION

Previous studies from our laboratory suggested that a form of path integration provides the spatial memory of shelter guiding escape navigation (Vale, Evans, and Branco, 2017). Path integration is an efficient and fast way of updating a vector, but it suffers from limitations such as degrading in accuracy over time unless it is calibrated with other cues, lacking the representation capabilities to compute routes to shelter in complex environments, and lacking the flexibility to infer new positions based on available cues. However, a vast literature details that the brain forms representations capable of resolving these shortcomings. I therefore hypothesised that, while path integration is likely a vital component of the computational toolkit required for escape navigation, it is unlikely to be the only computation used in cases of life or death.

I tested this by comparing escape navigation in the large spatial environment in a well-lit room and in near total darkness (dark conditions described in section 2.2.1.1, experimental protocol in 2.2.4)(n(light) = 44 mice, 116 trials; n(dark) = 21 mice, 160 trials). The start position of mice in each condition was not different, as displayed in figure 3.1, where start position is plotted in each condition. As path integration depends on internal representations of movement, vestibular cues, and proprioceptive cues, if path integration is indeed the dominant computation underlying escape navigation we should observe

no difference between these conditions (see example trials from each conditions in figure 3.2).

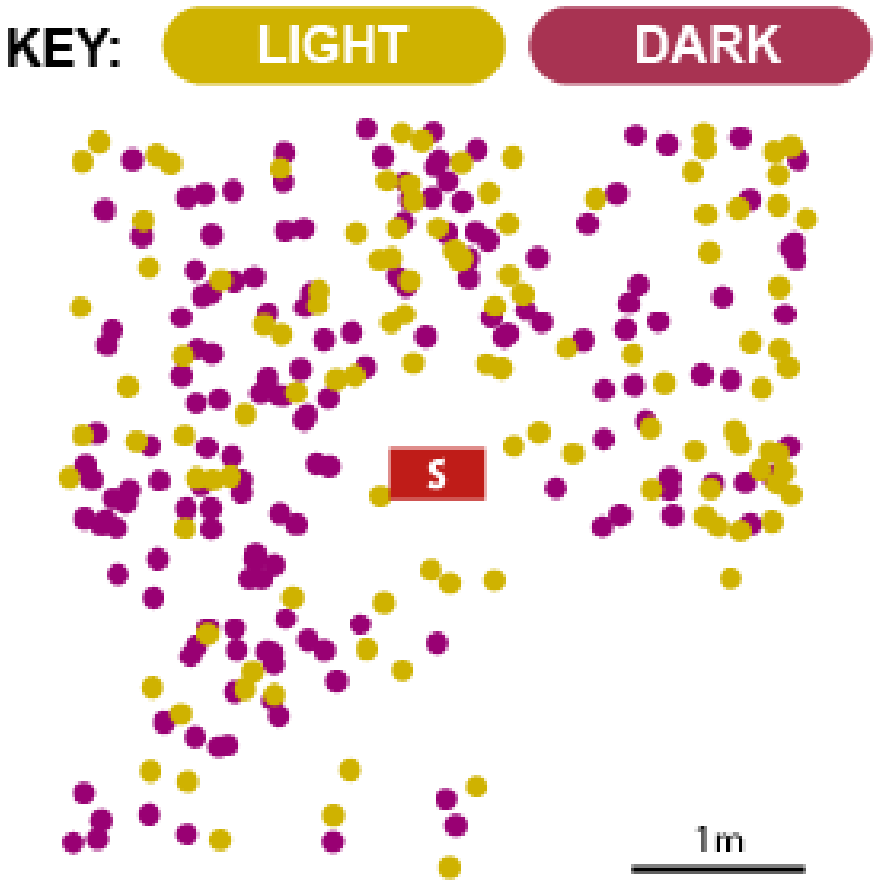

Figure 3.1: **Positions of mice in light and dark conditions upon stimulus delivery.** The position of mice relative to the shelter at the time of stimulus delivery. There is no obvious difference or bias in the start positions relative to the shelter in either condition.

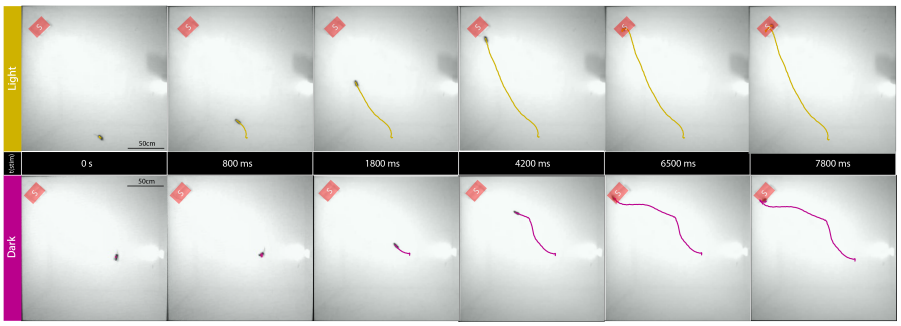

Figure 3.2: **Example trials from light and dark conditions over large spatial scales.** Escape to shelter is typically fast and direct in well-lit conditions, while long range escape to shelter in the dark involves more stops, changes of head angle and movement direction, and is slower.

3.1.1 *Threat perception to ultrasound stimuli is similar between light and dark conditions*

To ensure a comparison in navigational abilities could be made between the two conditions, I first verified that the ultrasound stimuli delivered were perceived as similarly threatening in light and dark conditions by comparing the reaction times of mice in these two settings. Two crucial metrics are required to assess threat perception: the probability of response and the reaction time. The probability of response provides a measure of how well mice detect the stimulus, while the reaction time has been shown to correspond to threat level and vigour of response by both behavioural experiments and optogenetic experiments mimicking threatening stimuli (Evans et al., 2018). I found no difference in reaction time when manually labelling trials, blind to condition, whether the first observable response (light mean 0.396 ± 0.846s, dark mean 0.387 ± 2.09s, p = 0.966 independent two-sample t-test), the initiation of orienting towards the shelter (light mean 1.02 ± 1.27s, dark mean 1.08 ± 2.40s, p = 0.849 independent two-sample t-test), or the first acceleration of the body were used as measures (light mean 1.35 ± 1.30s, dark mean 1.36 ± 2.44s, p = 0.967 independent two-sample t-test) (figure 3.3b). Similarly, manual measures of flight probability were not different between the two conditions. These behavioural readouts of stimulus response suggest no difference in threat perception between light and dark condition.

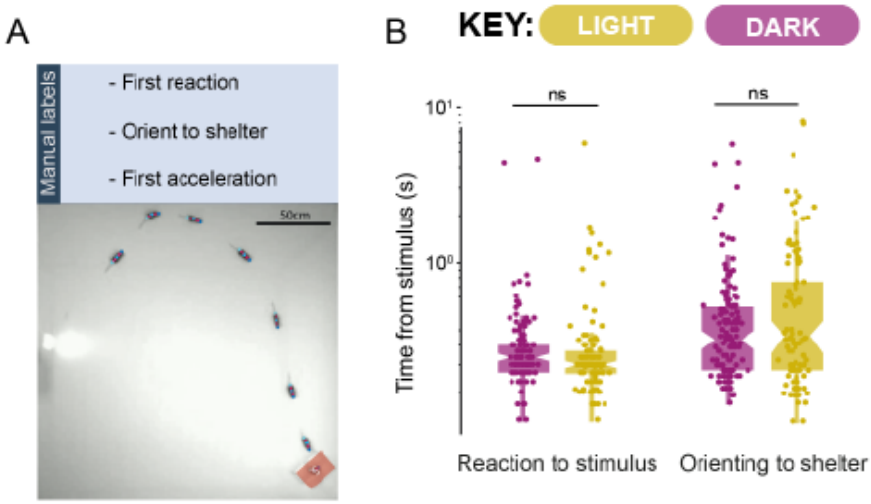

Figure 3.3: **Similar reaction times in light and dark conditions indicate similar threat perception.** A) Left hand panel shows the different features of flight that were manually labeled, along with an example trial with tracking overlay. B) Right hand panels show the reaction time related manual labels, with no significant difference between light and dark conditions in either first observable reaction to the stimulus (e.g. a startle response, p = 0.966, independent t-test) or the time of the first orienting away from the path at stimulus onset (p = 0.849, independent t-test).

3.1.2 *Two-phase flight is maintained in both light and dark conditions*

As characterised by previous studies in our laboratory (Vale, Evans, and Branco, 2017), the early observable features of flight are head orienting towards shelter followed by an initial acceleration in this direction. Both occurred at similar latency in light and dark, indicating both stimulus detection and urgency of response are similar in the two conditions. During the first second the first sign of stimulus detection, as detected through manual labelling, the head-angle to shelter decreased by a similar amount in light and dark (light mean: 48.1 ± 52.9°; dark mean: 46.1 ± 52.9°, p = 0.749 independent two-sample t-test), suggesting that the cognitive processes ongoing during this early phase are comparable between the two conditions, both in terms of their underlying computations and their accuracy. Together the reaction time, probability of response, time to orienting and time to peak acceleration all indicate that the threat level is perceived similarly between light and dark conditions, and that the response to the threat is similar to the two-phase orient-accelerate process reported by Vale and colleagues.

3.1.3 *Escape navigation in the dark is slower and less accurate*

With a valid comparison between escape navigation in light and dark conditions established, I next investigated the differences between escape navigation in light and dark conditions over large spatial scales. In particular, previous data from Vale and colleagues suggested that visual cues are not used during flight as navigation in the dark is not impaired and rotation of local visual cues surrounding the environment had no effect on flight trajectory. However, these studies were in an environment of around 1m in spatial scale: do these findings apply over larger spatial areas where the navigational challenge is greater and more calibration of flight trajectory is required?

According to the hypothesis that path integration is the dominant computation underlying escape navigation, there should be little observable difference between escape in light and dark conditions as visual cues are not necessary for path integration. However, I observed that mice tended to navigate less well in the dark, as can be seen in the example trials in figure 3.2. Mice took longer to reach the shelter in the dark (light 7.28 ± 0.75s, dark 13.5 ± 0.77s, p = 6.8 x 10^{-8} independent two-sample t-test), despite starting from similar distances to the shelter at stimulus delivery (see figure 3.4). This difference in navigational ability between light and dark conditions could have several different causes. Mice could simply navigate along the same trajectory in either condition, but more slowly in the dark. Alternatively, they could take longer to reach the shelter not because they are moving more slowly, but because they are taking a less direct path

to the shelter in the dark. I therefore aimed to describe, characterise and understand where this observed difference in navigational ability arose from.

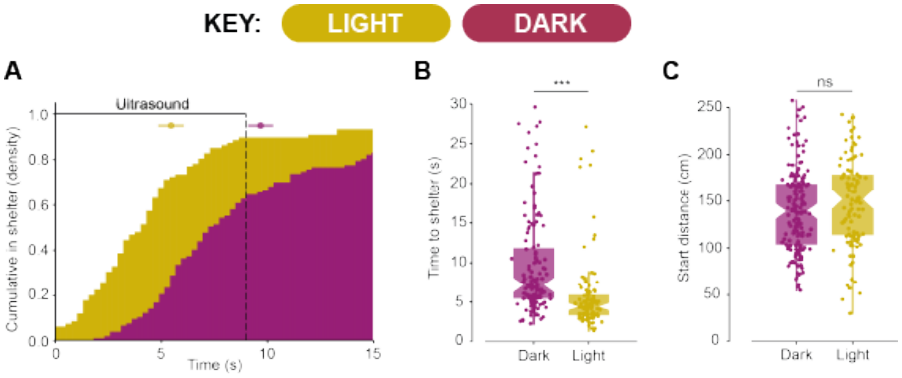

Figure 3.4: **Mice take longer to reach shelter in the dark.** A) Cumulative histogram of the proportion of mice that have reached the shelter through time post-stimulus. Black dotted line shows the point at which the ultrasound stimulus ends. B) Box-whisker plots of the time of shelter arrival for each trial. Mice arrived at the shelter later in the dark ($p = 6.8 \times 10^{-8}$, independent t-test). C) Box-whisker plots of the starting distance to the shelter, taken at the time of the stimulus. The difference in time to shelter is not explained by the trials being from a greater distance as this start distance was similar between light and dark conditions ($p = 0.701$, independent t-test).

I analysed whether mice were travelling less quickly in the dark. As shown in figure 3.5, mice were substantially slower when navigating to shelter in the dark (mean speed in light 0.394 ± 0.223, mean speed in dark 0.156 ± 0.087, $p = 2.82 \times 10^{-25}$ independent two-sample t-test, figure 3.5A, B, and D). Despite this difference in mean speed, this did not appear to be driven by a difference in urgency, as indicated by a similar time to peak speed, with the peak speed being reached faster in the dark, likely due to it's lower magnitude (light peak speed time 2.72 ± 3.25, dark peak speed time 1.80 ± 2.09, $p = 0.033$ independent two-sample t-test, figure 3.5C). Similarly, manually labelling the number of pauses mice took in flight showed a substantially higher number in the dark (light mean 0.192 ± 0.573 stops per flight, dark mean 0.849 ± 1.164 stops per flight, $p = 4.53 \times 10^{-7}$ independent two-sample t-test, figure 3.5E). I observed that mice often cast their head while paused, suggestive that they were trying to sample the environment for evidence of the shelter. Therefore, part of the difference in time to reach the shelter is driven by a difference in speed of travel and tendency to pause, presumably to gather sensory evidence of the shelter, between the two conditions.

I next asked whether the difference in speed was the only difference leading to a delay in reaching the shelter in the dark. To do so, I compared the trajectories of mice in each condition. As shown in the example trajectories in figures 3.2 and 3.6, I observed that mice tended to be less direct in their trajectory in the dark. To quantify this,

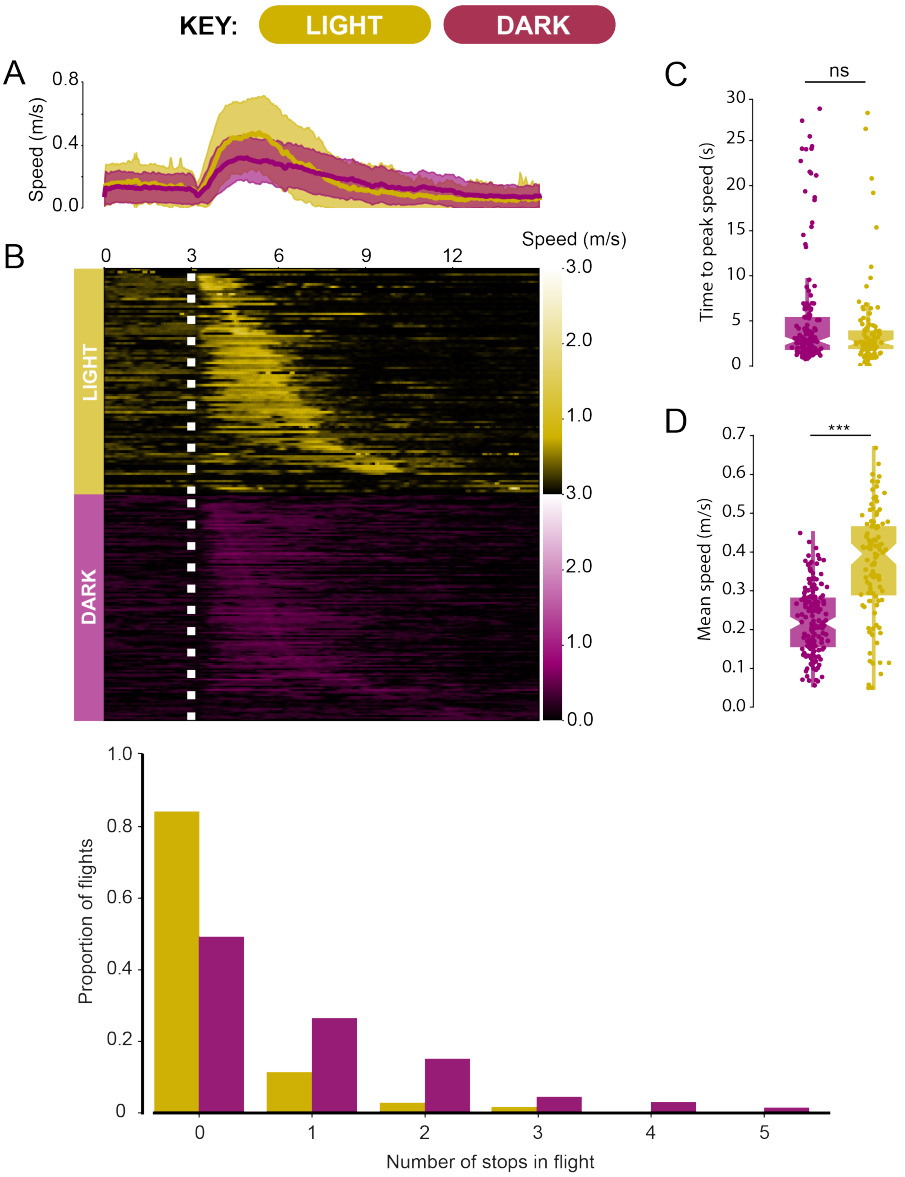

Figure 3.5: **Mice escape faster in the light** A) Line plots of the mean speed at each point in flight, with standard error of the mean overlay. B) Heatmap of speeds in each condition through flight. White dotted line represents stimulus onset. C) The time to the peak speed, taken from the stimulus onset to the end of the stimulus ($p = 0.033$). D) The mean speed in each condition. The was a highly significant difference between light and dark conditions, with mice in the light on average travelling faster during flight ($p = p = 2.82 \times 10^{-25}$, independent t-test). E) Histogram of the number of stops in each flight, as determined through manual labelling of trials ($p = = 4.53 \times 10\text{-}^{7}$, independent t-test).

I computed the tortuosity of flights, defined as the distance travelled during flight divided by the start distance to the shelter, therefore providing a measure of how direct a flight is.

I observed a large difference in the tortuosity of flights between light and dark conditions (light mean 1.51 ± 0.67, dark mean 2.60 ± 3.21, p = 0.00044 independent two-sample t-test). As can be observed in the

example trajectories in figure 3.6, while many flights were similarly accurate in the dark to the light, in some flights the initial error from orienting is only corrected late on, presumably when other non-visual cues, such as olfactory cues, become available. Comparatively, trials in the light that have a similar initial error are corrected towards the shelter. This aligns with the hypothesis that the difference observed in tortuosity is due to the ability to engage different cognitive processes when visual cues are available.

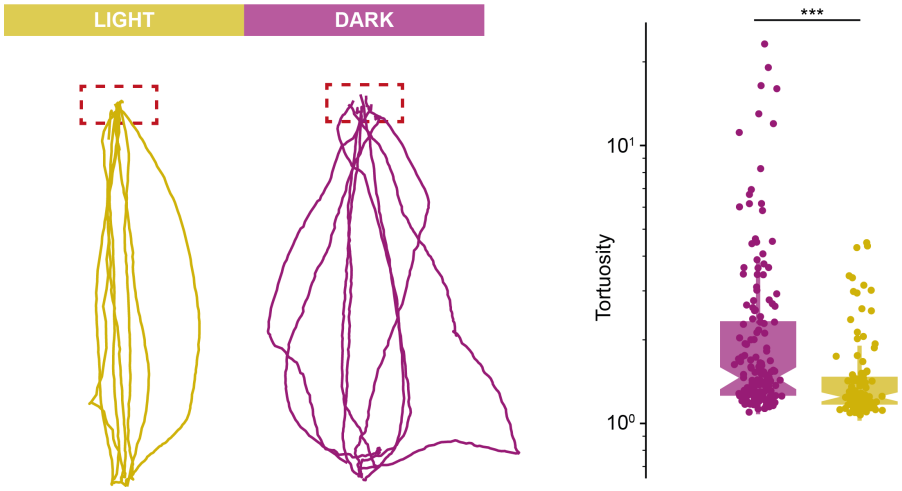

Figure 3.6: **Trajectories towards shelter in the dark are less direct than in the light.** A) Example trajectories which have been placed into the same coordinate frame to allow comparison. B) Box-whisker of the tortuosity between light and dark conditions (light mean 1.51 ± 0.67, dark mean 2.60 ± 3.21, p = 0.00044, independent t-test).

Together, this data suggests that the mice take longer to reach the shelter in the dark, and that this is driven by a slower flight speed, an increased tendency to pause to gather sensory evidence of the shelter, and less direct trajectories towards the shelter in the dark. However, as previously observed, flights in both conditions still matched the observation of Vale and colleagues that escape occurs in two phases: an initial orienting and a secondary acceleration towards the shelter. I therefore hypothesised that, over longer distances, a further phase occurs in which visual cues increasingly guide navigation. I next tested this by analysing flight trajectories and observing whether a later phase of flight could be identified.

3.1.4 *Flight over large spatial scales involve two distinct cognitive processes*

I hypothesised that Vale and colleagues finding of two phases of flight in fact correspond to one cognitive process driven primarily by path integration, and that a second cognitive process is initiated over larger spatial scales that involves the integration of visual cues to guide flight. To quantify when this hypothesized second cognitive process

starts, I analysed the properties of trajectories of flights through time in each condition.

According to the hypothesis that there is an additional cognitive process only active later in flight, mice should initially start travelling along a trajectory suggested by path integration, and later correct towards the shelter only in the light where visual cues can guide the trajectory. I developed two metrics to measure this: the distance to the initially enacted path, and the distance to the shelter path. The distance to the initial path is computed by taking the coordinates of the mouse at onset of movement and 1 second later, computing the vector between these points, and then computing the distance to this vector at each point in flight. A similar computation is performed for the distance to the shelter path, except the vector used is computed between the mouse position at movement onset and the shelter. An example trial with corresponding plots of these metrics is shown in the first row of figure 3.7.

A clear divergence in these metrics is seen between 1 and 2 seconds post movement onset. After this point, in the light mice diverge faster away from their initial path and converge towards the shelter path (figure 3.7). Conversely, in the dark mice take longer to diverge from their initial path, and do not converge towards the shelter path as clearly as in the light. While the distance to the shelter path in the dark is dominated by the flights that are very inaccurate, the difference in the distances to the shelter path and the time to deviation away from the initial path provide suggest that after around 1-2 seconds a distinct process is initiated that increases the accuracy of flight when visual cues are available.

This data suggests that flight can indeed be decomposed into a further, previously unidentified phase in which cognitive processes that are only available with the presence of visual cues begin to be used. While this doesn't preclude path integration also being used, we next investigate how the conditions under which this other cognitive process is used.

3.1.5 *Differences between light and dark correspond to two phases of flight*

Having identified differences between the properties of flight between light and dark, and found preliminary evidence that two distinct cognitive processes are enacted in flight that explain these differences, I next sought to test this by seeing whether the differences in flight correspond to the timing of the hypothesised cognitive processes. To do so, I subdivided flights into two phases 3.7. Based on the distance to the shelter path and the distance from the initial path, I estimated that both of these values had diverged between light and dark conditions at 1.3 seconds post movement onset, and therefore used this value as the threshold for splitting flights into distinct phases.

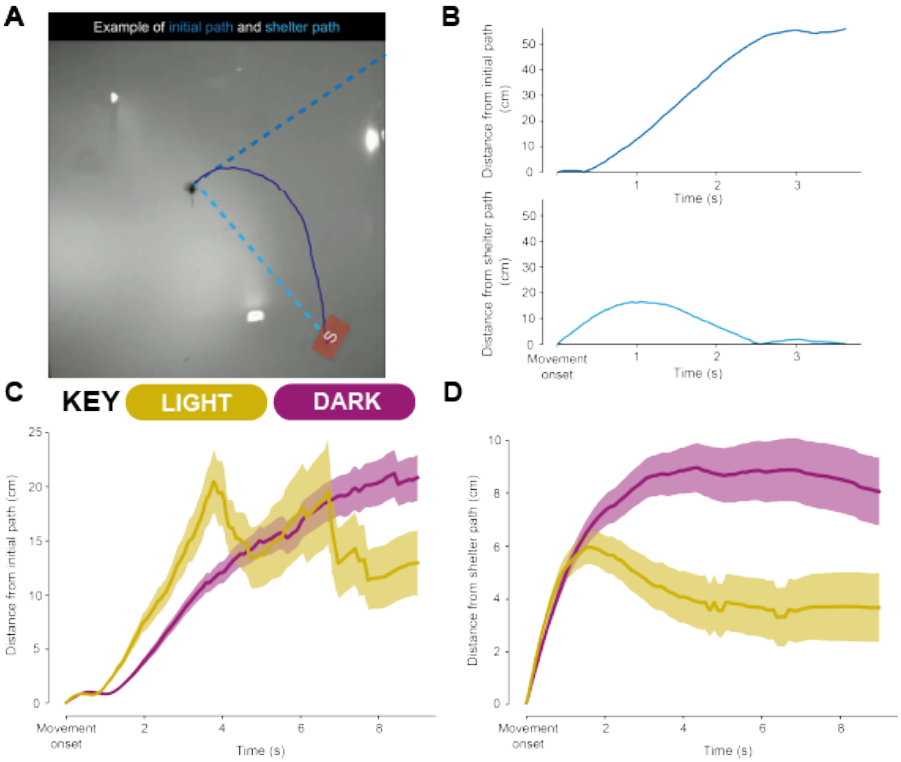

Figure 3.7: **Flights can be split into two distinct phases which are different between light and dark conditions.** A) Top row shows an example trial with the dark blue line depicting the initial path, and the light blue line depicting the path to shelter. B) Distance to the two lines in (A) through the trajectory. These two measures are then averaged for each condition. C) Average of distance from the initial path, with standard error of the mean overlay. D) Average of the distance to the shelter path. In both C and D, a clear divergence is observable between 1-2 seconds post movement onset.

Most of the differences between flights in light and dark conditions were focused in the latter phase of flight, after the 1.3s threshold (figure 3.8). In the dark the latter phase of flight, characterised by movement towards the shelter, was slower than in the light (dark late phase mean speed 0.338 ± 0.352 ms^{-1}, light dark phase mean speed 0.522 ± 0.203 ms^{-1}, p=8.794 x 10^{-7} independent t-test). Comparatively, there was no difference between light and dark in the early phase of flight (dark early phase mean speed 0.167±0.181 ms^{-1}, light early phase mean speed 0.244±0.199 ms^{-1}, p = 0.913 independent t-test). To determine whether these differences are due to the latter phase of flight, a 2-way ANOVA was performed, showing a significant difference between early and late phase when condition is accounted for (p = 0.0000320).This suggests that the observed differences in speed between light and dark conditions are primarily because of distinct cognitive processes that can only be used in the light.

A similar difference can be observed in head angle changes. When comparing the early and late phases of flight as defined previously, the difference in head angle changes per metre travelled is focused mostly in the latter phase of flight, with head angle changes occurring

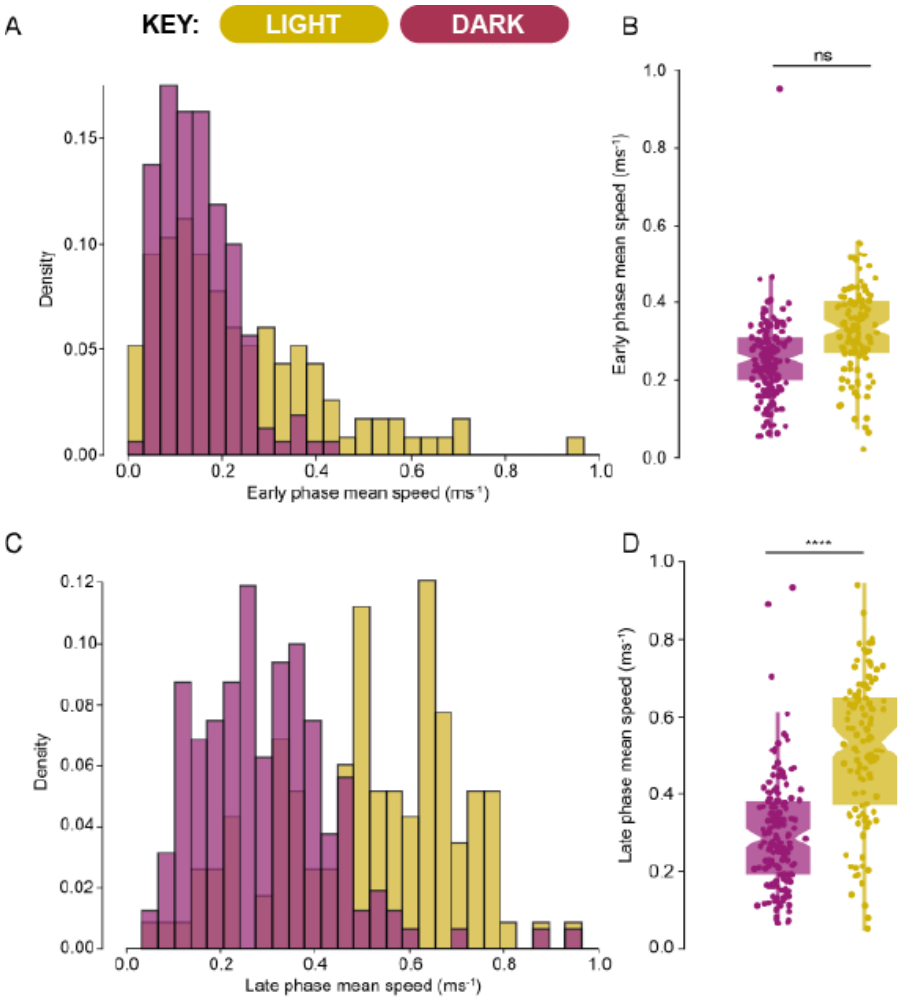

Figure 3.8: **Increased speed in light conditions is focused in the later phase of flight.** A/B) Histogram and box-whisker of speeds in the early phase of flight, split by condition (p = 0.913, independent t-test). C/D) Histogram and box-whisker of speeds in the late phase of flight, split by condition (p = 8.794 x 10^{-7}, independent t-test).

more in this phase in the dark (figure 3.9). While a small difference is present between light and dark conditions in the early phase of flight (early phase head angle change per distance travelled in light: 28.0 ± 21.6° per metre; in dark: 34.1 ± 23.7° per metre; p = 0.0282 $^{-5}$, independent t-test; figure 3.9 A and B), there is a much larger difference between the two conditions in the later stage of flight (late phase head angle change per distance travelled in light: 17.2 ± 24.0° per metre; in dark: 30.6 ± 28.3° per metre; p = 5.31 x 10 $^{-5}$, independent t-test; figure 3.9 C and D). To determine whether these differences are due to the latter phase of flight, a 2-way ANOVA was performed, showing a significant difference between early and late phase when condition is accounted for (p = 0.000511). Importantly, these differences weren't necessarily about the accuarcy of the movements, as there was no difference between major difference between the head angle to shelter in the two conditions (figure 3.9 E). This data therefore shows that the differences in head angle changes are focused in the latter phase.

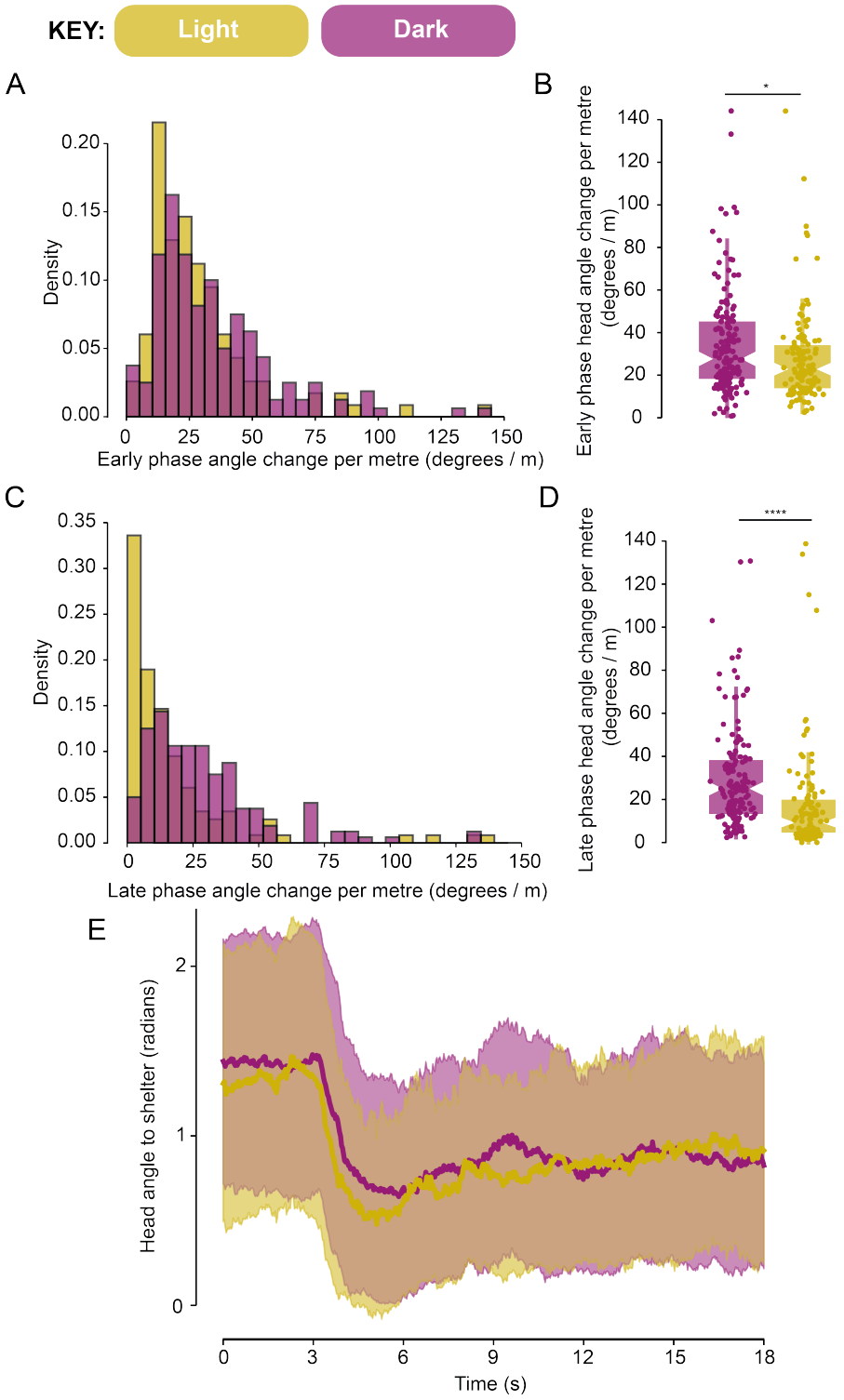

Figure 3.9: **Head angle changes are greater in the dark and are focused in the late stage of flight.** A/B) Histogram and box-whisker of head angle change per metre travelled in the early phase of flight ($p = 0.0282$, independent t-test). C/D) Histogram and box-whisker of head angle change per metre travelled in the late phase of flight. There is no significant difference between angle change before the phase change, but there is after ($p = 5.31 \times 10^{-5}$, independent t-test).

Overall in this section I have shown that flight is slower, less direct, and involves more pauses and head angle changes in the dark. I hypothesised that this was due to distinct cognitive processes being used when visual cues are available, and provided evidence for this by identifying a time-delay of around 1.3s post movement onset at which point the trajectories of flight diverge, with correction towards the shelter and away from the initial path occurring in the light, but not in the dark. Together, this suggests that the observed difference in time to reach the shelter between light and dark conditions is due to the availability of cognitive processes that refine flight trajectories. I next sought to design experiments that test this hypothesis directly.

3.2 A SHIFT FROM PATH INTEGRATION TO OTHER COGNITIVE STRATEGIES OCCURS THROUGH FLIGHT

So far, I've shown that over larger spatial scales visual cues play a more important role in guiding escape navigation, particularly focused during a second phase of flight. I therefore hypothesised that these data could be explained by distinct cognitive processes underlying the two phases of flight: first, a path integration dominated process underlies the initial orienting to shelter; and second, a cognitive map and visual cue based computation uses the available visual cues to refine the trajectory to the shelter in the second phase of flight.

I next tested this hypothesis by building an arena of similar spatial scale with a slowly rotating central portion. The aim was to dissociate the influence of the cues used by path integration, such as proprioceptive and vestibular inputs, and other cues such as visual and auditory cues present in the environment. The arena was modelled on the Barnes maze, with a diameter of 1.6 metres.

This arena allowed several different experimental configurations. First, the shelter could be placed on the outer part of the arena, such that only the mouse rotates. In total darkness, this setup provides a test of the sensitivity of the vestibular system to slow rotation and how its detection is integrated into the initial phase of escape navigation. Alternatively, the shelter could be placed on the edge of the inner portion of the arena, such that the mouse and the shelter rotate in tandem. In this case, if the mouse is integrating the vestibular input into its estimate of the shelter location, we expect the mouse to initially orient towards the initial position of the shelter. Any updating of the trajectory thereafter should be different between light and dark conditions.

Each experiment consisted of several baseline trials to ensure the mouse responded to the stimulus and to compare to the rotation trials (total of 48 mice, 15 in inner light rotation, 9 in dark inner, 24 in dark outer). Mice were then passively rotated while exploring. Movement of the mice was reduced and confined to the centre of the arena as

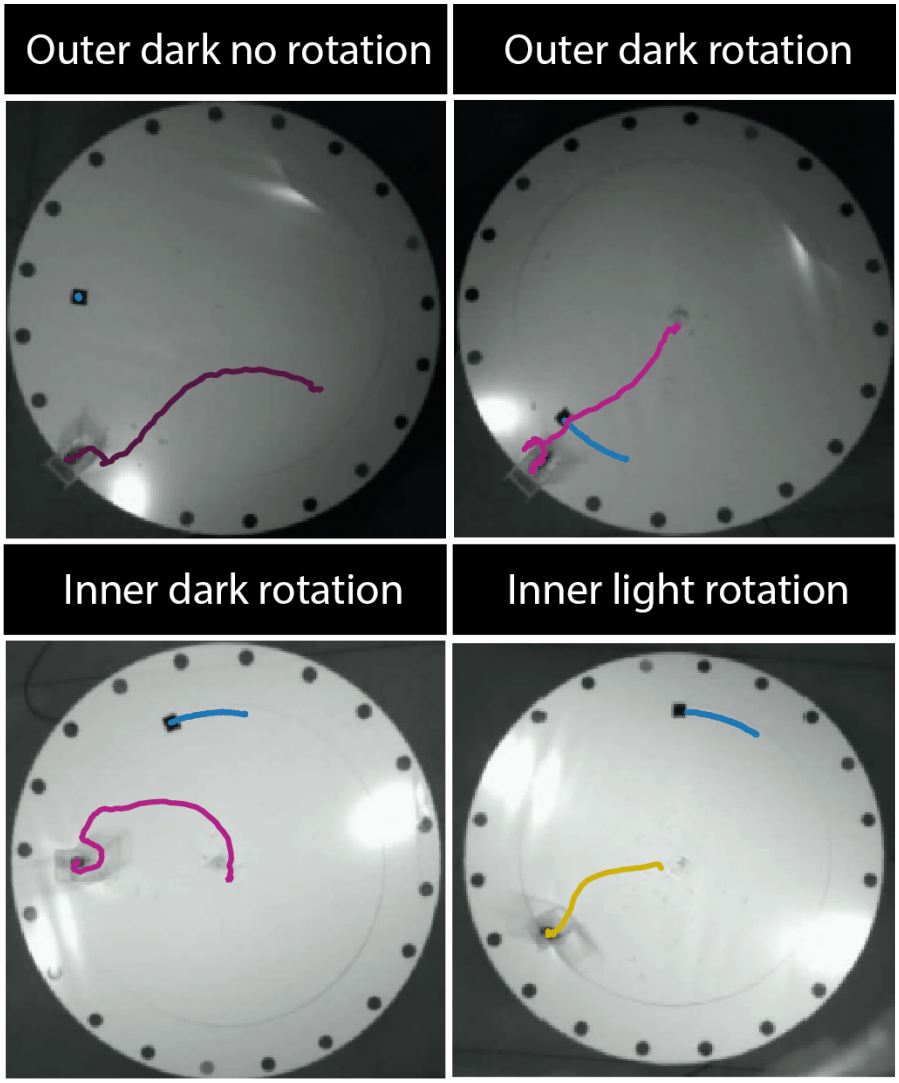

Figure 3.10: **Example trials from each rotation condition.** Example trials from each experimental condition in the rotation experiments. Blue trajectories show the rotation marker. Dark purple = dark trials with no rotation. Light purple = dark trials with rotation. Gold trajectories = light trials with rotation. Each trajectory shows the body position of the mouse from stimulus onset to reaching the shelter.

much as possible without physical interference by placing a petri dish with a small amount of bedding in, which the mice like to investigate. Mice were rotated during this period of investigation.

3.2.1 *Mice are highly sensitive to vestibular input and use it to compute the flight trajectory*

I first tested whether vestibular cues are integrated into the mouse's estimate of the shelter location by slowly rotating mice in near total darkness, with the shelter on the external, static portion of the maze (n = 15 mice, 55 trials). As the predominant cue related to the rota-

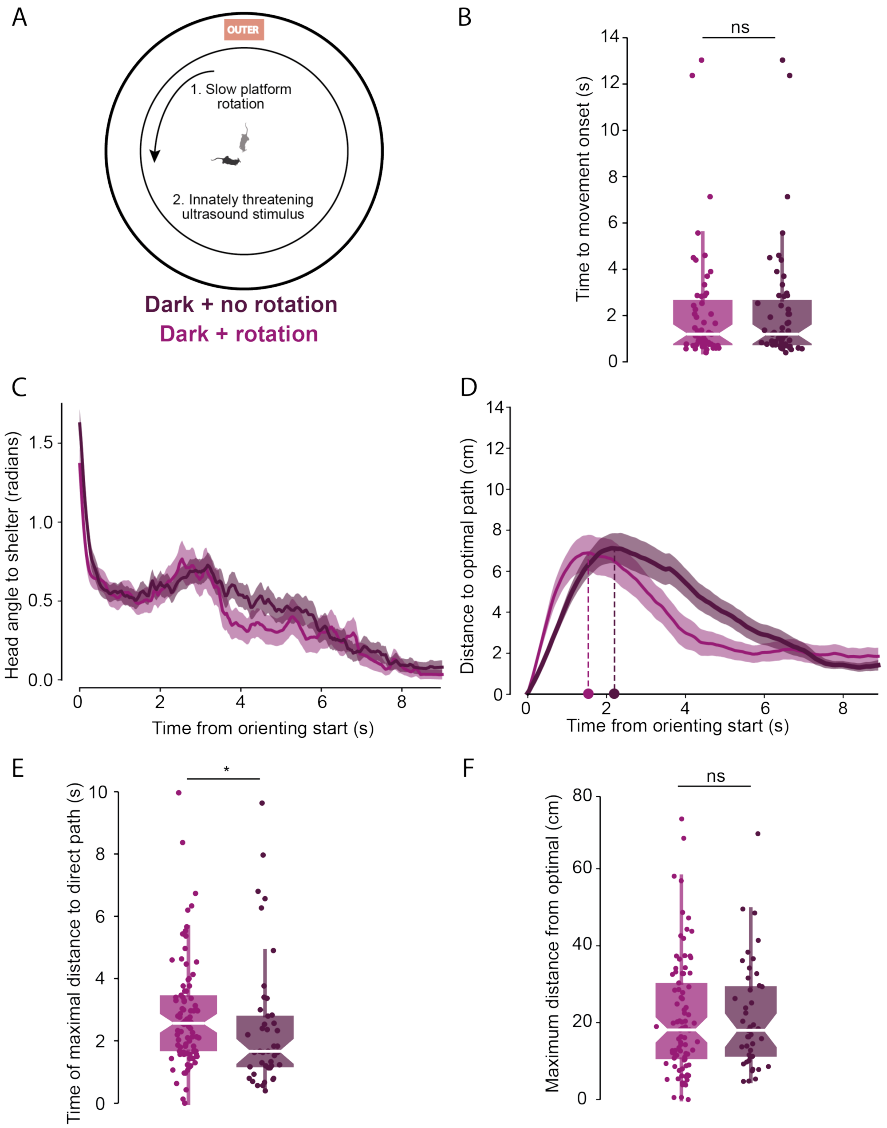

Figure 3.11: **Mice integrate vestibular cues to guide escape navigation.** A) Schematic depiction of the rotation experiment with the shelter on the external portion of the maze. B) Time to movement onset, as determined through manual labelling, with no significant difference between the rotation and no-rotation trials. C) Head angle to shelter (in radians) through time for rotation and non-rotation trials. D) Distance to the shelter path through time in rotation and no rotation trials. E) Time of the maximal distance to the shelter path, with this time occurring later in the rotation trials (p = 0.0070, independent t-test). F) Maximum distance to the shelter path. No difference was detected, indicating mice navigated equally well in both rotation and non-rotation trials (p = 0.366, independent t-test).

tion was vestibular, this tested the sensitivity of the mouse vestibular system and the ability of mice to integrate very fine differences in angular velocity into their estimate of the shelter location.

Mice were remarkably good at detecting this slow rotation and updating their estimate of the shelter (see example trajectories in figure 3.10). Comparing the head-to-shelter angle between control and rota-

tion trials, I found a rapid decline in the head angle to the shelter that was indistinguishable between conditions (figure 3.11c). The navigational abilities of mice in each condition were comparable, as assessed by comparing the distance to the shortest possible path to shelter. The maximal distance to this path in flight was not different (rotation mean 21.6 ± 13.78cm, non-rotation mean 21.2 ± 15.2cm, p = 0.366 independent t-test, figure 3.11e), though the time to reach this maximum was faster after rotation (figure 3.11f)(light mean 2.44 ± 2.09s, non-rotation mean 2.90 ± 1.85s, p = 0.0070 independent t-test). This more rapid time to reach maximal speed could be due to mice noticing being rotated and therefore being more sensitive to threatening cues, though this was not reflected in a difference in the time to movement onset, and so could be due there being greater variability in the start position of mice in the non-rotation condition (figure 3.11b).

Differences can also be observed when comparing the trajectories of mice navigating in the dark after being slowly rotated, with the shelter on the internal or external portion of the maze. When the shelter is on the internal portion, it moves in tandem with the mouse, whereas when on the external portion, it stays stationary. Mice navigate directly back to the original shelter location in absolute space - so directly to the shelter when placed on the external portion, and to where the shelter used to be when on the internal portion. This is shown by the increased distance to the optimal path when the the shelter is rotated in tandem with the mouse (dark inner maximum distance to optimal path 31.3 ± 15.6cm, dark outer 21.6 ± 13.8cm, p = 0.00188 independent t-test, figure 3.13). Together, this data suggests that mice integrate even very slow rotations through a highly sensitive vestibular system and use this information to update an estimate of the shelter location.

3.2.2 *Mice are able to use visual cues to update flight trajectory*

Having established that the vestibular system plays a role in guiding escape navigation, I next tested the second aspect of the hypothesis generated in the first set of experiments in this thesis: whether this ability of mice to integrate vestibular rotation input can be adjusted by visual cues through flight. To do so, I further compared escape trajectories when the shelter was placed on the inner rotating portion of the maze and the external non-rotating portion of the maze. In order to directly navigate to the shelter location, mice must know that the shelter has also rotated and take this into account.

After rotation in the dark with the shelter resting on the internal, rotating portion of the maze, mice escaped to the original shelter location, and only discovered the new position of the shelter after some exploration or after taking time to use other cues such as olfactory cues (n = 10 mice, 46 trials)(see figure 3.10 for an example trial). This

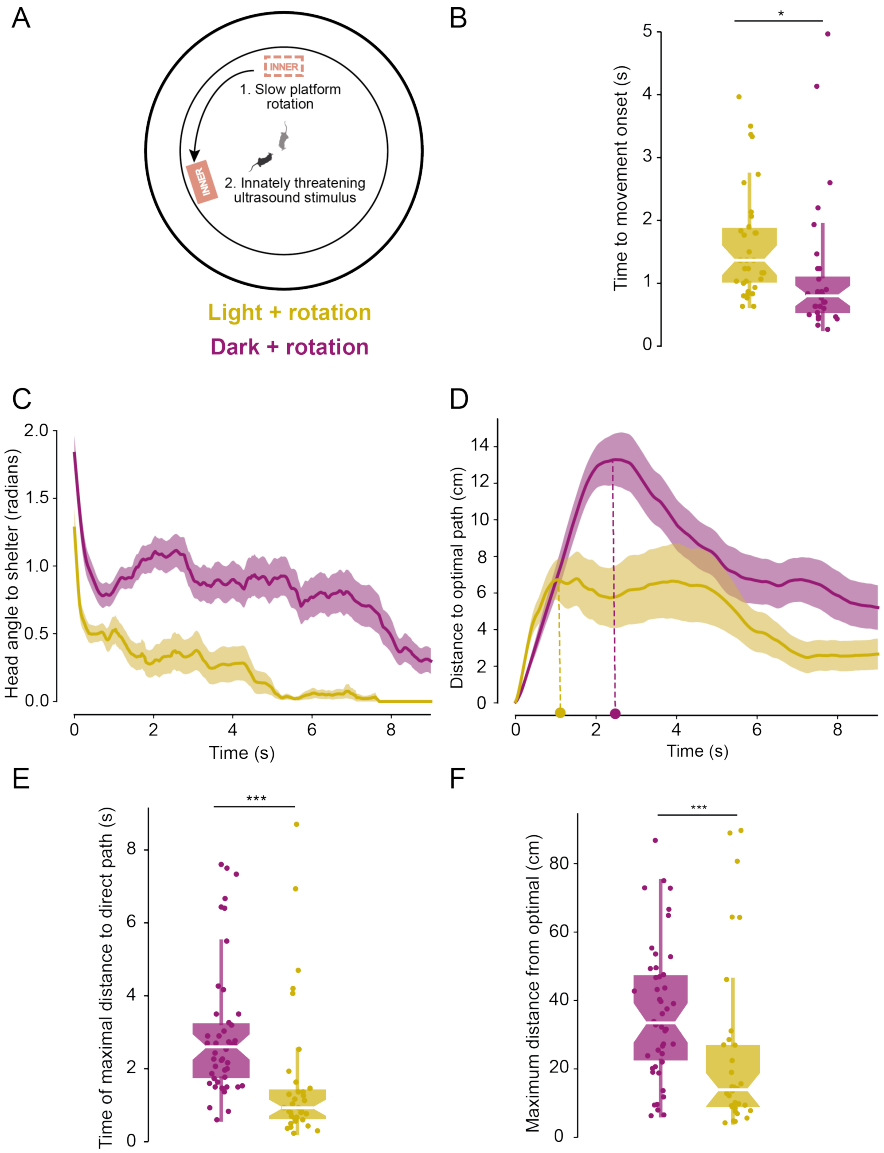

Figure 3.12: **Visual cues are used to correct towards the shelter after rotation.** A) Schematic depiction of the rotation experiments with the shelter on the internal portion of the maze. B) Time to movement onset in seconds, as determined by manual labelling. In the light, mice took significantly longer to initiate movement. C) Head angle to shelter in light and dark condition through flight. D) Distance to the shelter path, in its original position, in light and dark. E) Time taken to reach the maximal distance to the shelter path. It took significantly longer in the dark than in the light, indicating visual cues are used to update flight early ($p = 8.78 \times 10^{-7}$, independent t-test). F) Maximum distance to optimal path (cm), with this distance being larger in the dark, indicating mice navigated less accurately ($p = 2.3 \times 10^{-4}$, independent t-test).

is reflected by mice taking a longer time to reach the shelter after rotation when the shelter is placed on the internal portion of the maze than when it is on the outer, non-rotating portion (dark shelter outer time to shelter 4.48 ± 1.76s, dark shelter inner rotation time to shelter 6.54 ± 3.22s, $p = 7.59 \times 10^{-4}$, independent t test. Figure 3.13A and

B). Similarly, the maximum distance to the optimal path is greater in trials where the shelter was on the internal portion of the maze than on the external portion, a result corroborated by the head angle to the shelter through flight (maximum distance from optimal, shelter outer 21.58 ± 13.79cm, shelter inner 31.27 ± 15.64cm; p = 0.00576 independent t-test, figure 3.13). These data suggest that mice are able to integrate the rotation and use this information to guide navigation under the prior that the shelter does not move.

By contrast, in the light mice were much faster to correct their path, and were able to use the available visual cues to navigate to the new shelter location (n = 15 mice, 34 trials). This is shown by the more rapid reaching of the maximal distance to optimal path (light mean 1.65 ± 1.89s, dark mean 2.98 ± 1.81s, $p = 8.78 \times 10^{-7}$ independent t-test, figure 3.12E). Moreover, in the dark, in addition to the correction occurring at a later point in flight, the maximal distance to the optimal path was larger (light mean 23.8 ± 24.4cm, dark mean 36.5 ± 19.5cm, $p = 2.3 \times 10^{-4}$ independent t-test, figure 3.12F). The direction of the error was also correlated to the direction of the rotation when the shelter moved (figure 3.15B). This difference in post-rotation navigation between light and dark conditions suggests visual cues are used during flight, a finding contrary to previous reports based upon the rotation of available cues.

Direct comparison of the different rotation conditions further supports this hypothesis that path integration is used initially to guide escape navigation. In the dark, mice make larger errors when the shelter rotates alongside the mouse (compared maximum distance to shelter p values: 0.015 for inner light; 0.042 for outer dark; 0.038 for non-rotation dark. Tests are 1-way ANOVA with pairwise, values reported previously, figure 3.14D). The similarity in initial orienting can be seen as the head angle to shelter decreases similarly sharply in each condition (figure 3.14A). This then later diverges as mice are not able to adjust to the inner rotation in the dark. Furthermore, the direction of the correction is correlated with the angle of the rotation, suggesting the mice are estimating where the shelter is based on this rotational information (figure 3.15B). This suggests that mice initially orient through a vestibular based mechanism, and can then use visual input to adjust their path.

Further observations match the hypothesis that the cognitive process guiding this correction are the same as those observed in the light versus dark comparison in the previous section. The time period over which the head orienting response matches in light and dark approximately equals that seen in the large spatial environment. In the large environment experiments, mice took around 1s to react to the stimulus and complete the head orienting movement, regardless of the distance to the shelter. The secondary acceleration and movement phase then accounted for the difference in navigational abilities. Similarly, the correction of mice in the light occurs after a similar time delay

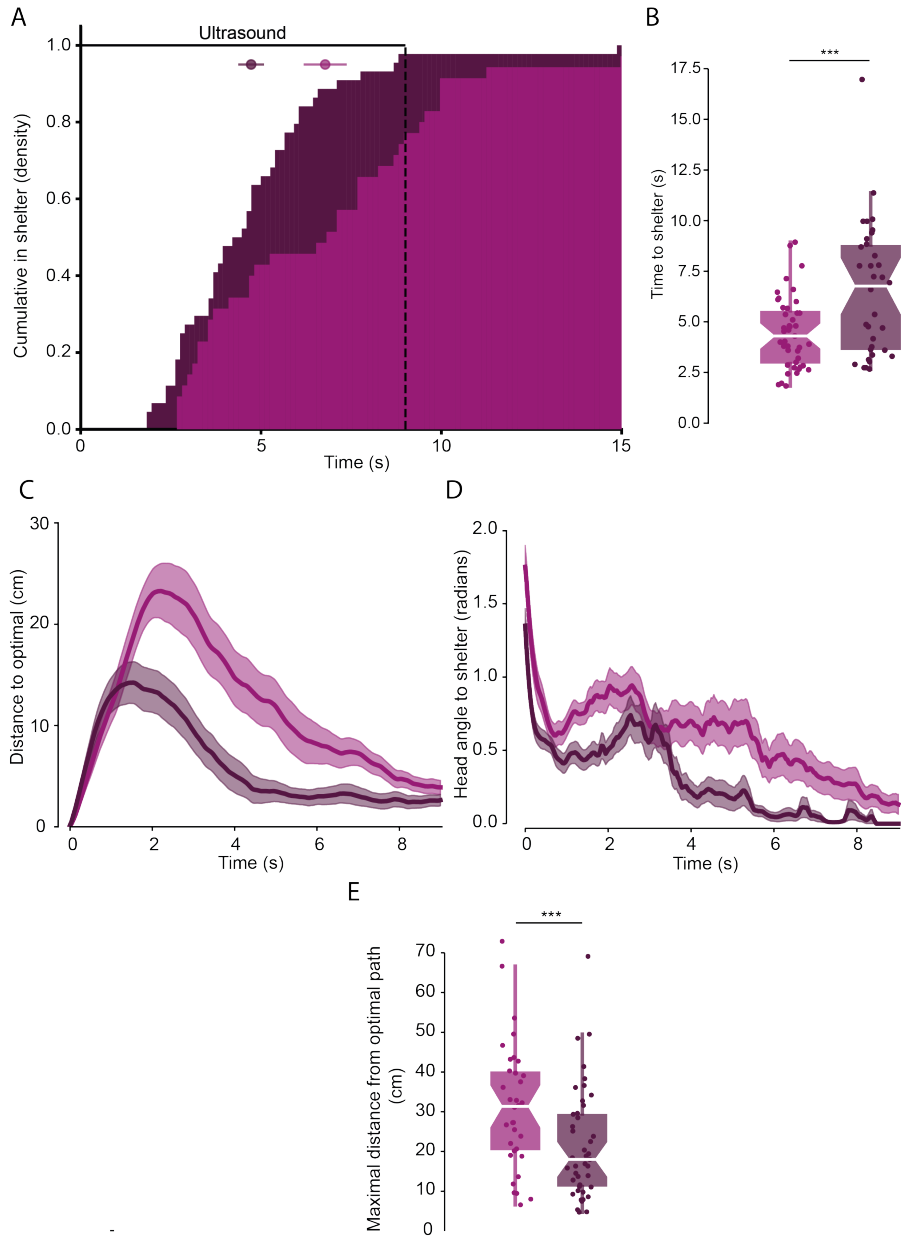

Figure 3.13: **Detected rotation in the dark directs mice to original shelter location.** A) Comparison of time to shelter, plotted cumulatively. B) Time to shelter, plotted as a box-whisker plot. A/B) show that mice take longer to reach the shelter in inner rotation trials ($p = 7.59 \times 10^{-4}$, independent t-test). C) Distance to the shelter path through flight. D) Head angle to shelter through time. E) Maximum distance from the optimal path, with this distance being significantly larger in inner rotation trials ($p = 0.00576$, independent t-test).

during the second phase of flight after rotation (figure 3.12D, E, 1.65 ± 1.89s), with the small difference in mean value largely driven by outliers. This data is suggestive that similar cognitive processes underlie the correction towards the shelter in the light over large spatial scales, and after slow rotations in the light.

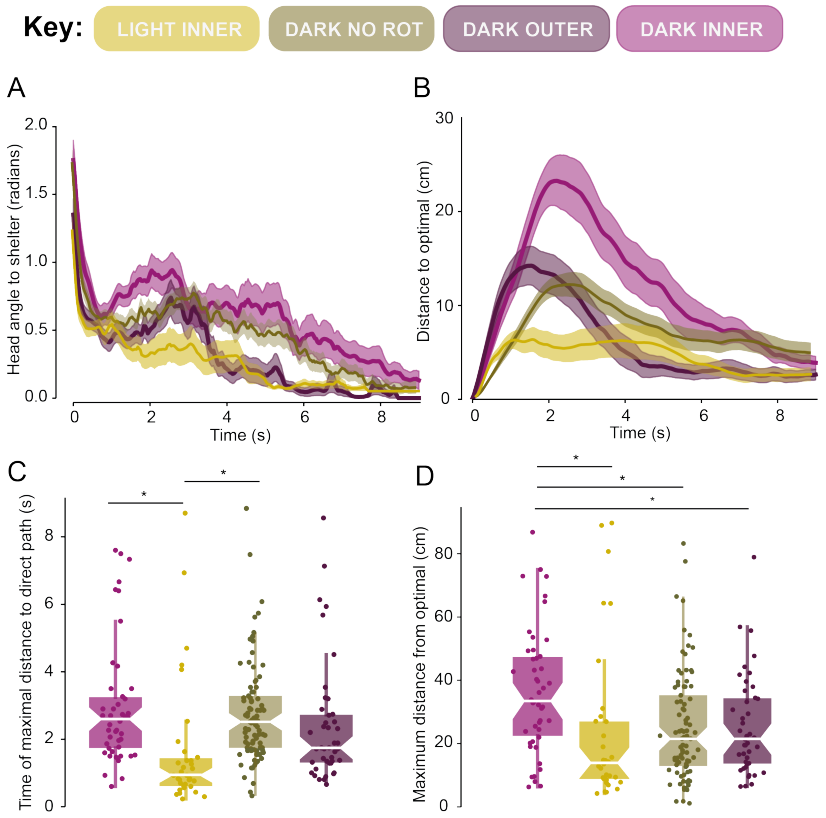

Figure 3.14: **Comparison of different rotation conditions indicates dark inner rotation has the largest effect on navigational ability.** A) Head angle to the shelter through time across conditions. B) Distance to the optimal path through time by condition. C) The time of the maximum distance to the shelter path. Statistical significance refers to 1-way ANOVA test. D) Maximum distance to the shelter path. Statistical significance refers to 1-way ANOVA with pairwise comparison test. Compared maximum distance to shelter p values: 0.015 for inner light; 0.042 for outer dark; 0.038 for non-rotation dark. Tests are 1-way ANOVA with pairwise, values reported previously.

3.3 VISUAL TAXON IS NOT NECESSARY FOR ESCAPE NAVIGATION

The previous data, taken together, suggest that the initial orienting to the shelter is not dependent on visual cues, but that the ability to refine trajectories over larger spatial scales is. I next aimed to test the hypothesis that the visual cue used is simply the sight of the shelter - so called 'taxon navigation' - by removing the shelter immediately prior to stimulus presentation. To do so, the shelter was attached to a piece of string, enabling the shelter to be moved from behind a curtain. Trials were alternated between baseline trials where the shelter does not move, and probe trials where the shelter is raised.

Mice escaped directly to the shelter location in probe trials (n = 11 mice, 16 pairs of trials). As can been seen in figure 3.16, baseline trials without shelter movement and trials in which the shelter was raised were largely indistinguishable in their trajectories. There was no de-

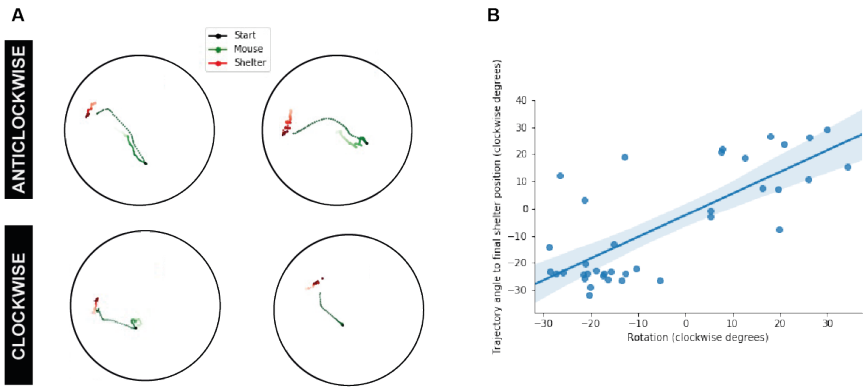

Figure 3.15: **Error direction depends on rotation direction.** A) Example trials from clockwise and anticlockwise rotations. Green dots show mouse trajectory, red dots show shelter movement (lighter red being earlier in the trial). B) The correlation between the rotation angle and the error angle of the trial. Error angle was calculated by finding the mean angle between the mouses location and the shelter through the first half of flight.

tectable difference in the flight trajectories between baseline and shelter raise conditions when the distance to shelter, tortuosity (baseline 1.28 ± 0.281, shelter raise 1.35 ± 0.476, p = 0.664 independent t-test), and time to reach shelter were compared (figure 3.17). The only difference observed is slightly faster speed during the midpoint of flight in the shelter raise trials, reflected in a more rapid decrease in the distance to the shelter, perhaps due to a 'panic' when the mice realise the shelter isn't where they remembered (figure 3.17). In accordance with this hypothesis, mice often spent time exploring the former shelter location, as if they expected a shelter to be there, rapidly changing direction searching for it.

This experiment clearly demonstrates that the strategy adopted by mice in light conditions is not simply visual taxon navigation - that is, mice don't simply see the shelter and run towards it. While such as a strategy may also be used in escape navigation in other conditions, it is not necessary for it, suggesting the strategy used to exploit visual cues to guide navigation to shelter also incorporate the spatial relationships between the visible cues.

3.4 ESCAPE WITH MULTIPLE SHELTERS

So far I have arrived at a hypothesis that the initial orienting and acceleration can be achieved through the integration of vestibular input, and a later phase is driven by visual cues and a cognitive map, and have also provided evidence supporting this hypothesis. However, an ambiguity remains in this hypothesis as to what happens when two shelters are present in the environment. Can the presence of another

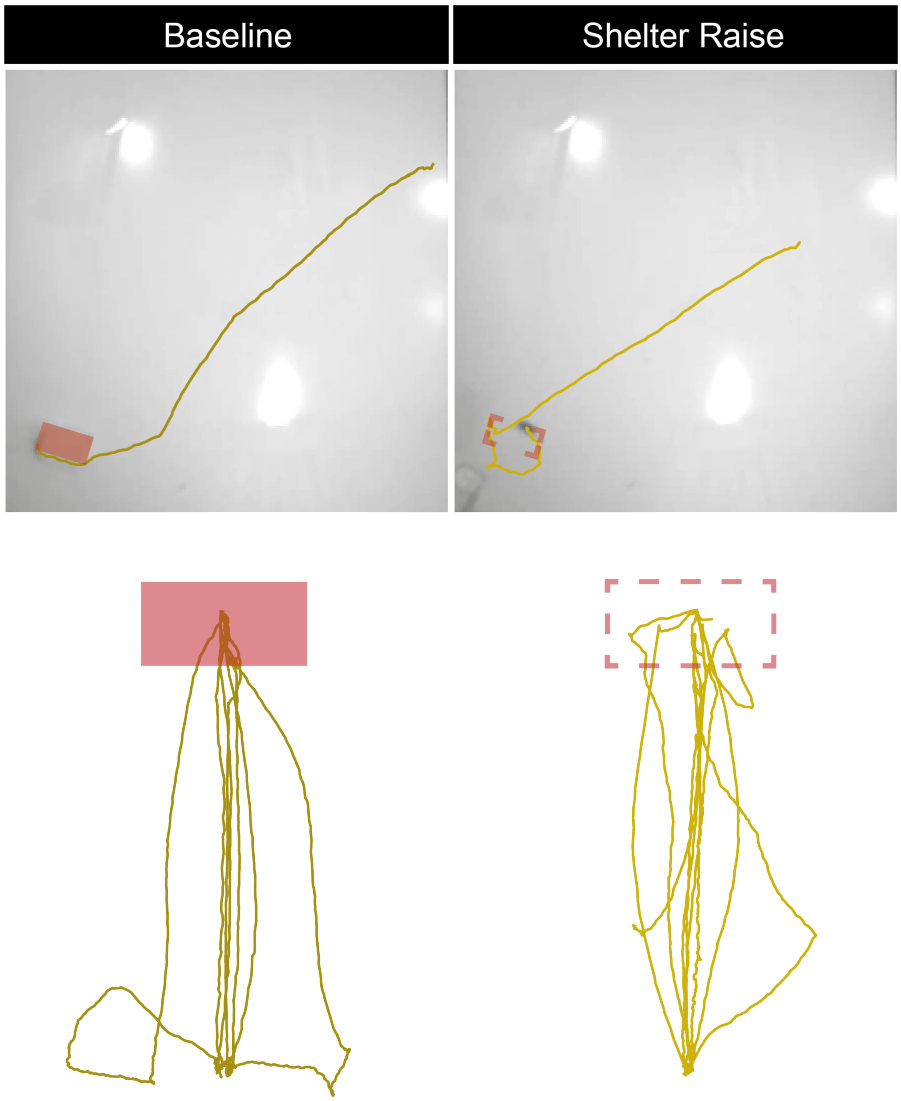

Figure 3.16: **Example trajectories from shelter raise experiments.** Summary of the shelter raise experiments, testing whether sight of the shelter is required for accurate escape. Top left panels show example trials from each condition overlapped onto the image of the arena. Below are all trials from each condition fitted to the be same orientation and length to enable comparison of trajectories.

shelter, presumably represented in the cognitive map, override the path integration driven initial estimate of the shelter location?

To test this, I replicated the experiment in the large arena, but with two shelters being present (n = 11 mice, 52 trials). Prior to any stimulus presentation, I waited for mice to visit both shelters to ensure they knew of their presence.

I first tested whether mice had a bias for escaping to the most recently visited shelter, which would be predicted by a simple path integration based algorithm that simply added up the vector travelled since the mouse was last in safety. Mice did indeed show a preference for the previously visited shelter, escaping to it more frequently than

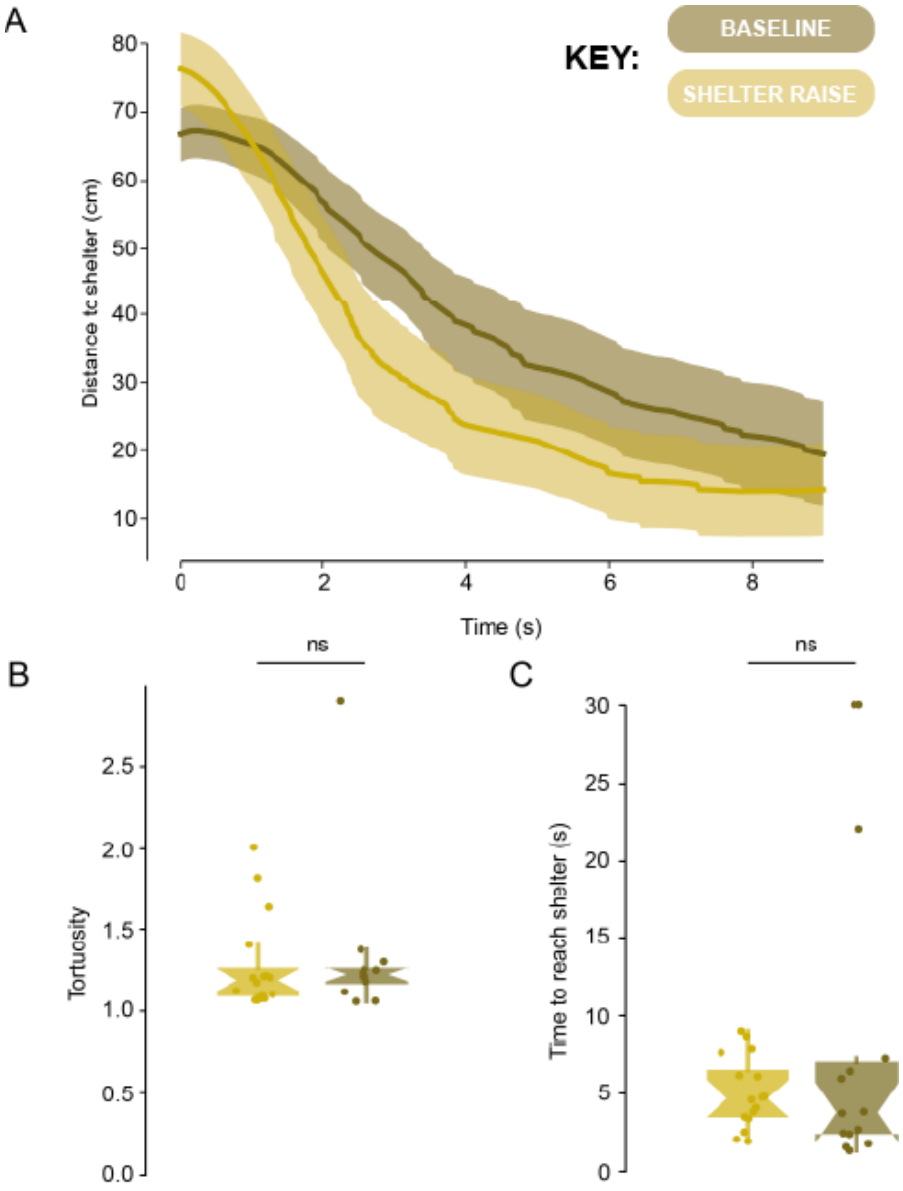

Figure 3.17: **Visual taxon navigation is not required for accurate escape navigation.** A) Distance to the shelter path plotted through time, with standard error of the mean overlay. B) Tortuosity box-whisker, with no significant distance between shelter raise and baseline trials (p = 0.664 independent t-test). C) Shelter raise trials and baseline trials show no difference in time to shelter.

the closest shelter (probability of escaping to previous shelter: 0.75, probability of escaping to nearest shelter: 0.654, figure 3.18).

However, this bias for the previously visited shelter was able to be overridden when the non-previously visited shelter was much closer. By plotting the difference in the distance to each shelter and then computed a density estimation of the likelihood to escape to either the previously visited or non-previously visited shelter, the propensity to escape to the previous shelter drops as the difference in the distance favours the nearest shelter more. Mice are equally likely to visit each shelter at around 1.5m difference between the two in favour,

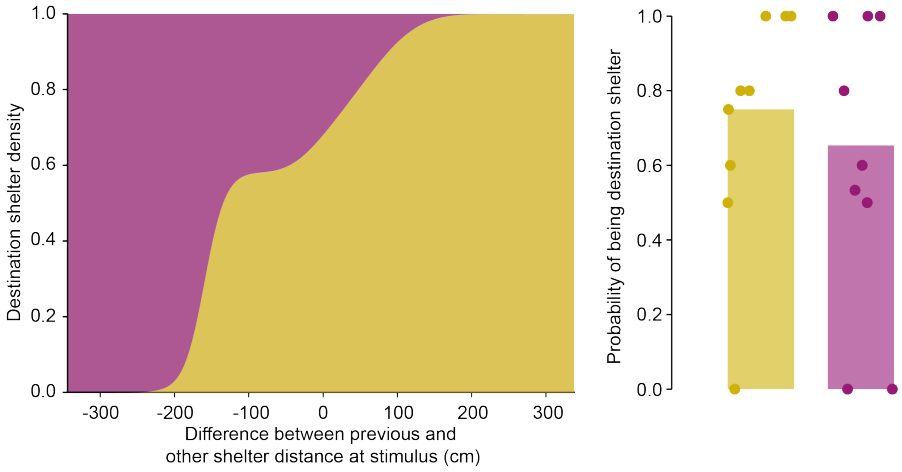

Figure 3.18: **Mice display a bias for the previously visited shelter.** A) Density estimate of propensity to end flight at previous or non-previous shelter as a function of the different in distance between the shelters. B) Probability of escaping to the previous shelter (yellow) and nearest shelter (pink).

where the non-previously visited shelter is closer. These data suggest that mice have an inherent preference to escape to the most recently visited shelter, as would be suggested by a path integration based strategy, but that knowledge of the presence of another shelter can be used to override this when a clear advantage can be had by escaping to the closest shelter. This provides further evidence for an interaction between a rapidly computed, path integration based vector to the shelter, and more cognitively sophisticated route planning using environmental knowledge.

3.5 VISUAL CUES ALONE CAN GUIDE ESCAPE NAVIGATION

We have seen that escape trajectories in both large spatial environments and after slow passive rotation are consistent with a two-phase flight, in which distinct computations underlie each phase. In particular, path integration is most consistent with the initial phase of flight, while some combination of a cognitive map and taxon navigation likely guides the latter phase. If this hypothesis is correct, the initial orienting phase should either be delayed or be less distinct without path integration, but mice should still be able to navigate to shelter when visual cues are available. Mice should also be unable to navigate to shelter under these conditions in the absence of visual cues.

To test this, I developed a test inspired by the Morris water maze, but adapted using escape navigation to dry conditions. Accordingly, I named the task the 'Dry Morris Maze' (DMM). In this paradigm, the mouse is first allowed to explore the large 2x2m arena and find the shelter (see section 2.2.7). Baseline escapes are taken when the mouse leaves the shelter to confirm that the mouse is capable of escape un-

der the conditions studied previously and provide a comparison. The mouse is then offered its home cage, left open next to the raised platform, which it voluntarily enters. To remove the possibility of path integration upon re-entry, the mouse is disoriented by placing the cage in an opaque box which is rotated behind a curtain. The shelter is removed from the environment, and the arena is cleaned with ethanol to minimize olfactory cues. The mouse is then reintroduced into the environment inside a transparent container allowing it to familiarize itself with the distal environmental cues. After 5 minutes, the mouse is released from the container and within a few seconds exposed to a looming visual stimulus or ultrasound stimulus to induce escape. The purpose of this test is to disrupt the self-motion cues hypothesised to underlying the initial phase of flight, and observe whether visual cues alone can guide escape navigation.

3.5.1 *Navigation in the Dry Morris Maze*

I first tested mice in this task in the presence of visual cues (n = 22 mice and trials). I found that mice initiate an escape response upon presentation of the stimulus after removal from the environment, disorientation, and removal of the shelter (see example pairs of baselines and probe trials in figure 3.19). Escapes were directed towards where the shelter was formerly located, and as can be observed in the example trajectories, mice tended to explore the former shelter area for an extended period of time, even after finding it is no longer present. This shows that mice can use visual cues alone to guide their escape navigation to the shelter, and that path integration is not necessary for escape navigation.

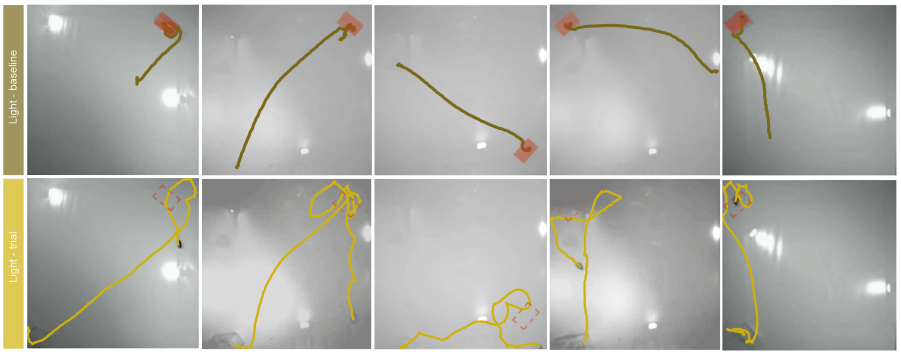

Figure 3.19: **Example pairs of DMM trials with visual cues available.** Top row: baseline trials in the light. Bottom row: paired DMM trials. Tracking shown from beginning to the end of the stimulus.

3.5.2 *Dry Morris Maze navigation is dependent on visual cues*

As path integration could not be used for navigation in the DMM, in light conditions navigation must be guided either by visual cues, as

intended, or by other cues that are still present in the environment, such as odour and auditory cues. To control for the possibility that these cues were used, I next performed the DMM in near total darkness (n = 9 mice and trials in dark, 22 in light).

In these conditions, despite navigating to shelter accurately in baseline, mice could not find the shelter location (see example pairs of baselines and probe trials in figure 3.20). Compared to DMM in the light, mice took significantly longer to reach the shelter location in the dark (mean time to enter previous shelter locations in dark 131 ± 77.2s, in light 42.7 ± 96.6s, $p = 0.0241$, independent t-test). Moreover, taking the first 30 seconds post-stimulus, mice spent significantly less time in the shelter quadrant in dark conditions than in the light (light time 19.3 ± 6.70s, dark time 2.89 ± 5.49s, $p = 7.49 \times 10^{-7}$, independent t-test). Similarly striking results can be seen when comparing various measures of the directness of flight between the two conditions. While baseline trials in the dark are very direct towards the shelter and show a low distance to the optimal path, which rapidly tends towards zero, in the probe trials the distance to the optimal path was higher and rose through the time that the stimulus was being delivered (figure 3.22d). Similarly, the distance to the optimal path in DMM trials in the light stay relatively low, and while they never tend to zero as mice leave the shelter location after reaching it, the distance to optimal is substantially lower than in the dark. Taken together, these data demonstrate that mice use the visual cues available to guide escape navigation, and that in dark conditions, path integration cues are necessary to navigate to shelter.

How does this difference in navigation between light and dark manifest in the properties of flight itself? In the dark, do mice simply walk randomly, or do they run continually in a particularly direction? To investigate this, I plotted the distance from the initially enacted path through time in light and dark DMM trials (figure 3.22B). In the light, the distance from this initially enacted path only changed very slowly, and stayed relatively low through the period of the stimulus. In contrast, in the dark, mice rapidly deviated away from their initially enacted path, suggesting their trajectories were not goal directed. Similarly, while in the light the distance to the optimal path remains relatively low through the stimulus presentation (3.22C), in the dark mice diverge from this path substantially. A similar difference can be observed between baseline trials in the dark and DMM trials in the dark. Taken together, these data show that mice can use visual cues alone to navigate to shelter, and that path integration is necessary to navigate to shelter in the dark.

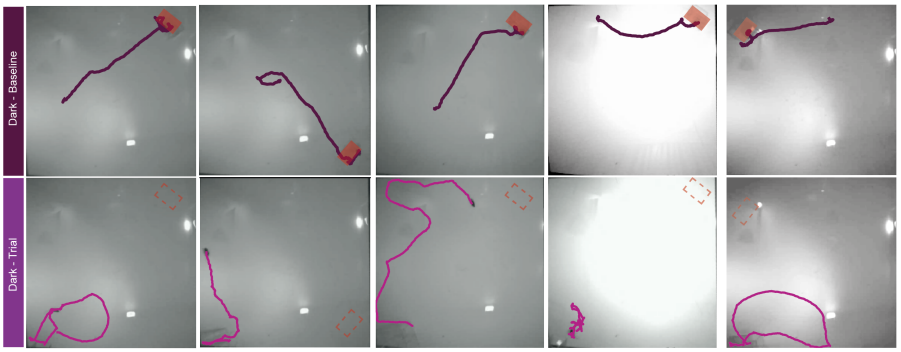

Figure 3.20: **Example pairs of DMM trials from dark conditions.** Top row: baseline trials in the dark. Bottom row: paired DMM trials. Tracking shown from beginning to the end of the stimulus.

3.5.3 *Delayed reaction time and mild impairment to navigation in the Dry Morris Maze*

I next investigated how this form of escape navigation - dependent only on visual and auditory cues - related to the previously presented data in the comparison between navigation in light and dark conditions in the large arena. According to the hypothesis arrived at through a comparison of escape in light and dark conditions - that escape occurs in two cognitive stages - the influence of visual cues only comes online after an initial path-integration based phase, and mice in the DMM task should have a delayed response as the influence of the vision based strategy takes time to 'come online'.

In line with this hypothesis, mice do indeed show a delayed reaction time. Manually labelling flights showed that the onset of movement was delayed in DMM trials in the light compared to baseline (baseline reaction time 0.351 ± 0.0507s, trial reaction time 0.642 ± 0.250s, p = 0.00501 paired t-test, figure 3.21C, n = 7 mice and trial pairs). Similarly, the time to reach peak speed within the stimulus was longer in DMM trials, reflecting this delay for cognitive processing (trial delay 4.78 ± 2.27s, baseline 1.96 ± 0.997s, p = 6.42 x 10^{-5} paired t-test). This delay in reaction time therefore suggests that the cognitive map-based strategy guides the second phase of flight and takes some time to come online, taking a secondary role to the rapidly computed path integration-based estimate of the shelter location.

3.5.4 *Delayed reaction time in DMM is not due to DMM experimental procedure*

To further test whether the delay in reaction time was due to loss of path integration, I performed a set of experiments in which, rather than remove the mouse from the environment, I trapped them under the transparent container during exploration. This experiment served two main purposes: to test whether the delay in reaction time was

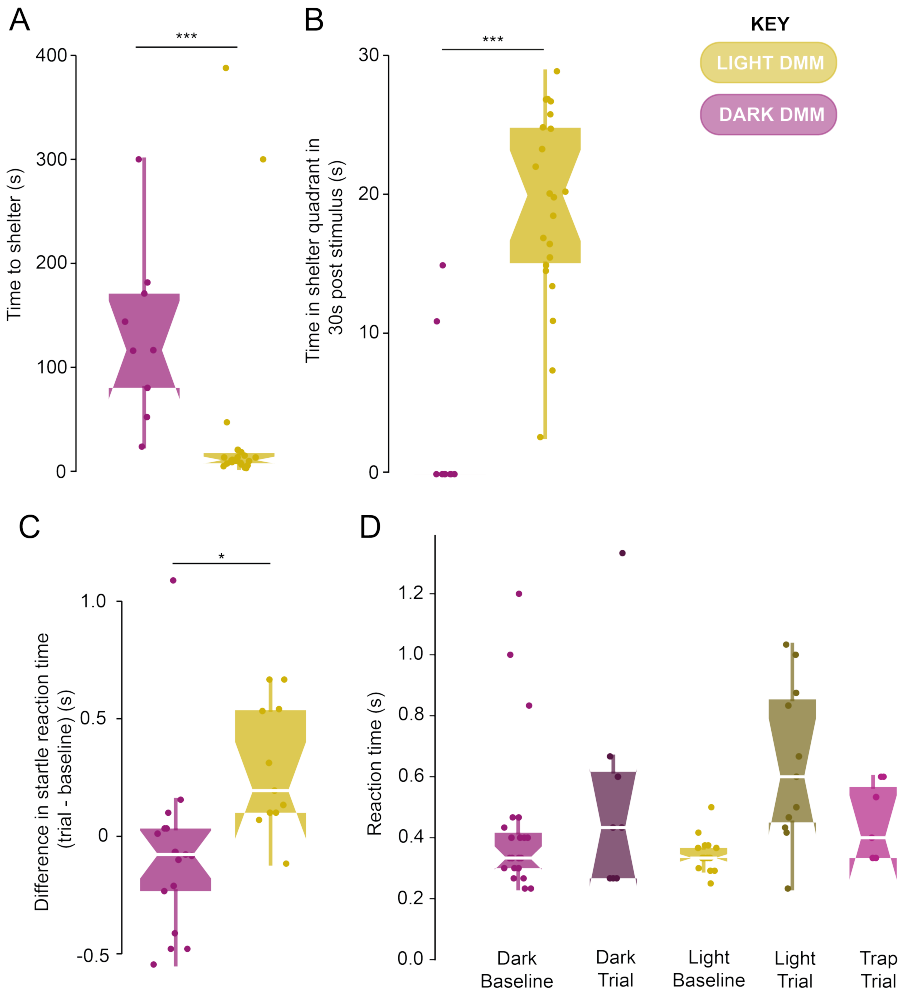

Figure 3.21: **Mice take longer to reach shelter in the dark DMM condition and explore shelter area for less time.** A) Mice take much longer to reach the shelter in absence of visual cues in the DMM task ($p = 0.0241$, independent t-test). B) Mice spend much less time in the shelter quadrant during the 30 seconds after the stimulus in the DMM task in dark conditions ($p = 7.49 \times 10^{-7}$, independent t-test). C) The difference in reaction times between baseline and DMM trials is greater in the light ($p = 0.00501$ independent t-test) D) Comparison of the reaction time for each experiment type (light and dark in baseline and DMM conditions, with the trap experiment (shown in detail later)).

due to a kind of general stress associated with being confined in a small container, and to observe the influence of path integration on flight more directly. This experiment is necessary because the difference in reaction time could have difference causes: it could reflect a longer processing time due to needing to initiate different cognitive processes, or it could reflect a difference in threat perception due to the experimental procedure.

The experimental procedure involved waiting for the mouse to explore the area underneath the box, at which point the box was released and trapped the mouse. I waited 5 minutes to replicate the

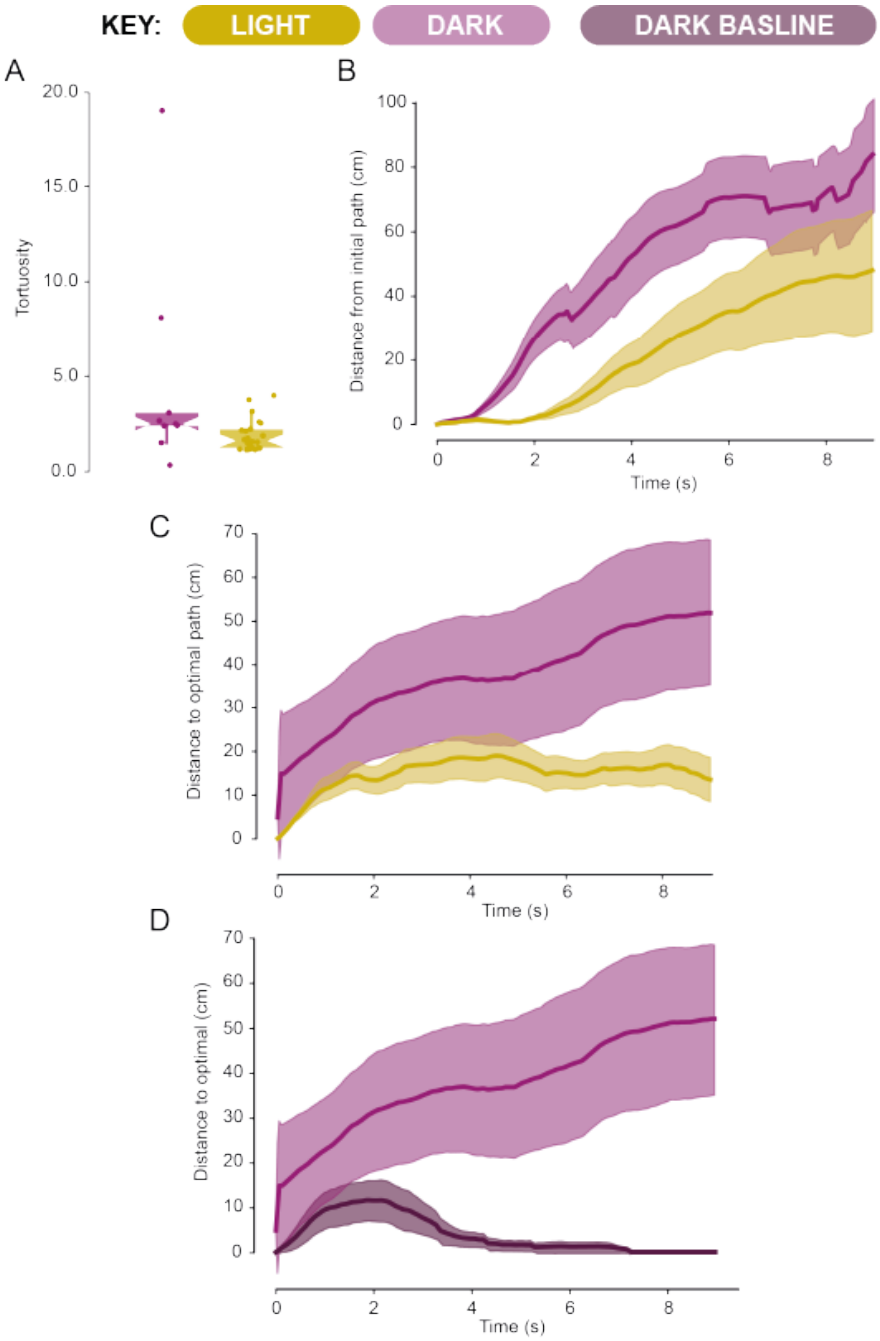

Figure 3.22: **Mice take less direct routes in DMM in the dark.** A) Tortuosity of DMM trials in light and dark conditions. B) Distance from the initial path in light and dark DMM trials. In the dark, this distance increases very quickly as mice are essentially walking randomly. C) Distance from the optimal path in light and dark trials. D) Distance from the optimal path in dark and dark baseline trials.

entrapment and cue exposure process in the Dry Morris Maze, and then lifted the box and delivered an innately threatening stimulus.

After entrapment, mice accurately navigated to shelter. The rate of decrease in the shelter distance was not different between trap trials and baselines (mean rate trap trials 0.654 ± 0.240ms^{-1}, mean rate baseline trials 0.685 ± 0.122ms^{-1}, p = 0.781 paired t-test, figure 3.24B).

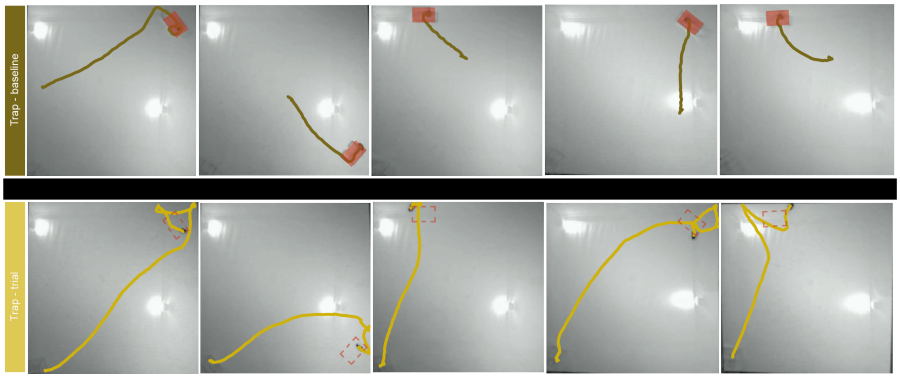

Figure 3.23: **Example pairs of trials from trap experiment.** Tracking overlaid onto pairs of frames from trials in the trap experiment. Top row shows baseline trials taken for comparison with the trap trials. Bottom row shows the paired trap trial, taken after entrapment in a transparent box and removal of the shelter to prevent visual taxon navigation.

Similarly, the tortuosity of flights was consistent between trap trials and baseline trials (mean tortuosity trap trials 1.35 ± 0.198, baseline trials 1.36 ± 0.0935, p = 0.935 paired t-test, figure 3.24A). This shows that there was no detrimental effect of the trap procedure itself on the ability of mice to navigate, suggesting the same cognitive functions that are available in baseline trials are also available in trap trials.

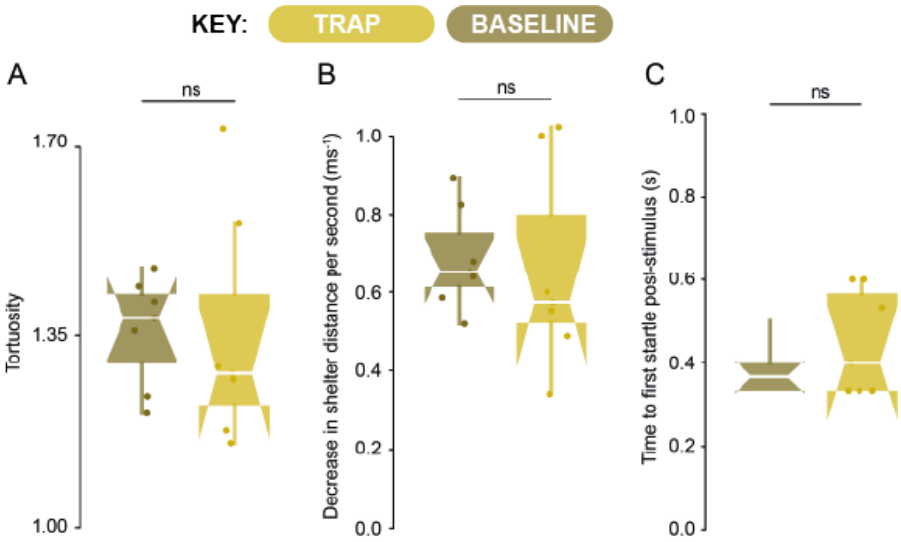

Figure 3.24: **Example pairs of trials from trap experiment.** A) Tortuosity of trap trials and baseline trials. No significant difference was observed (p = 0.935, paired t-test). B) Decrease in shelter distance per second in trap trials and baseline trials. No significant difference was detected (p = 0.781, paired t-test). C) Time to first startle post stimulus, as determined by manual labelling. No significant difference was detected (p = 0.231, paired t-test).

Moreover, mice displayed a reaction time much faster than in the DMM trials, with this reaction time comparable to baseline trials (mean reaction time trap trial 0.448 ± 0.117s, baseline trial 0.381 ± 0.056s, p = 0.231 independent t-test, figure 3.24C). Crucially, these es-

capes maintained both the reaction time and orienting response, indicating that the difference seen in DMM is not due to the experimental procedure of entrapment per se, but rather because the mouse can no longer use path integration.

These experiments also revealed a mild impairment to navigation in DMM trials. By directly comparing trap experiments to DMM, we can reveal the impact of path integration on escape navigation. While the distance to the shelter path was similar throughout, it took mice longer to reach shelter after DMM, perhaps indicative of lower confidence in their assessment of the shelter location (figure 3.25). This suggests path integration plays an important role and increases the confidence associated with escape navigation.

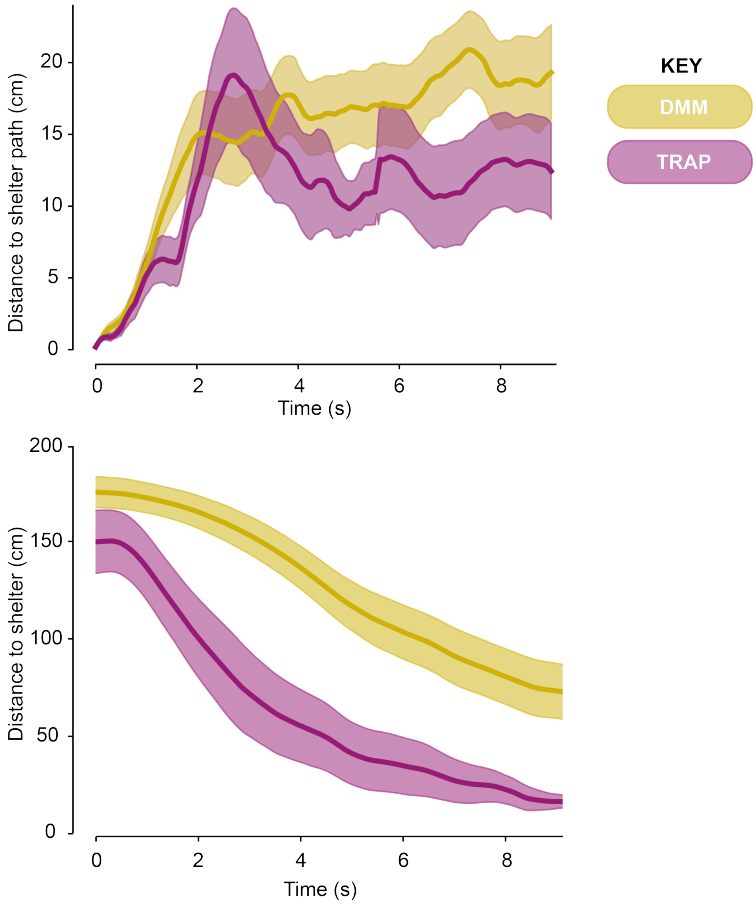

Figure 3.25: **Mice navigate less well in DMM than in trap.** A) The distance to the shelter path through flight in DMM and trap (cm). B) The distance to the shelter through flight in DMM and trap (cm).

These data indicate that the delayed reaction time observed in DMM not due to the experimental process itself. Crucially, it is due to the lack of availability of path integration, which in normal escape provides a rapidly available estimate of the shelter location in order to initiate defensive navigation as soon as possible.

3.6 PRELIMINARY CONCLUSIONS FROM BEHAVIOURAL DATA

Mice initiate escape navigation rapidly, and yet are able to navigate over long distances towards the shelter very precisely. In this chapter, I have asked how mice compute the vector to the shelter upon presentation of imminent threat. I addressed this in a series of behavioural experiments, each designed to reveal an aspect of the algorithm used to compute the shelter vector.

PRELIMINARY EXPERIMENTS Beginning with experiments in a large arena with a physical circular wall, I found that mice curve their trajectory to the shelter to ensure they can reach the intermediate safety of the wall during flight. While this necessitated constructing an alternate arena for the proceeding experiments, it nevertheless provided the first indication that mice incorporate knowledge of the structure of the environment into computing their escape trajectory. As these were preliminary experiments, I did not draw firm conclusions from them, but this observation guided my next experiments.

LONG RANGE ESCAPE NAVIGATION IN LIGHT AND DARK Taking this observation, I adjusted the environment and compared escape between light and near total darkness to assess the impact of visual cues on escape navigation over long spatial distances. I found that mice could use visual cues to refine their trajectories towards the shelter and correct for the small initial errors that are exacerbated at such ranges. This use of visual cues was confined to a previously unidentified phase of flight. However, this use of visual cues does not preclude the continued use and influence of path integration in this latter phase. While other explanations for these results are possible, I found no evidence in favour of any of the alternatives. The results are unlikely to be explained by a difference in threat perception because the reaction times were similar across conditions.

ESCAPE NAVIGATION AFTER ROTATION I next tested the relative roles of vestibular input into escape navigation by slowly rotating mice prior to stimulus presentation. I found that mice could detect this rotation with very high sensitivity, and integrate this into their estimate of the shelter location. As such, if the shelter rotated in tandem with the mouse, mice escaped to the original shelter location. However, with visual cues available, mice could use the visual sight of the shelter to correct their course during flight. I therefore next explored the ways in which visual cues guide escape navigation, and whether such visual taxon navigation was necessary for the influence of visual input on escape trajectories.

DISTINGUISHING BETWEEN USING A COGNITIVE MAP AND TAXON NAVIGATION How could the mouse compute the vector to the nest without direct path integration? It is likely that path integration is a more computationally efficient way of navigating than using knowledge of the relationships between environmental features - in this case the visual cues available in the arena - to determine the location of a shelter. An alternative is to store a representation of the relationships between different environmental features and use the observable cues to infer where the shelter should be - in other words, to store a so called 'cognitive map'. It is, however, unclear whether such a computationally demanding strategy would be used to guide escape navigation.

To distinguish between these two strategies, I designed a behavioral test that removed the possibility of using path integration to navigate, termed the 'Dry Morris Maze'. In this task, in baseline trials, path integration is possible; in DMM trials, path integration is not possible. Mice could only navigate to the shelter location in this task when visual cues were available, but did so with a delayed reaction time relative to baseline trials, indicating a more demanding cognitive strategy was being used. When the experimental conditions were replicated without removing path integration by trapping the mice, the difference in reaction time was no longer present. It would have been necessary to run these experiments in the dark to fully verify that changes in reaction time are not related to other, non-path integration aspects of this tasks. However, overall this data suggests that mice can use a cognitive map alone to navigate to shelter, but that this comes with extra computational costs.

CONCLUSIONS FROM BEHAVIOURAL DATA Together with the experiments previously described, this data provides strong evidence that mice can use a cognitive map to navigate to shelter. Moreover, the data suggest that they use visual cues during escape navigation, either using a map-like representation or a taxon navigation strategy. This two-stage escape strategy has several benefits. The initial path integration phase is rapid to compute and provides a robust estimate of the direction the mouse should turn and run towards. However, especially over large spatial scales, the accuracy of this approach can be refined by the use of visual cues, which is only required after the initial phase. The mouse innate escape response seems to have harnessed the benefits of each of these approaches, enabling escape to be initiated very rapidly while taking into consideration the environmental complexities present and enabling the use of the visual information available to refine the flight trajectory. Mouse escape thus strikes a balance between robustness and speed of response, and accuracy and efficiency over larger spatial scales.

4

DEPENDENCY OF ESCAPE ON THE HIPPOCAMPAL FORMATION

4.1 HIPPOCAMPUS LESIONED MICE SHOW DISRUPTED ESCAPE NAVIGATION

Having identified an ability of mice to use cognitive maps during escape navigation, I next sought to investigate whether the brain structures typically associated with these aspects of cognition are involved in escape navigation. The neural correlates of spatial cognition are among the best studied systems in rodent neuroscience, with decades of research detailing how neurons represent the spatial environment. This research has centred on the hippocampal formation, in which a variety of spatially responsive cells are present. My neural investigation therefore focused on disrupting the hippocampal system and observing the effect on escape navigation.

4.1.1 *Hippocampus lesioned mice show erratic exploration but maintain shelter preference*

I first tested the role of the hippocampus in escape navigation by inducing a broad lesion by injecting ibotenic acid, which degrades tissue and therefore disrupts neural function. Several weeks post-injection, to allow the acid to take effect and for the mice to recover, I tested their ability to navigate to shelter using escape navigation (n = 4 mice, 148 trials). Post-experiment histology verified that the injections had degraded tissue in the desired brain region (figure 4.1a). To test the ability of hippocampus lesioned mice to navigate to shelter, they were introduced to the large square arena and the same protocol as the comparison between navigation in the light and the dark was used.

Hippocampus lesioned mice displayed an erratic pattern of exploration, suggestive that the lesion had impacted spatial cognition (figure 4.1). Lesioned mice spent on average longer outside of the shelter (fraction of time inside shelter lesioned mice 0.343 ± 0.355, non-lesioned mice 0.512 ± 0.357, p = 0.00349 independent t test). However, this longer duration outside the shelter was not due to a lower preference for the shelter, as the mean duration of stay in the shelter was in fact longer in lesioned mice (mean shelter stay duration lesioned mice 178 ± 586s, non-lesioned mice 29.4 ± 71.5s, p = 0.00721 indepen-

dent t test). This indicates that while mice still had a preference for being in the shelter, which could be because they lacked the spatial memory of the shelter location and therefore could not navigate back towards it. However, when they happen upon the shelter by chance during exploration, they still perceive it as a safe location.

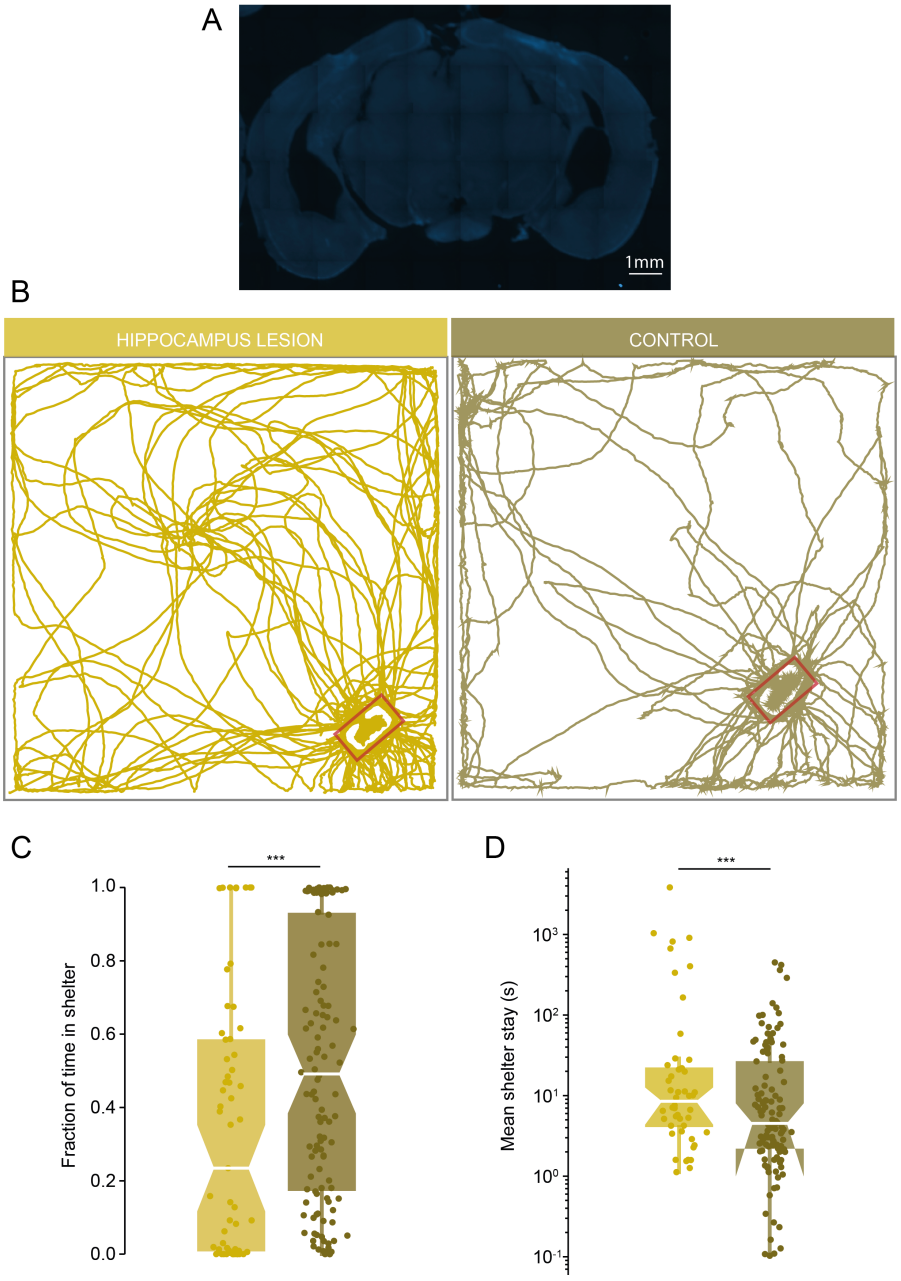

Figure 4.1: **Maintained shelter preference, but disrupted exploration patterns, in hippocampus lesioned mice.** A) Histological section of hippocampus lesioned mouse, with ibotenic acid lesioned hippocampus shown. B) Comparison of tracking data from an experimental session in hippocampus lesioned mouse and a randomly selected control. C) Fraction of time in shelter in each experimental session, with more time spent in the shelter in control mice ($p = 0.00349$, independent t-test). D) Mean shelter stay in hippocampus lesioned and control mice, with hippocampus staying in shelter on average for longer ($p = 0.00721$, independent t test).

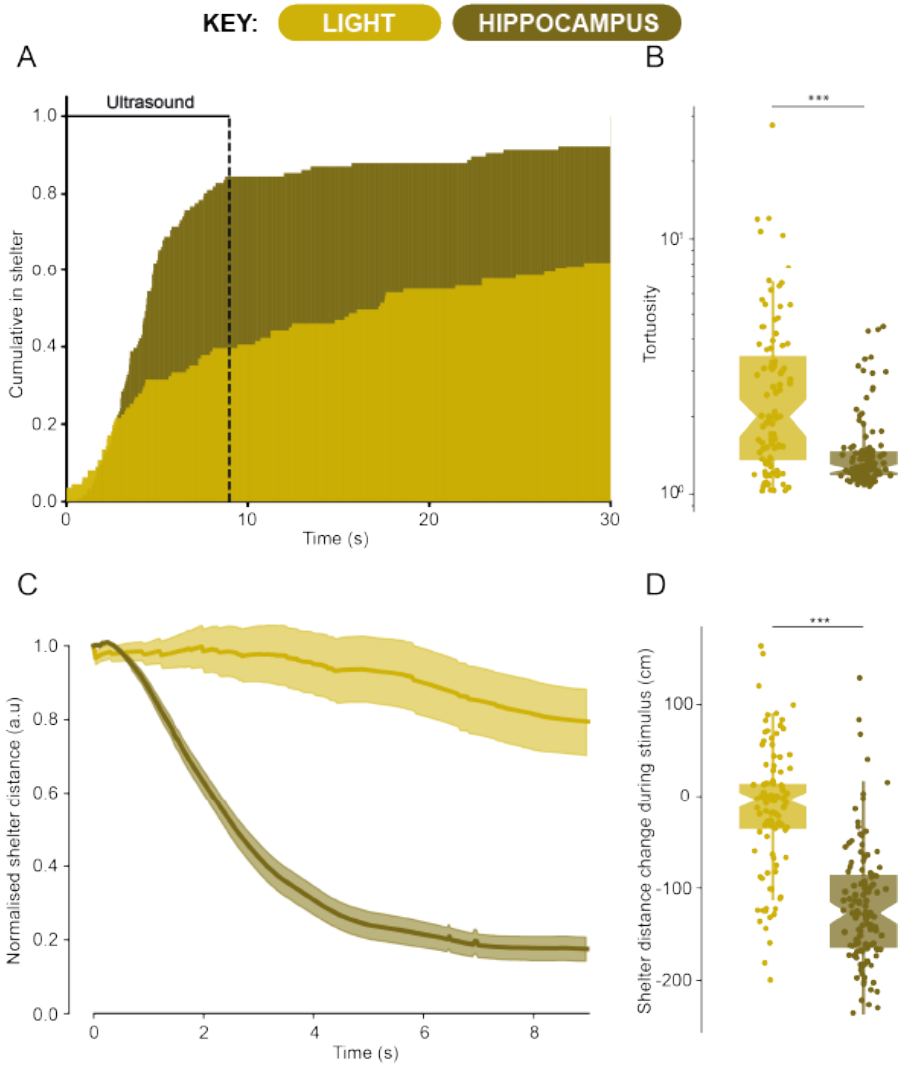

Figure 4.2: **Hippocampus lesioned mice show decreased ability to navigate to shelter during escape.** A) Time to shelter plotted cumulatively, with the hippocampus lesioned mice taken substantially longer to arrive back to shelter post stimulus onset (lesion mean 200.0 ± 556.5s, lesion median 17.2, IQR 126.6s; control mean 7.28 ± 0.75s, control median 4.4, IQR 3.51s, $p = 3.28 \times 10^{-4}$, independent t-test). B) The tortuosity of flights in hippocampus lesioned and control mice, with hippocampus lesioned mice taken much less direct routes back to shelter ($p = 3.64 \times 10^{-5}$, independent t-test). C) Shelter distance normalised to the start distance through flight . D) Shelter distance change during the stimulus ($p = 1.31 \times 10^{-25}$, independent t-test).

4.1.2 *Disrupted escape navigation in hippocampus lesioned mice*

Given the observed preference for the shelter, I asked whether normal defensive responses were intact in lesioned mice. Innately threatening visual and auditory stimuli were delivered as in the previous studies in the large environment: stimuli were only delivered after 7 minutes post introduction to the arena, after the mouse had first visited the shelter, and when the mouse was not already running towards the

shelter. For comparison, I used the experiments collected in the large arena for the light and dark experiments as controls as they underwent the same experimental protocol.

Compared to non-lesioned control mice, lesioned mice took substantially longer to reach the shelter post-stimulus (lesion mean 200.0 ± 556.5s, lesion median 17.2, IQR 126.6s; control mean 7.28 ± 0.75s, control median 4.4, IQR 3.51s, $p = 3.28 \times 10^{-4}$ independent t-test, figure 4.2a). This increase in time to reach the shelter was primarily driven by escape trajectories being less direct in lesioned mice. Compared to control mice, the trajectories of lesioned mice had a greater tortuosity (lesion mean tortuosity 2.83 ± 3.30, control mean tortuosity 1.51 ± 0.67, $p = 3.64 \times 10^{-5}$ independent t-test, figure 4.2b). Similarly, the shelter distance normalised to distance at the time of stimulus onset decreases far faster in the control mice than in lesioned mice (figure 4.2c). During the period of the stimulus, in control mice the shelter distance decreased by over 2 metres, but scarcely decreased at all in hippocampus lesioned mice (control shelter distance change -267.1 ± 146.4cm, lesioned mice -31.5 ± 150cm, $p = 1.31 \times 10^{-25}$ independent t-test, figure 4.2d). Therefore, despite a comparable preference for the shelter, lesioned mice show a substantial deficit in navigating back to shelter post-stimulus, largely due to escape trajectories not being directed towards the shelter in hippocampus lesioned mice.

4.1.3 *Hippocampus lesion mice maintain defensive responses*

The defensive responses of lesioned mice were more challenging to quantify than those in non-lesioned mice. Part of the criteria used to identify the onset of escape is the orienting towards the shelter. However, as lesioned mice did not typically orient post-stimulus, and their erratic exploration pattern meant precisely defining the onset of a defensive response was not straightforward.

So how do the defensive responses of lesioned mice compare to the non-lesioned mice? Direct comparison of reaction times between these two groups is challenging as the defensive response is different, and so it is unclear which part of the action should be taken to compare through manual labelling, as was used previously. However, a clear readout can be found in the acceleration post-stimulus which can serve as a proxy for stimulus response.

However, lesioned mice did accelerate post-stimulus. As can been seen in the raster plots in figure 4.3a, lesioned mice clearly increased speed post-stimulus, though the onset of this speed increase was more variable than in the light-dark comparison in (pre-stimulus mean speed 0.157 ± 0.114ms^{-1}, post-stimulus mean speed 0.238 ± 0.197 ms^{-1}, p = 0.000181 independent t-test, figure 4.3). Comparison of the speed of lesioned mice pre- and post-stimulus periods shows an increase in speed, indicating that mice did indeed perceive the threat-

ening stimulus and adjust their actions accordingly (mean speed increase 0.0785 ± 0.156ms^{-1}, figure4.3c). Despite this maintained response, these responses were clearly disrupted compared to non-lesioned mice, with post-stimulus acceleration significantly lower in lesioned mice (lesioned mice acceleration 0.022 ± 0.12ms^{-2}, control mice acceleration 0.14 ± 2.58 ms^{-2}, p = 1.13 x 10 $^{-17}$, independent t-test, figure4.3). This acceleration post-stimulus provides evidence that hippocampus lesioned mice do show a defensive response to innately threatening stimuli, and that the disrupted spatial navigation is not due to a deficit in stimulus detection, but that it takes a significantly different form to that observed in control animals.

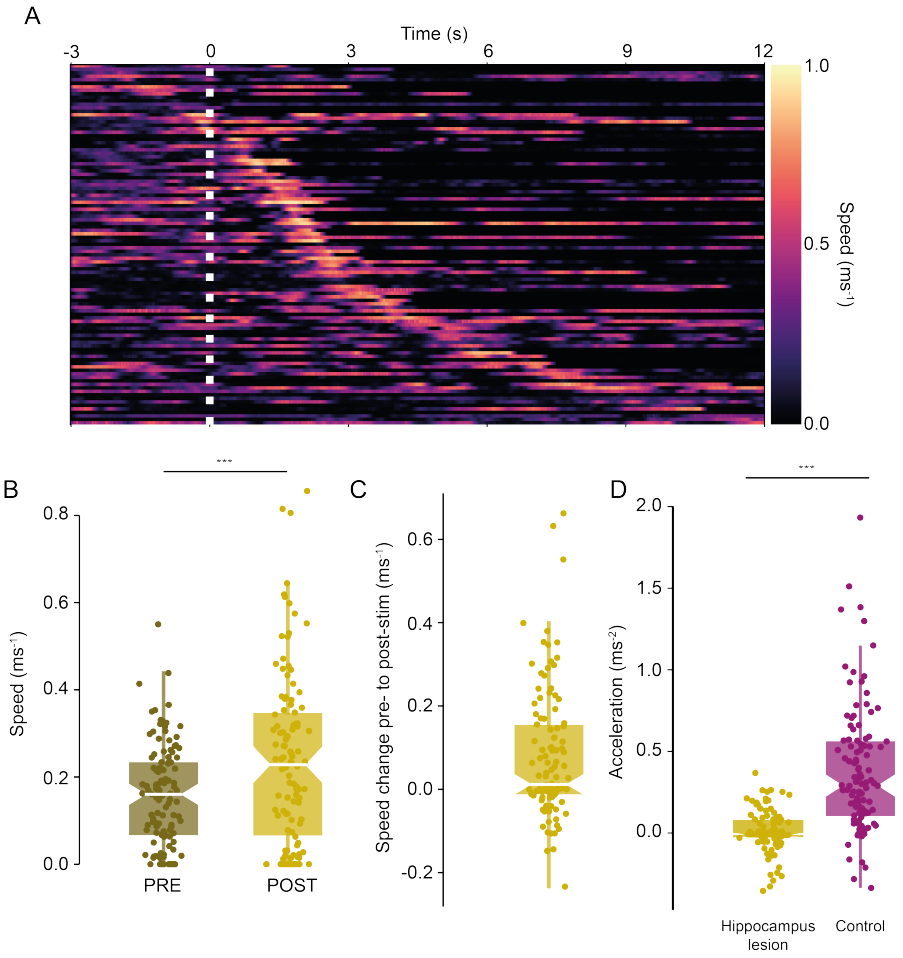

Figure 4.3: **Hippocampus lesioned mice accelerate post-stimulus onset.** A) Raster plot of speed through time post-stimulus. Stimulus onset at t=0, denoted with white dotted line. B) Mean speed box-whiskers in the 3 seconds pre- and post-stimulus (p = 0.000181 independent t-test). C) Change in speed between pre- and post-stimulus. D) Acceleration in lesioned versus control, non-lesioned mice after the stimulus.

4.2 DISRUPTING CODING IN THE SUBICULUM DISRUPTS SHELTER MEMORY

Having established the involvement of the hippocampus in escape navigation in a non-specific way, I next aimed to investigate subregions of the hippocampus for more specific effects on escape navigation. To do so, I was guided by previous anatomical, electrophysiological and behavioural research suggesting a role for the subiculum in navigation. The subiculum in particular seemed to be a promising avenue for further study because it projects to the retrosplenial cortex (Cembrowski et al., 2018), which our laboratory has recently shown to have a vital role in encoding the head-angle to the shelter (Vale et al., 2020). I therefore hypothesized that the subiculum may be routing information about the structure of the environment and the location of the shelter from the hippocampal formation to the retrosplenial cortex in order to guide orienting actions upon stimulus presentation.

4.2.1 *Muscimol infusions into subiculum precludes acceleration to innately threatening stimuli*

To test this hypothesis, I began by implanting cannula over the subiculum to enable the infusion of the pharmacological agent muscimol to disrupt neural activity (muscimol infusions n = 8 mice, 74 trials; control infusions n = 3 mice, 14 trials). Muscimol or control solution was infused by anaesthetising implanted mice, attaching tubes and a syringe to the implanted cannula, and slowly injecting muscimol through the syringe. 30 minutes after the mouse recovered from anaesthesia, the mouse was introduced into the large arena and the experiment proceeded in light conditions according to the escape navigation assay outlined previously, with both visual looms and ultrasound sweep stimuli used to elicit defensive responses. After assessing escape navigation in this paradigm, histological samples were retrieved to verify successful infusion and its anatomical location and spread.

Subiculum disrupted mice showed a striking phenotype compared to control infused mice. Rather than accelerate and escape, subiculum disrupted mice typically froze, particularly in response in looming stimuli. To ultrasound stimuli, these mice usually stopped but also included an inaccurate orienting movement, such that it displayed more of a startle response than a stereotypical freezing response. By contrast, control mice, which underwent the same procedures except for being infused with a solution identical but with DiI substituted for muscimol, displayed no observable differences in escape navigation to untreated mice, rapidly and accurately escaping to shelter. This phenotype is captured by the longer time to reach the shelter post-

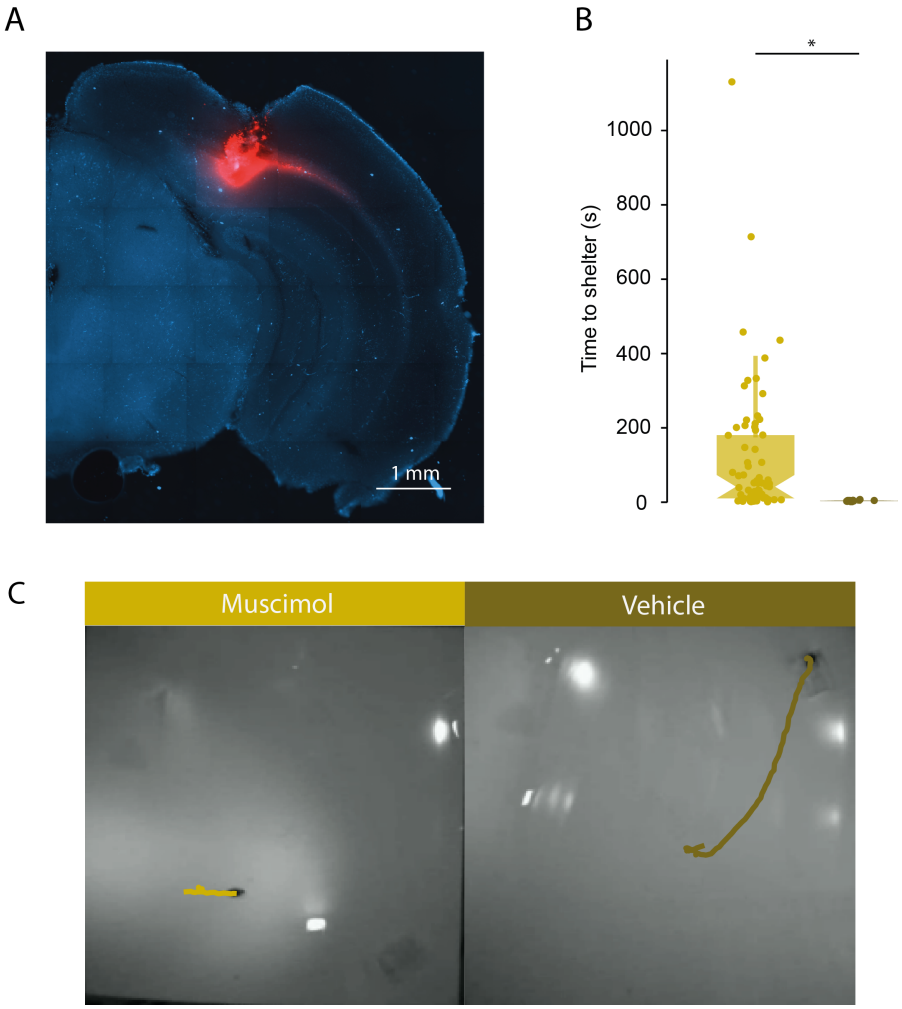

Figure 4.4: **Subiculum muscimol infusion disrupts escape to shelter.** A) Histological section through subiculum, red marker shows example muscimol/vehicle infusion site. B) Time to shelter box-whisker for subiculum muscimol trials (light yellow) and control vehicle infusions (dark yellow)(p = 0.0300, independent t-test).

stimulus in muscimol infused mice (muscimol 111 ± 179s, control 4.06 ± 1.61s, p = 0.0300 independent t-test).

What drove this longer time to reach the shelter? the change in shelter distance during the stimulus (muscimol distance change -26.5 ± 126cm, control distance change -205 ± 66.5cm, p = 1.99 x 10^{-6}, independent t-test), and the change in speed post-stimulus (muscimol speed change -0.0106 ± 0.0987ms^{-1} control 0.0788 ± 0.0873ms^{-1}, p = 0.00240, independent t-test)(figure 4.5). Similarly, in cases where post-experiment histological checks revealed an unsuccessful infusion of muscimol, the behavioural phenotype associated with muscimol infusion was not present. Together this data suggests that infusing muscimol into the subiculum induces a deficit in escape navigation, with mice instead electing to freeze or startle in response to innately threatening stimuli.

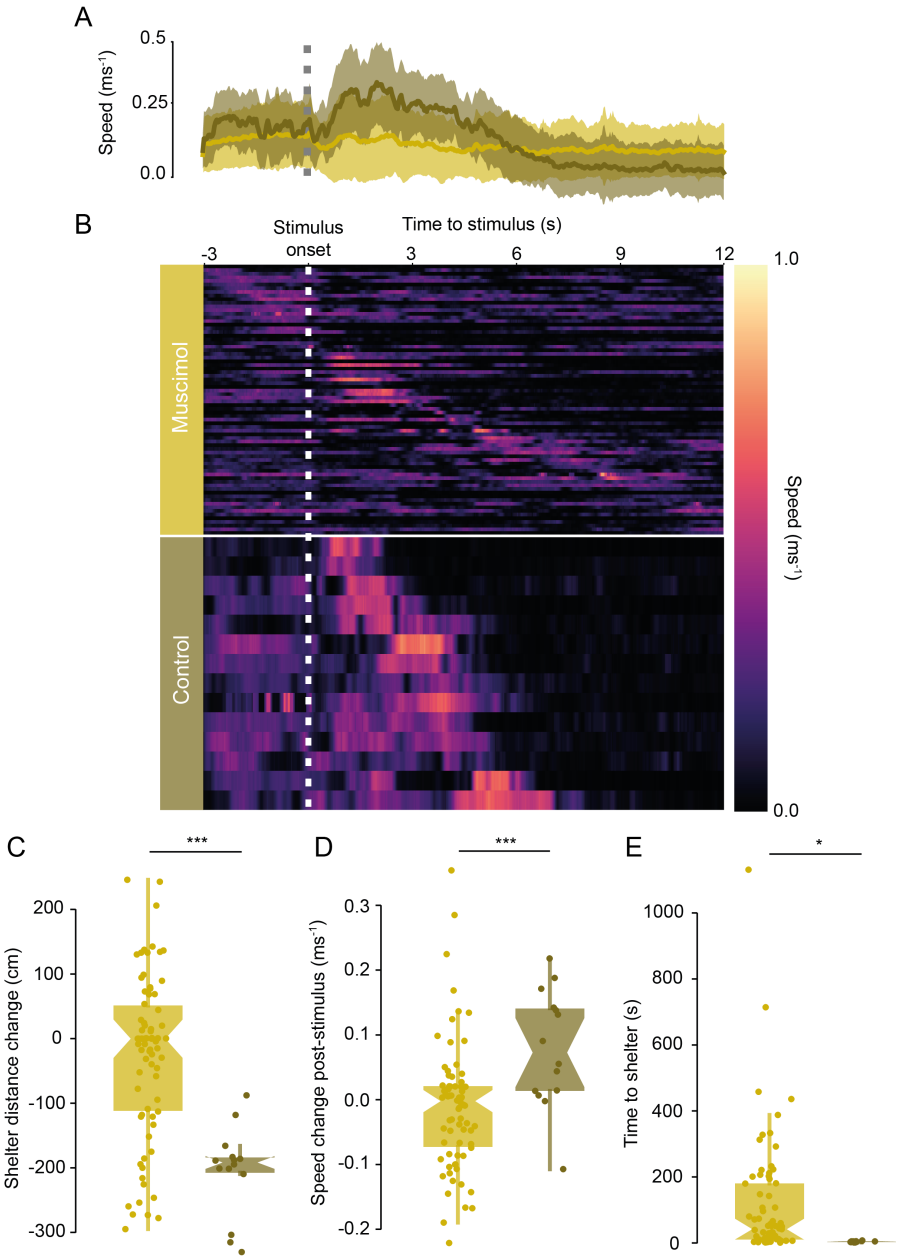

Figure 4.5: **Subiculum muscimol infusion precludes acceleration to threatening stimuli.** A) The speed of mice through time, with standard error of the mean overlay. Stimulus onset at t=0, denoted with gray dotted line. B) The speed of mice through time in raster plot form to show all trials. Stimulus onset at t=0, denoted with white dotted line. C) Change in shelter distance during stimulus ($p = 1.99 \times 10^{-6}$, independent t-test) D) Change in speed post-stimulus ($p = 0.00240$, independent t-test)

4.2.2 *Optogenetic disruption of subiculum coding precludes acceleration without innately threatening stimuli*

Therefore, the behavioural effect of silencing the subiculum with muscimol aligns with my hypothesis that the subiculum conveys information about the spatial structure of the environment to the retrosplenial cortex to guide escape navigation. I next aimed to use methods

that would allow the specific silencing of particular subgroups of neurons based on anatomical or gene expression, which is not possible with muscimol. I therefore tested whether optogenetic stimulation or chemogenetic silencing, and hence disruption of coding, of subiculum would replicate the effect of silencing with muscimol on escape navigation.

To do so, I implanted fibre optic cannula over the subiculum after injecting a genetic construct expressing channelrhodopsin 2 (ChR2)(n = 2 mice). Post-surgery, I then waited 3 weeks to allow the ChR2 construct to infect cells and express the opsin, and to allow any post-surgery inflammation to subside. After these three weeks, the mice were then tested in the Barnes arena used in (Vale et al., 2020; Vale, Evans, and Branco, 2018; Vale, Evans, and Branco, 2017). For these experiments, this arena was used as it already had a laser equipped, which is not the case for the larger environment used in the other studies in the present thesis.

I first checked for in tact escape responses in these mice. Likely as a result of the surgery, these mice did not respond to looming stimuli. Mice are known to occasionally suffer deficits in eyesight following surgery, often due to factors such as the fixation of the head or drying of the eyes. While no such signs were observed during surgery, surgery seems the most likely explanation for their lack of response. Instead, I therefore used ultrasound sweep stimuli to induce escape navigation in these experiments (n = 19 trials).

Implanted mice accurately navigated to shelter upon presentation of auditory stimuli. After characterising this response, I then stimulated ChR2 in the subiculum with a blue laser 1 second prior to ultrasound stimulus delivery (n = 5 trials) (figure 4.6). Consistent with the muscimol data, mice did not navigate to shelter in this condition, though were clearly still startled by the stimulus. I next asked whether this effect was due to the effect of the ChR2-subiculum stimulation on navigation, or whether it was a more general motor phenotype.

I addressed this by stimulating with the laser alone, with no innately threatening stimulus (n = 12 trials). In this case, mice stopped or did not accelerate, displaying a similar phenotype to that seen during the muscimol trials. I then attempted to vary the intensity of the laser to find a point at which laser stimulation alone did not elicit a phenotype, but laser stimulation and ultrasound stimulation did elicit a phenotype. I was unable to find such a laser intensity. This suggests that the precluded acceleration phenotype observed to both stimuli and optogenetic stimulation was due to the optogenetic stimulation alone, rather than being due to disrupting a response to the stimlus.

I therefore concluded that while replicating the muscimol phenotype with optogenetics was interesting, the lack of independence between laser only stimulation and laser + ultrasound stimulation meant that, under the time constraints, my efforts were better focused elsewhere.

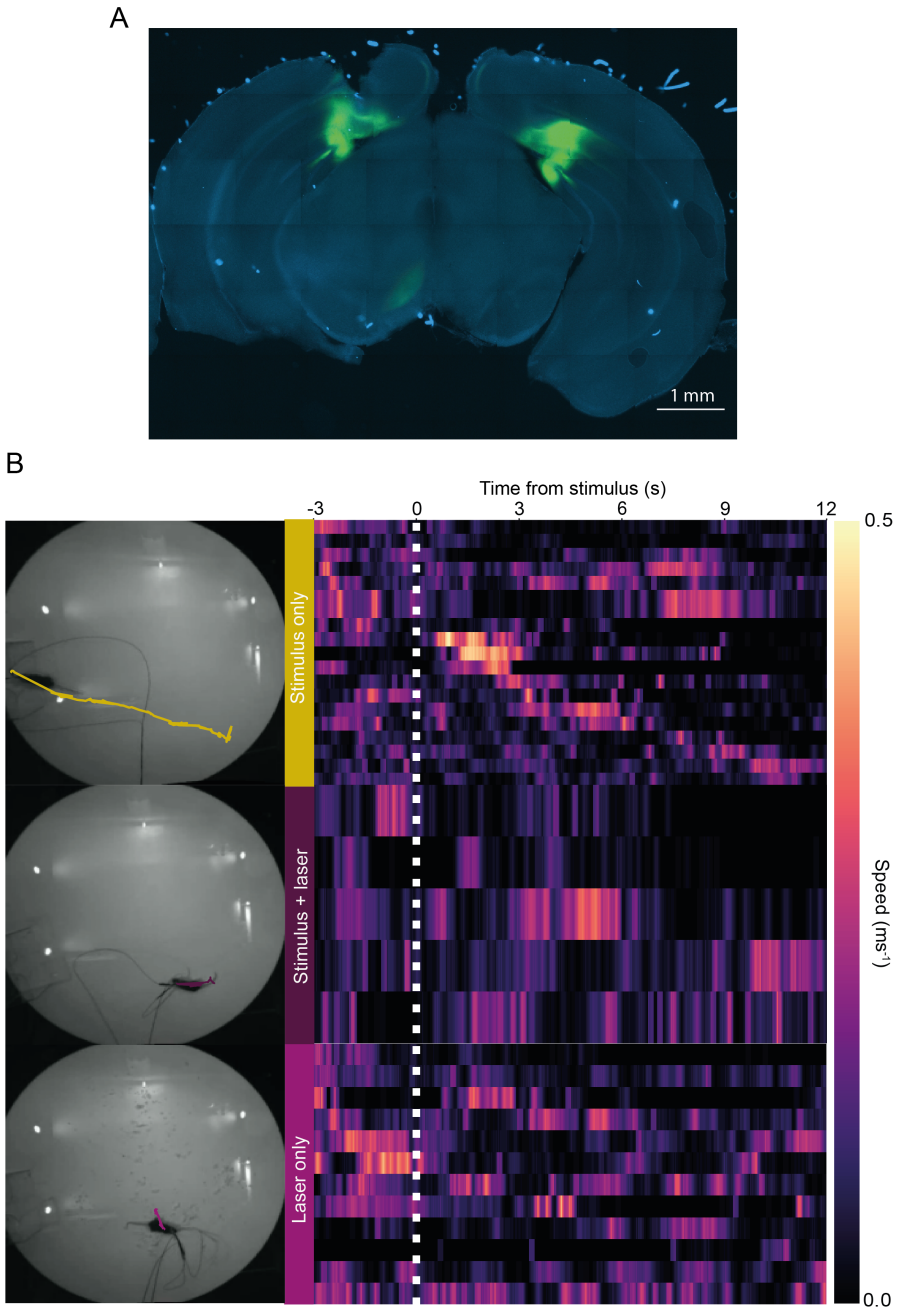

Figure 4.6: **Subiculum optogenetic stimulation disrupt escape and precludes acceleration.** A) Histological verification of virus injection and implant location. B) Left hand panels are example trials, with speed heatmap raster plots on the right

4.3 PRELIMINARY CONCLUSIONS FROM NEURAL LESION DATA

In the first results section, I showed that mice are able to initiate escape rapidly and accurately by combining two algorithms for computing escape routes, on based on path integration, the other guided by visual cues during flight. In this section, I aimed investigate the role of areas typically associated with these abilities in guiding escape navigation through a series of selective lesions, using different methods.

IBOTENIC ACID HIPPOCAMPUS LESION I began with a broad lesion to the hippocampus using ibotenic acid. Ibotenic acid is a non-specific agent that works through degrading tissue, hence leading to large scale changes in brain function. I began with using this approach to test in the broadest way possible whether the hippocampal formation guides escape navigation.

I found that hippocampus lesioned mice maintained their preference for the shelter. After entering the shelter, lesioned mice actually spent longer inside than non-lesioned controls. There are several possible interpretations of this finding. Given the known role of the hippocampus in spatial memory, a reasonable hypothesis would be that a lack of spatial memory, as has previously been described in hippocampus lesioned mice and was detailed in the introduction, means hippocampus lesioned mice struggle to navigate back to shelter during exploration. This meant that despite spending longer in the shelter per visit, they spent less time inside the shelter in total. Alternatively, given the ventral hippocampus has previously been associated with anxiety, it is possible that disruption of this system underlies the observed phenotype.

A deficit in spatial memory could be observed in defensive responses. While mice accelerated after the delivery of innately threatening stimuli, suggesting the stimuli were still detected and perceived as aversive, they clearly showed a decreased ability to navigate to shelter. This manifested through a longer time to reach the shelter after the stimulus, an increased tortuosity of flight suggesting a less direct path being taken, and the shelter distance scarcely changing during the stimulus.

This suggests that the hippocampus as a whole is likely to be involved in some form in guiding escape navigation. Interestingly, if the hippocampus were only involved in the latter phase of flight, the observed phenotype should be similar to that observed in dark conditions in the behavioural experiments presented previously. However, both phases of flight seem to be effected by a hippocampal lesion, suggesting that the hippocampus may be involved in guiding both of the identified phases of flight.

I next sought to narrow down the scope of lesion undertaken to further refine this finding.

MUSCIMOL SUBICULUM INFUSION To narrow down the lesion onto particular subregions of the hippocampus, I next infused muscimol into the subiculum. I targeted the subiculum due to recent work from our laboratory showing that the retrosplenial cortex guides the initial orienting towards the shelter (Vale et al., 2020). As a major projection from the hippocampus to the retrosplenial cortex is via the subiculum (Cembrowski et al., 2018) and the subiculum has previously been linked to spatial memory and encoding variables of inter-

est such as head-direction, the subiculum was an obvious target to link these two sets of findings.

I opted to use muscimol as my next means of disrupting neural activity as it is less abrupt than ibotenic acid, is able to be narrowly infused into specific targeted sub-regions, but still has a substantial effect on neural activity. I found that subiculum muscimol infused mice showed a clear reduction in their ability to navigate to shelter relative to control mice infused with vehicle. This manifested as a lack of acceleration or change in shelter distance post stimulus onset.

It is significant that a much smaller disruption than ibotenic acid lesion led to a similar phenotype. In addition to providing further evidence of the importance of the hippocampal formation broadly, it also suggests a particular sub-region through which the broader lesion could be mediating its effect. While other sub-regions were not investigated, that this sub-region aligns with an anatomically plausible mechanism of action suggests the subiculum is worthy of further study with respect to escape navigation.

OPTOGENETIC DISRUPTION OF NEURAL ACTIVITY IN THE SUBICULUM A limitation of using muscimol to disrupt neural activity is its lack of specificity for any particular cell-type. Instead, virus driven approaches allow genetically or anatomically defined cell-types to be isolated from the rest of a neural population. For this reason, I next sought to develop a virus driven approach to disrupting neural activity in the subiculum by stimulating with ChR2.

I found that while stimulating with ChR2 in tandem with an innately threatening stimulus disrupted escape navigation, and precludes acceleration, this phenotype was also seen with optogenetic stimulation alone. It's also possible that this finding is induced by light leaking from the brain or fibre, such that the mouse is in fact responding to the light rather than the light acting via neural activity. This finding therefore limits the interpretation of the results. However, it is nevertheless interesting that optogenetic stimulation alone partly replicates the finding that muscimol infusion yielded. This is likely due to non-escape related factors, but could nevertheless have to link to the phenotype observed in muscimol infusions.

CONCLUSIONS FROM NEURAL LESIONS Together this suggests that the hippocampus is likely to play a role in guiding escape navigation, a result that was partly surprising when I set out on these experiments. This initial expectation was based upon work from our laboratory suggesting that the retrosplenial cortex guides the initial orienting response by interacting with the superior colliculus, suggesting that circuits downstream of the hippocampus could guide escape navigation without it (Vale et al., 2020).

However, my findings suggest that the hippocampus may play an ongoing role in shaping the retrosplenial cortex activity required to undertake this role. The implications of this are further explored in the discussion section of this thesis.

Part IV

DISCUSSION

5

DISCUSSION

5.1 SIGNIFICANCE OF FINDINGS

The results presented in this thesis have been split into two: a first set of experiments identifying the behavioural level algorithms used to guide escape navigation; and a set of experiments aimed at identifying neural structures that correspond to the high-level cognitive involvement observed in the first experiments.

5.1.1 *Behavioural experiments*

I identified evidence that high-level spatial representations of the kind associated with cognitive maps guide instinctive escape navigation under normal conditions. Moreover, I found that these representations alone can guide escape navigation when the possibility of path integration is removed, but at the cost of a delay in reaction time. Prior studies of escape navigation in mice had first characterised the response of mice to instinctively threatening stimuli (De Franceschi et al., 2016; Yilmaz and Meister, 2013), and then focused on whether a spatial memory guides the escape (Vale, Evans, and Branco, 2017).

While it had been previously investigated that rodents can use cognitive maps to navigate to safety (Barnes et al., 2005), these studies suffer the limitation the initiation of the escape action is not tightly controlled in time, and the motivation of the mice is unclear. With escape behaviour induced by an instinctively threatening stimulus, the timing of the onset of the behaviour and the motivation of the animal is controlled much more tightly, allowing the introduction of features of the behaviour such as reaction time that were previously precluded. Moreover, as the motivation of the mouse is clearer, there is greater confidence that movement post-stimulus is related to the assay in hand.

This ability to precisely control stimulus onset times and intensity led to several insights. I was able to be confident that threat perception was comparable between light and dark conditions, meaning the properties of navigation could be isolated and investigated without this potential confound. Moreover, control over stimulus timing allowed precise measurement of reaction time, in turned enabling an assessment of the reaction time associated with different cognitive

processes across different assays. This provided a crucial insight that, when path integration was unavailable through experimental manipulations, mice used a cognitive map based strategy that required a longer reaction time, providing evidence in favour of the hypothesis that flight occurs in two phases with distinct cognition underlying each.

Prior to this study, most studies of escape from instinctively threatening stimuli had been undertaken over a spatial area of under 1 metre, and often in arenas as small as 30cm^2. This limited the observations that could be made from exploratory data and the interpretations that could be made from experimental data as several experimental confounds are present at this spatial scale, including olfactory cues and an increased ability to use other sensory features of the shelter to navigate. Furthermore, over this spatial scale, the navigational challenge is less difficult and the complexity of escape trajectories more simple. By expanding the spatial scale over which mice escaped in my assay to 2m, I was therefore able to observe differences between navigation in light and dark conditions that would otherwise have been missed, including that mice perform more head angle changes and pauses in the dark. In smaller spatial arenas, these features of escape navigation would have been missed as corrections in the navigational path are less necessary and can be guided by sensory features of shelter.

A further strength of this study is the range of behavioural controls and assays used, each of which provide data supporting the hypothesis of two-stage escape navigation. By introducing slow rotations of mice prior to escape, I was able to show that the mouse vestibular system is highly sensitive at integrating rotation and updating an estimate of the shelter location. This likely interacts with the path integration based system that will also depend on vestibular information, in addition to self-motion and proprioceptive cues. I further showed that visual cues can then be used to update trajectories when it becomes apparent that the shelter has moved in tandem with the mouse, which mice seem to have a prior against happening, likely as shelters in their natural habitat do not move in this way.

Other assays were also developed to test specific aspects of escape navigation. For instance, to tease apart the observation that reaction time increases when path integration was unavailable, I developed an assay in which the mouse is trapped rather than removed from the environment, and verified that under these conditions reaction times were normal. Similarly, to test whether the visual cues used in the latter phase of flight identified were in fact dependent on a cognitive map, I developed an assay in which the shelter was raised to remove the possibility of using visual taxon navigation. This study therefore combines a more exploratory initial stage, in which navigation in the light and dark was compared over large spatial scales in order to generate further hypotheses, and further testing of more specific hypotheses with carefully designed controls.

Together, these experiments provide further evidence for the role of sophisticated cognition in behaviours that has previously been viewed as more rudimentary. These data therefore add to longstanding debates, outlined in the introduction to this thesis, about instinctive behaviours and cognition.

5.1.2 *Involvement of the hippocampus in escape navigation*

I undertook preliminary experiments enacting different lesions to the hippocampal formation in order to identify substructures particularly related to the behavioural findings. The hippocampus, to my knowledge, had not previously been investigated in the context of instinctive escape behaviour. Part of the reason for this gap in the literature arises from the relatively recent identification of goal directed navigation to shelter in rodents in response to instinctively threatening stimuli. Prior to this, the link between defensive responses to these stimuli and goal directed navigation had been observed in other species, but only in recent years has a reliable method of inducing such goal directed navigation been detailed.

I found that various lesions to the hippocampal formation severely effected escape navigation. Most abruptly, large scale degradation of hippocampal tissue via ibotenic acid injection led to severe deficits in navigational abilities, despite a preference for the shelter being maintained. Post-stimulus acceleration suggested this was not due to a reduction in the ability of mice to detect the stimulus. This therefore provides the first strand of neural evidence that the cognitive map is actively engaged in guiding escape navigation.

I then focused on particular a sub-region of relevance within the hippocampus, the subiculum, for investigation with less abrupt disruptions. This region was chosen for two key reasons. First, it has been associated in particular with coding head-orientation, which, as the initial phase of flight involves orienting towards the shelter, seemed likely to be involved in escape navigation. Second, recent work from our laboratory has identified the retrosplenial cortex as a key node in the escape navigation system, playing a vital role in the orienting response (Vale et al., 2020), and the subiculum provides a major source of input to the retrosplenial cortex from the hippocampus (Cembrowski et al., 2018). These observations suggested that subiculum may be particularly relevant to escape navigation.

In two sets of experiments, I found that disrupting neural activity in the subiculum also disrupted escape navigation. However, only in the muscimol infusions, and not in the optogenetic stimulation, did appropriate controls lead to a firm conclusion. In the optogenetic disruption experiment, optogenetic stimulation alone reduced acceleration post-stimulus. However, this finding did align with the muscimol infusion data. The more clear finding comes from compar-

ing muscimol subiculum infusion with vehicle infusion. Here, mice could no longer navigate to shelter. This suggests that in addition the hippocampus more broadly being involved in escape navigation, the subiculum may be of particular importance.

How would this work on a circuit level? One possibility is that the hippocampus serves to 'update' the estimate of the orienting angle to the shelter, and then further refines flight after this initial orienting. Recent evidence from our laboratory suggests that the retrosplenial cortex is particularly important for orienting to shelter (Vale et al., 2020). Given the projections described from the hippocampus, and specifically the subiculum, to the retrosplenial cortex, it's possible that disrupting the hippocampus also disrupts the retrosplenial cortex estimate of orienting angle. This would mean that even the early phase of flight was disrupted by hippocampus lesions, despite the influence of visual cues and a cognitive map on escape trajectories occurring later. In this way, the path integration based orienting response could still be dependent on the hippocampus for updating or calibration. If this representation were disrupted, the result could be a freezing response, analogous to previous findings by our laboratory that freeze mice when exposed to instinctively threatening stimuli with no shelter present (Vale, Evans, and Branco, 2017). This proposed model for a relationship between the hippocampus and retrosplenial cortex in guiding escape is a clear future direction of study.

These data are complementary to the behavioural findings, as they provide further evidence of a role for a 'cognitive map' in guiding escape navigation. That these two independent strands of information converge - a finding unexpected when I started this study - is significant as previous studies had not investigated this, and had an underlying assumption that relatively 'simple' instinctive behaviours would not involve such high level cognition. More broadly, the interaction between high-level cognition and motivation or instinctive variables is an area of increasing study, and escape navigation could be a very useful model system towards our further understanding. In particular, escape navigation is useful because there are clear, rich, experimentally tractable behavioural outputs, while the motivational component and trial onset is tightly controlled, which is typically untrue of other attempts to study motivation and how it interacts with high level cognition.

Moreover, these data relate two longstanding lines of neuroscientific research: spatial navigation and instinctive defensive behaviours. While previous studies, such as those from our laboratory, have also successfully achieved this, this study for the first time implicates the traditional hub of spatial navigation research - the hippocampal formation - with guiding instinctive defensive behaviour. Together, these strands of evidence push further the argument that escape navigation is not a simple 'reflexive' response, but rather involves sophisticated cognition.

5.2 LIMITATIONS OF THE STUDY

When designing and planning out this study, I had intended the neural and behavioural data to carry approximately equal weight. However, owing to time constraints, it was not possible to extend the preliminary neural lesion data in the way I would have liked. In this way, one limitation of this study is that the behavioural weight is not sufficiently linked to the neural data to draw firm comparisons. However, both sets of data are useful and interesting and push forward our understanding in important ways.

BEHAVIOURAL EXPERIMENTS The limitations of the behavioural study primarily relate to data acquisition and the supervised way in which reaction time was measured. Manually labelling reaction time is not optimal as it is a potential source of bias, despite trials being labelled blind to condition. Ideally, reaction time would be extracted in an unsupervised and hence unbiased way. Increasingly, automated extraction of such features is becoming possible through increased capacity to record from different camera angles leading to 3 dimensional tracking, and machine learning algorithms allowing the automatic extraction of different behavioural states from these features. Similarly, having 3D tracking and animal pose information through flight could have yielded further insights into the differences between flight in light and dark conditions, and the different reactions of mice in the DMM versus baseline trials. And finally, were the experiments in silencing the subiculum to be expanded on, this form of tracking may allow the unsupervised extraction of behaviours such as freezing, which in this study were mainly only indirectly observed through speed and acceleration data, rather than mouse pose.

In terms of experimental design, an intrinsic limitation is that it is extremely hard to control for and hence rule out all other possible sources of navigational information. This includes the influence of learning through trials, though this was explicitly control for in similar conditions in previous experiments (Vale, Evans, and Branco, 2017). This limitation applies to all navigational studies, and I consider the controls applied in the present study to be more robust than those applied in most similar studies.

A further limitation is that all behavioural experiments were performed on young, male mice. While this facilitates within group comparison and was limited in this way for the experimental reason that these mice tend to explore more and hence permit greater numbers of trials, it potentially limits their generalisation to females and mice of different ages. Previous experiments from within the lab indicate that there is no major difference in navigational strategy in these groups, but this work is preliminary, unpublished, and does not directly replicate the present experiments.

NEURAL LESION EXPERIMENTS As the neural interventions were primarily preliminary studies, there are some limitations in the interpretation of these results. While a striking phenotype was observed in the ibotenic acid hippocampus lesioned mice, the scale of the intervention means there are likely to be confounding off-target effects. This uncertainty is reflected in the erratic exploration patterns of these mice. However, these concerns are partially resolved by similar phenotypes being observed with other interventions. Therefore, despite these limitations, as these are the first data collected from hippocampal lesioned mice in escape navigation induced by instinctively threatening stimuli, they provide a useful step forward in our understanding.

The more narrowly targeted disruptions to neural activity gave results that aligned with the broader hippocampal lesion. However, as is often the case with technically challenging experiments, the sample size may not have been large enough to truly tease out the specific phenotype present. This limitation arises because these experiments are particularly low throughput: they require two surgical procedures, spaced by a week, and only after the experiment is complete does the experimenter know whether it was targeted to the desired brain structure. While a striking phenotype was observed, a greater number of trials and mice than was possible for the present may have revealed further features of the observed phenotype.

The clearest limitation is present in the optogenetic stimulation experiments. Here, a phenotype that aligned with the previous observations from lesion experiments was observed when instinctively threatening stimuli alone were compared with stimuli + optogenetic stimulation to disrupt neural coding in the subiculum. However, when controlled for the optogenetic stimulation alone, mice also displayed the same phenotype, meaning firm conclusions could not be made.

5.3 FUTURE DIRECTIONS

This set of experiments opens up several strands of future research. In terms of behavioural research, a particularly promising line of research is to focus on the spatial memory component of the DMM assay. For instance, for how long is the memory of the shelter location maintained? This question was outside the scope of the present study, but could be addressed by varying the time delay between the mouse leaving the environment and being reintroduced to visual cues. In this way, this task could provide a way of testing long-term spatial memory and its consolidation, without the long training times typically associated with such studies.

A further question, more closely related to spatial navigation as a field than escape navigation specifically, is whether the cognitive map allows goal directed navigation from previously un-visited spaces.

In principle, inference of a new vector should be possible based on available visual cues, regardless of whether the position itself has been visited. The DMM escape navigation provides a good task to test hypotheses about how this may be encoded neurally as stimulus onset times and the motivation to navigate to the shelter location are well controlled, which normally provides an experimental challenge when addressing these questions.

As a limitation of the study was that neural experiments were prematurely curtailed, a wide expanse of experiments are possible with respect to the role of the hippocampus in guiding escape navigation. These range from further perturbations to the recording of neural activity in this brain region during escape navigation.

A clear future direction is to develop viral approaches to altering neural activity in the hippocampus and subiculum during escape navigation assays. An unsuccessful attempt to do so was made in the present thesis, but this line of enquiry would open up a new range of questions and would increase experimental power by enabling interleaved control and intervention trials and sessions. Moreover, such an approach would allow specific sub-populations within these structures to be manipulated, which was not possible with either muscimol or ibotenic acid manipulations.

Given the wealth of knowledge that exists about how the hippocampal formation represents space, recording these neurons during escape navigation assays should also be a priority for future research. A longstanding question within the spatial navigation literature is how spatial representations are used to guide goal-directed navigation, a question that could be addressed through the escape navigation paradigm used here. This would further extend our knowledge of spatial navigation, instinctive behaviours, and how instinctive drives interact with high-level cognition to guide intelligent behaviours.

5.4 CONCLUDING REMARKS

The present work progresses our understanding of the brain and intelligent behaviour by drawing together two independently well studied fields: instinctive defensive behaviours and spatial cognition. I began with a hypothesis over how the urgency required to escape from imminent threat interacts with the rich set of spatial representations in the brain. Through a series of behavioural experiments, I verified a hypothesis that a two-phase process occurs in escape navigation, initially depending on a reliable and rapidly computed path integration based estimate of the direction to shelter, followed by a later phase in which visual cues are used to compare to a cognitive map in order to refine escape trajectories. I then disrupted brain regions associated with the cognitive map during escape navigation assays, and showed

that they have an involvement in guiding defensive navigation to shelter.

Identifying a clear relationship between high-level spatial representations and instinctive escape behaviour furthers our knowledge of both fields and provides a means of investigating how instinctive drives interact with abstract cognition to drive intelligent behaviours in animals.

Part V

APPENDIX

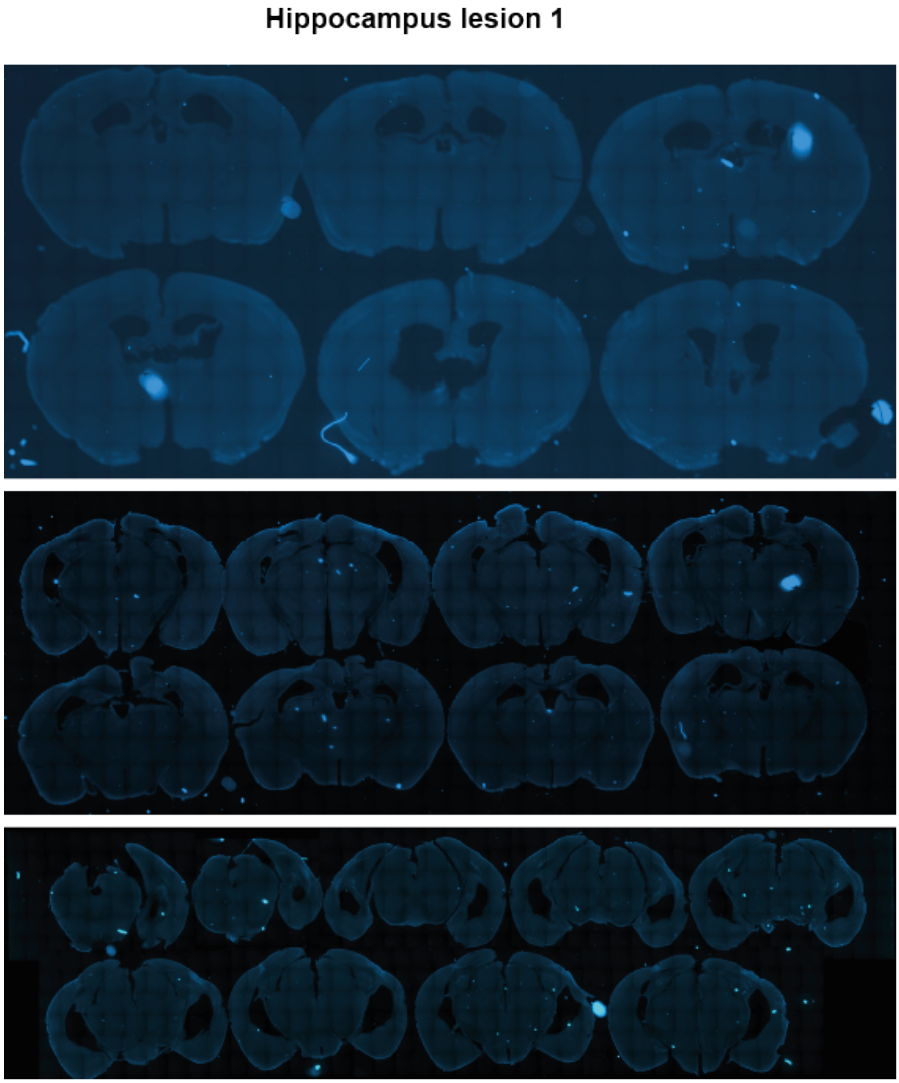

Figure .1: **Histological verification of hippocampus lesion 1**

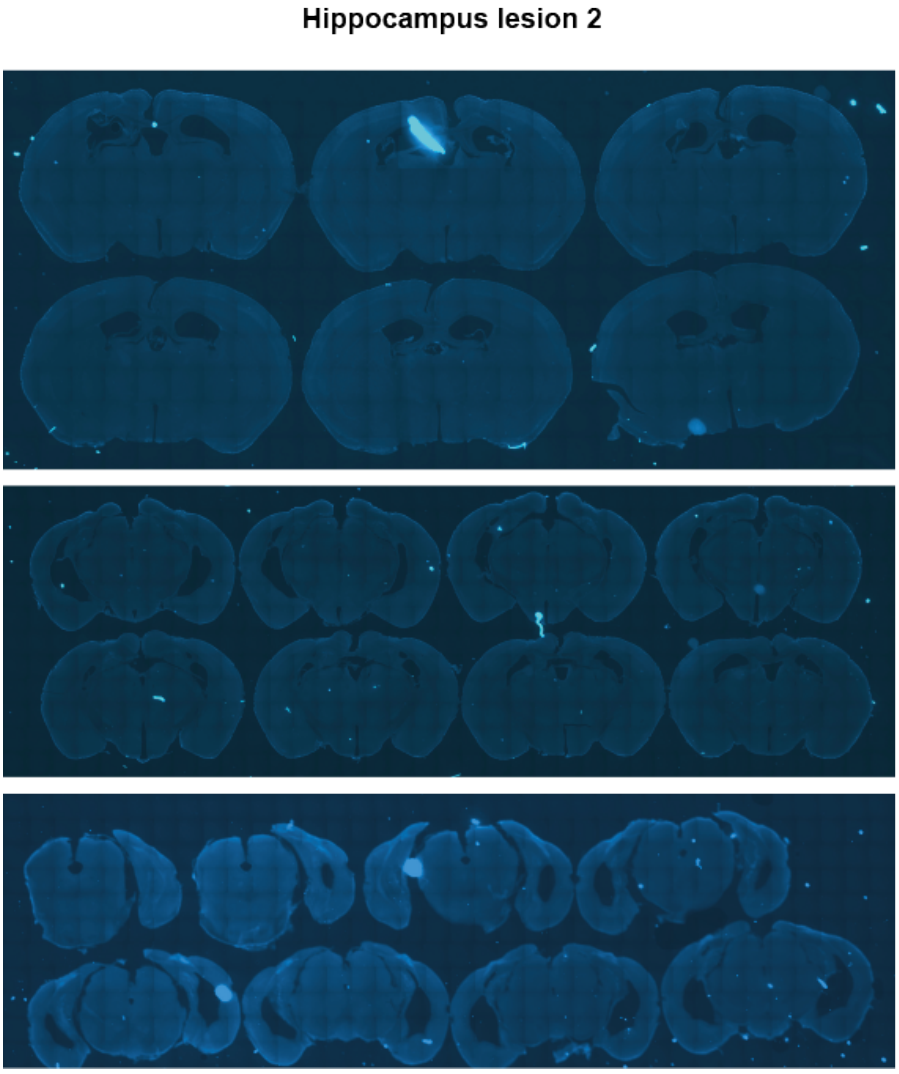

Figure .2: **Histological verification of hippocampus lesion 2**

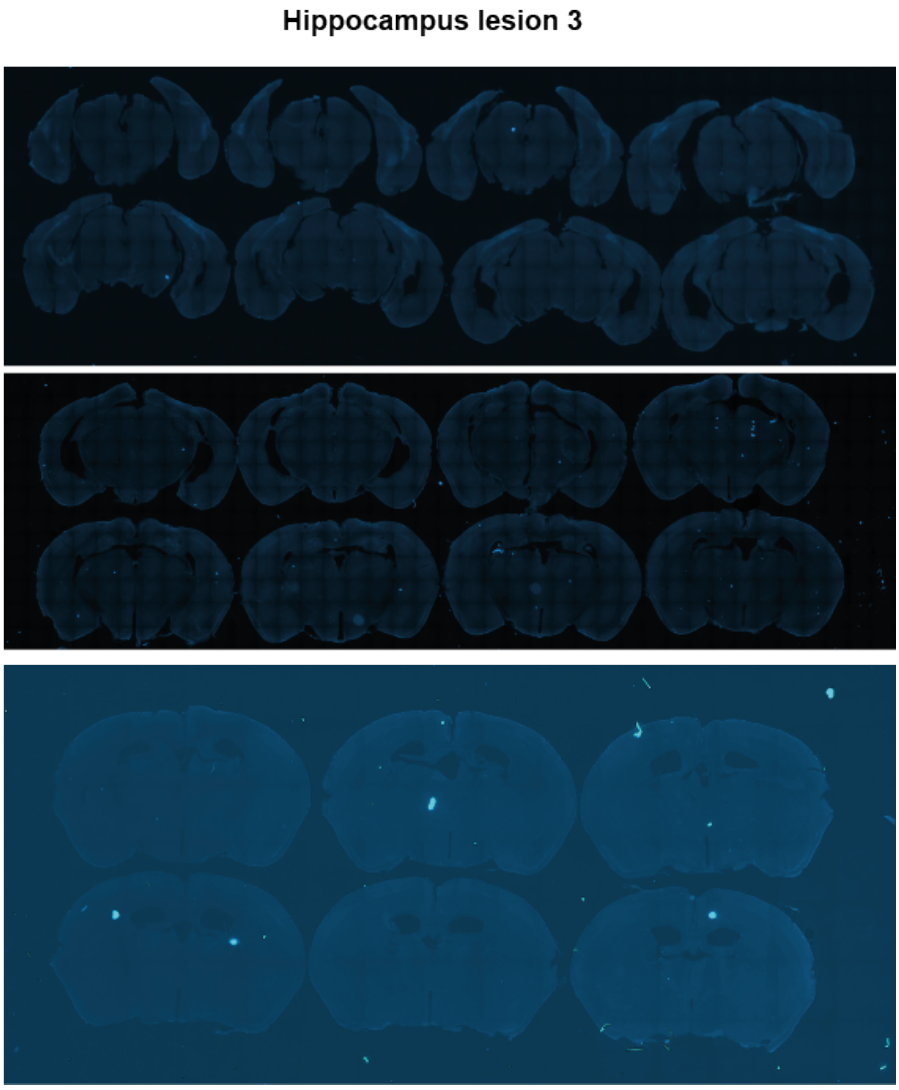

Figure .3: **Histological verification of hippocampus lesion 3**

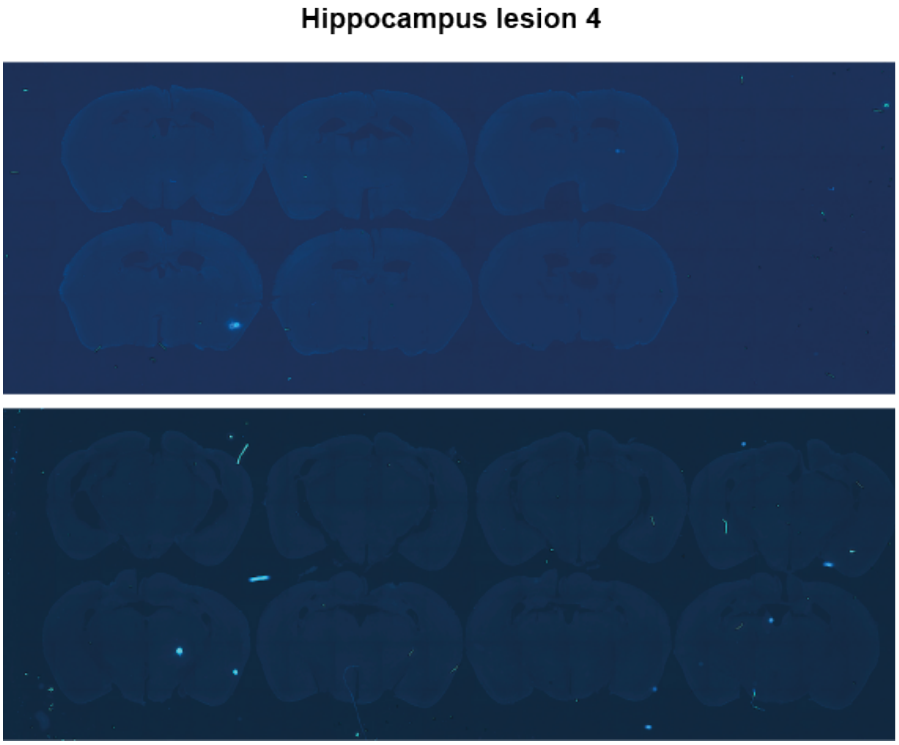

Figure .4: **Histological verification of hippocampus lesion 4**

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DECLARATION

This project was conducted primarily by myself, with experimental design and analysis supervised by Professor Tiago Branco. As this project involved several different skillsets, other members of the lab contributed in certain places. Histological processing for the image in figure 4.1a was done by Panagiota Iordanidou. Surgeries for the experiment presented in figure 4.1-4.3 were conducted by Ruben Vale. Code for the alignment of videos was originally written by Philip Shamash, which I then and then adapted to this project.

All other work presented is my own.

London, UK, April 2021

Matthew G. Phillips